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Chemiluminescence Immunoassay for S-Adenosylhomocysteine Detection and Its Application in DNA Methyltransferase Activity Evaluation and Inhibitors Screening

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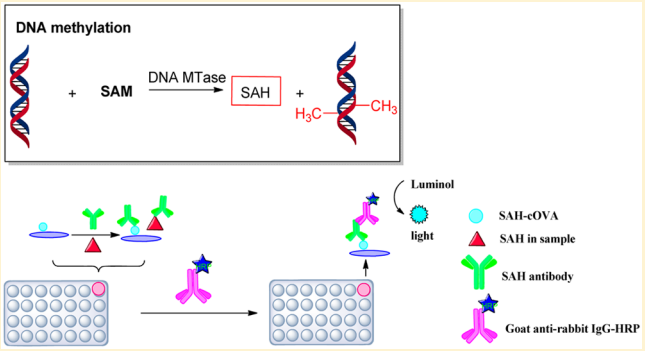
Supporting Information

ABSTRACT: Aberrant methylation by DNA transferase is associated with cancer initiation and progression. For high-throughput screening of DNA methyltransferase (MTase) activity and its inhibitors, a novel chemiluminescence immunoassay (CLIA) was established to detect S-adenosylhomocysteine (SAH), the product of S-adenosylmethionine (SAM) transmethylation reactions. We synthesized two kinds of immunogens for SAH and characterized the polyclonal antibodies in each group. The antibody with higher titer was used to develop a competitive CLIA for SAH, in which SAH in samples would compete with SAH coated on microplate in binding with SAH antibodies. Successively, horseradish peroxidase labeled goat antirabbit IgG (HRP-IgG) was conjugated with SAH antibodies on the microplate. In substrate solution containing luminol and H_2O_2, HRP-IgG catalyzed luminol oxidation by H_2O_2, generating a high chemiluminescence signal. The method could detect as low as 9.8 ng mL^{-1} SAH with little cross-reaction (3.8%) to SAM. Since higher DNA MTase activity leads to more production of SAH, a correlation between the chemiluminescence intensity and DNA MTase activity was obtained in the range from 0.1 to 8.0 U/mL of DNA MTase. The inhibition study showed that, in the presence of SAM as methyl donor, Lomeguatrib, 5-Azacytidine, and 5-Aza-2′-deoxycytidine could inhibit the DNA MTase activity with IC_{50} values of 40.57 nM, 2.26 μM, and 0.48 μM, respectively. These results are consistent with the published studies. The proposed assay does not depend on recognizing methylated cytosines in oligonucleotides (methyl acceptor) and showed the potential as an accessible platform for sensitive detection of DNA MTase activity and screening its inhibitors.



DNA methylation is one of the most commonly epigenetic events occurring in mammals. It happens in the C-5 position of cytosine in the CpG dinucleotides, which is catalyzed by DNA methyltransferases (MTase) using S-adenosylmethionine (SAM) as the methyl donor.[1,2] Abnormal DNA methylation can inactivate the tumor suppressor genes and has been associated with a variety types of cancers, such as colon cancer, lung cancer, prostate cancer, and gastric cancer.[3,4] DNA MTase has become an important biomarker in early disease diagnosis and potential therapeutic targets in cancer therapy. Therefore, a simple, sensitive, and accurate method for analyzing DNA MTase activity is necessary in cancer diagnosis and therapy.

Radioactive assay using [methyl-^{3}H]-SAM is the current standard method for evaluation of DNA MTase activity.[5,6] Over the past decade, various biochemical methods avoiding radioactive reagents have been proposed to detect DNA MTase activity. The methods include liquid chromatography/mass spectrometry,[7−9] colorimetry,[10−12] fluorescence assay,[13−17] and polymerase chain reaction (PCR).[18] These methods are mainly based on the structure difference between methylated and unmethylated DNA sequence. However, most of these assays require expensive equipment and time-consuming sample treatment and are not suitable for convenient quantification. In last several years, electrochemical methods were presented via oxidation signal change of 5-methyl cytosine (5-mC) or molecules embedded into hybridized DNA for recognizing methylated bases.[19] In 2012, Wang et al. utilized an anti-5-mC antibody for specific conjugation with 5-mC and thus improved the sensitivity of the electrochemical assay.[20] However, the simplicity of these tests is still not satisfactory, which indicated that the

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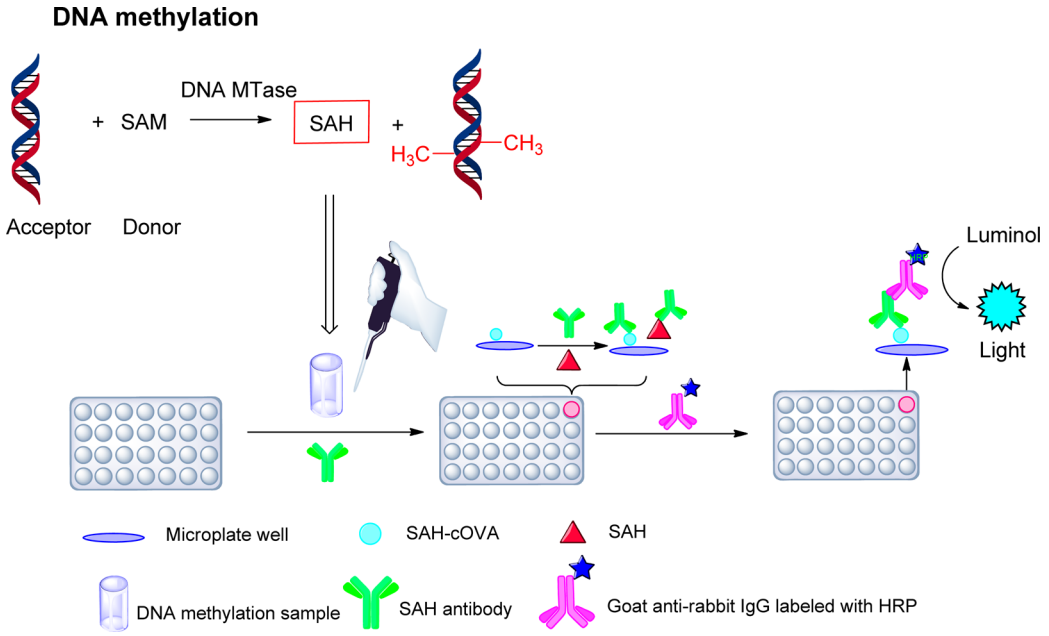
Scheme 1. Schematic Illustration for CLIA Detection of DNA MTase Activity



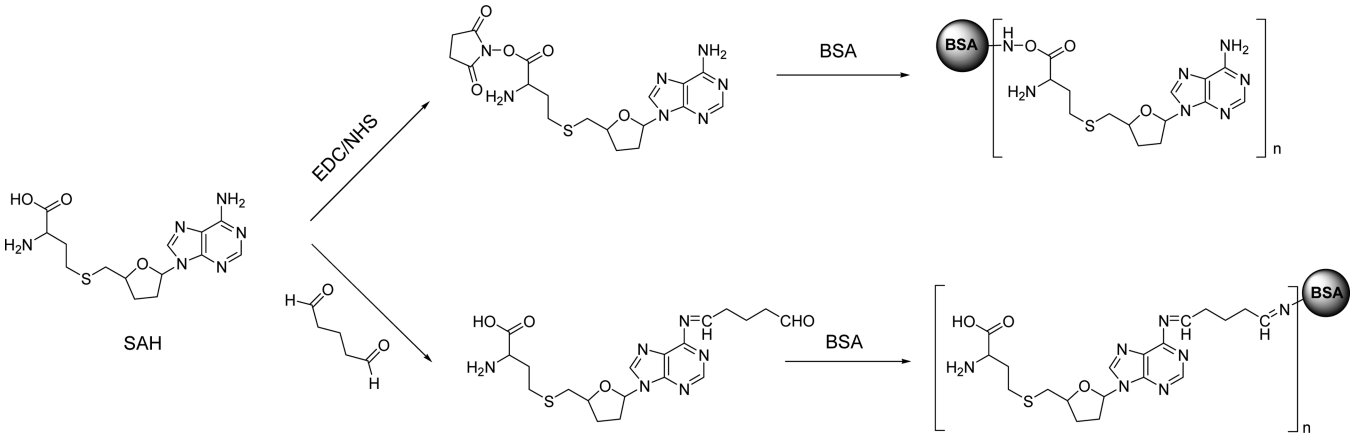


Figure 1. Synthesis of SAH immunogen.

electrochemical method could not be applied as a high-throughput screening in clinical diagnosis.

In this article, we reported a sensitive and rapid chemiluminescence immunoassay (CLIA) performed in a 96-well microplate. The method was based on detection of SAH, the product of SAM transmethylation reactions. Two kinds of SAH immunogens were synthesized to produce SAH antibodies in rabbits. The optimal antibody product was utilized to develop a competitive CLIA (Scheme 1). In this assay, SAH in analytes and coating antigen could simultaneously couple with a certain quantity of SAH antibody added in the well. A higher level of free SAH would result in less conjugation of SAH antibody with SAH coated on the microplate well. In consequence, the chemiluminescent (CL) intensity would decrease. Since higher DNA MTase activity leads to more production of SAH, the activity of DNA MTase could be determined by a decrease of CL intensity. To our knowledge, this is the first work to develop an immunoassay for analysis of DNA MTase activity using anti-SAH antibody. The proposed assay is nonradioactive and does not require expensive equipment or restriction endonuclease. Almost 90 samples could be determined within 4 h. Therefore, this study provides a fast and sensitive platform for analyzing DNA MTase activity and screening its inhibitors.

EXPERIMENTAL SECTION

Reagents and Apparatus. S-Adenosyl-L-methionine (SAM), S-adenosylhomocysteine (SAH), bovine serum albumin (BSA), and ovalbumin (OVA) were supplied by Sigma-Aldrich (St. Louis, MO). Goat antirabbit IgG labeled with horseradish peroxidase (HRP) was from Sungene Biotech Co. Ltd. (Tianjin, China). Luminol was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Human DNA MTase (DNMT1) was purchased from New England Biolabs (Ipswich, MA). Double-stranded DNA used in this work was provided from GENEWIZ Inc. (Suzhou, China). The sequences of the oligonucleotides were as follows: oligonucleotide 1 (oligo 1), 5′-CTA GCT ATG TGC CGA ATT TCA AGG ACA GTT GTA TGG CCC TCGT-3, and its complementary oligonucleotide 2 (oligo 2), 5′-A CGA GGG CCA TAC AAC TGT CCT TGA AAT TCG GCA CAT AGC TAG-3′. The oligonucleo-

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tides were dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and stored at −20 °C.

CLIA signal was read by a BHP9504-microplate luminometer (Beijing Hamamatsu Photon Techniques Inc., Beijing, China). White polystyrene microplates were supplied by Jet Biofiltration Products Co., Ltd. (Beijing, China).

Synthesis of Artificial Immunogens for SAH. In the SAH molecule, the carboxyl and amino groups could be both connected with carrier proteins to obtain artificial immunogens (Figure 1). The carboxyl groups in fragment of homocysteine were activated by EDC/NHS and coupled with BSA.[21] For this purpose, 15.3 mg of SAH, 35.0 mg of EDC, and 10 mg of NHS were dissolved in 5 mL of DMF, then stirred overnight at room temperature. The mixture was dropwised into 10 mL of PBS (pH 7.4) containing 60 mg of BSA and stirred for 8 h at room temperature. Also, the amino groups in the fragment of adenine were activated by glutaraldehyde. For this design, 15.3 mg of SAH was mixed with 35 mg of BSA in 3 mL of borate buffer (0.05 M, pH 8.5). Then, 60 μL of 25% glutaraldehyde solution was added and the reaction was allowed to proceed for 4 h at room temperature. The reaction mixture was dialyzed against PBS for 3 days and distilled water for another 3 days. The solution in dialysis bag was lyophilized and stored at −20 °C. For SAH immobilization onto the microplate, coating antigen was prepared similarly, in which cationized ovalbumin (OVA)[21] was used instead of BSA.

Immunoassay Procedure. The immunogens were employed to prepare polyclonal antibodies in New Zealand rabbits. The detailed immunization procedures were shown in Section 1, Supporting Information. A 96-well polystyrene microtiter plate was coated with 100 μL/well of coating antigen (1 μg mL^{-1}) and incubated at 37 °C for 2 h. The excessive coating antigen was removed, and the free binding sites in the well were blocked by 250 μL/well of 1% (w/v) OVA containing 0.5% (v/v) Tween 20. After incubated at 37 °C for 2 h, the wells were washed three times by PBS with 0.5% (v/v) Tween 20, and 50 μL of analyte solution was added, for example, SAH solution or DNA MTase methylation solution. The antiserum (1:20 000) was added (50 μL/well) and incubated at 37 °C for 0.5 h. After washing procedure, goat antirabbit IgG-HRP (1:20 000, 100 μL/well) were added and incubated for 30 min at 37 °C. The microplate was washed, and 100 μL of chemiluminescent substrate (0.5 mmol L^{-1} luminol, 0.2 mmol L^{-1} *p*-iodophenol, and 1 mM H_2O_2, 2:2:1, v/v/v) was pipetted into each well. Five minutes later, the CL signal in each well was recorded.

RESULTS AND DISCUSSION

Verification and Optimization of SAH Immunogens. The purified SAH immunogens in two groups were analyzed by UV (Figure S1) and UPLC−Q-TOF-MS (Figure S2). SAH showed an absorption peak at 259 nm, and BSA had a characteristic absorption peak at 278 nm, while both immunogens showed an absorption peak near 259 nm. These results indicated that the immunogens were successfully conjugated. The UPLC−Q-TOF-MS spectra indicated a more unique immunogen synthesized in the EDC/NHS group with the coupling ratio of SAH to BSA equivalent to 14:1.

Antibody titer is defined as the dilution factor of antibody that results in a CL signal 2 times higher than the blank group. As a result, the titer of SAH antibody was more than 200 000 in the EDC/NHS group and less than 5000 in the glutaraldehyde group. This result primarily indicates that the expose of adenosine is beneficial to obtain the high quality of SAH antibody.

Sensitivity and Specificity of SAH Antibody. Sensitivity of SAH antibody is expressed as the IC_{50} value in the SAH test, a SAH concentration leading to a 50% decrease of CL intensity compared with the blank group. Considering the higher titer, the SAH antibody in EDC/NHS group was used to develop an indirect competitive CLIA. According to the results of immunoassay optimization, the applied concentration of coating antigen was 1 μg mL^{-1} and the dilution factor of SAH antibody was 1:20 000 (Table S1). Thus, an optimum CLIA method was established, and the method showed high sensitivity toward SAH (Figure 2, IC_{50} = 106.7 ng mL^{-1}). The linear range for SAH detection was from 9.8 to 1155.0 ng mL^{-1}.

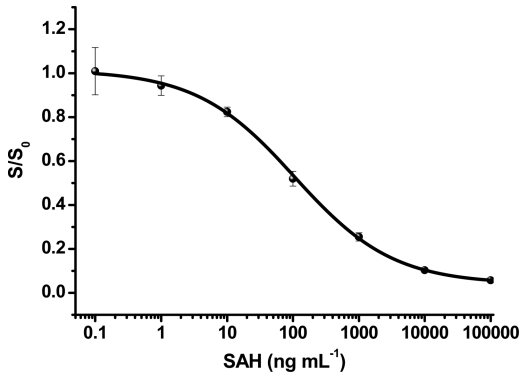

Figure 2. Decrease ratio of CL signal (S/S_0) in CLIA detection system versus concentration of SAH (n = 3).

Specificity of SAH antibody was measured by replacing SAH with structure analogues (SAM, adenosine and adenine), reactant compounds (DNA and DNA MTase), and DNA MTase inhibitors (5-Azacytidine, 5-Aza-2′-deoxycytidine, Lomeguatrib). The CLIA was performed as indicated above, and values of IC_{50} were calculated for each analyte to calculate its cross-reaction (CR) with SAH antibody: CR (%) = (IC_{50} of SAH/IC_{50} of analyte) × 100%. As was shown in Table 1, SAH antibody exhibited little cross-reactivity toward SAM (3.86%) and adenosine (1.84%) but almost no reaction with adenine, which suggested the ribofuranose as a part of determinant for SAH recognition. Furthermore, SAH antibody showed no binding with both DNA (2 μM, 33 μg mL^{-1}) and DNA MTase (16 U/mL), which guarantees that in DNA methylation reaction, only SAH is active toward SAH antibodies. Also, the three DNA MTase inhibitors exhibited little activity with SAH antibodies. This observation confirmed that the CLIA system would not yield false positive results when applied in screening DNA MTase inhibitors.

Assay of DNA MTase Activity. In eukaryotes, DNA methyltransferase (MTase) can specifically catalyze the transfer of a methyl group from SAM to cytosine within CpG dinucleotides. It plays an important role in the cell proliferation, gene transcription, and cancer origin. Several electrochemical methods have been developed to assay DNA MTase activity, based on the principle that the cleavage of methylated DNA by restrict endonuclease is hinderd by the methylation of CpG sequence. However, hybridization of DNA on an electrode needs a skilled operation. Also, the stability and repeatability of electrode modification is not excellent. Here we showed a

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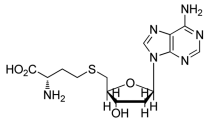
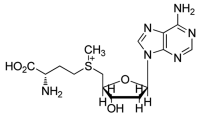
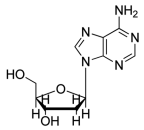
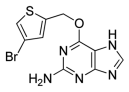
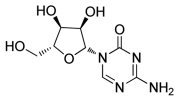
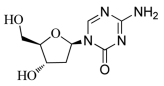
Table 1. Cross-Reactivity of SAH Antibody with Related Compounds

Analyte	Structure	IC_{50} (ng mL^{-1})	Cross-reaction (%)
SAH		106.7	100
SAM		2765.6	3.86
Adenosine		5785.6	1.84
Adenine		>100000	<0.03
Lomeguatrib		>50000	<0.30
5-Azacytidine		>100000	<0.03
5-Aza-2'-deoxycytidine		>100000	<0.03

chemiluminescent immunoassay that could be used to determine DNA MTase activity in a 96-well microplate without complicated modifications. And, the performance of this method does not require restrict endonuclease, so that the influence of restrict endonuclease would be avoided.

The methylation buffer is performed at 37 °C in 50 mM Tris-HCl (pH 7.8) containing 1 mM EDTA, 1 mM DTT, 560 ng mL^{-1} (1.4 μM) SAM, 0.5 μM DNA, and 4 U/mL DNA MTase. Under treatment with DNA MTase, the methyl group in molecule of SAM was transferred to the substrate of DNA. As a result, SAH was formed, and CL intensity in the competitive CLIA system was reduced. The presence of DNA MTase leads to more production of SAH; hence, its activity could be evaluated by decline of the CL signal. With methylation time extending from 0 to 2 h, the CL intensity decreased (Figure S3), which could be ascribed to the fact that more SAM was catalyzed to be SAH. Then, the intensity reduced gradually. After 3 h, it did not decrease any more. These data indicated that almost all SAM has been methylated. As a result, the concentration of SAH achieved a stable value. Thus, we selected the optimal methylation time of 3 h before immunoanalysis. This result was in accordance with that from electrochemical assay for DNA MTase activity determination.[22]

The K_m values for SAM (K_m = 4.0 μM) and DNA (K_m = 0.63 μM) were measured by a Michaelis–Menten kinetic study (Figure S4 and Table S2). These results are consistent with the published data. The optimal concentration of SAM and DNA in the study of enzyme inhibitor activity should be below 4.0 μM and 0.63 μM, respectively.

The DNA MTase activity was then assayed with DNA MTase varying from 0 to 16 U/mL. Since the higher DNA MTase activity is, the more SAH would be produced. It was found that CL intensity declined as DNA MTase concentration increased (Figure 3). A linear relationship of CL intensity with DNA MTase activity could be observed in 0.1–8.0 U/mL (inset in Figure 3, in which Y means S/S_0, while X is the logarithmic value of DNA MTase activity). The range covers

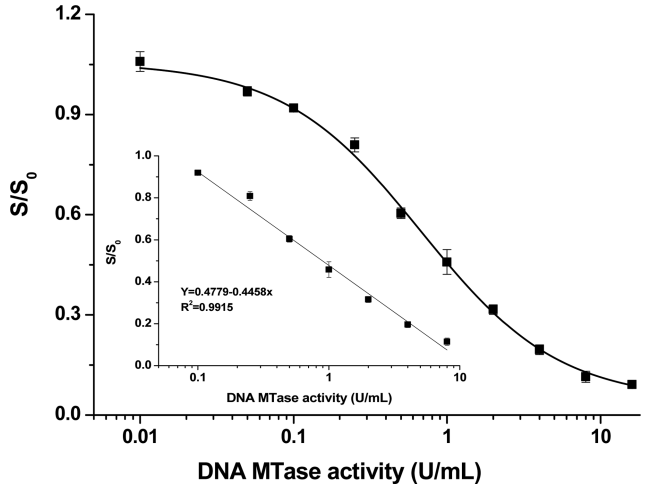


Figure 3. Competitive CLIA calibration curve under different DNA MTase activity (n = 3).

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the DNA Mase activity leading to a 10%−90% decrease of S/S_0. The detection limit of DNA MTase is defined as DNA MTase activity that results in a 5% decrease of S/S_0 observed from the competitive calibration curve, that was 0.07 U/mL (Figure 3). The RSD (relative standard deviation) for three independent assays was below 10%, indicating an acceptable repeatability of the proposed assay.

To investigate the selectivity of the proposed DNA MTase assay, alkaline phosphatase (ALP) and HRP were used instead of DNA MTase in the methylation reaction. The selectivity experiments were conducted in a similar way. As shown in Figure 4, the addition of DNA MTase (DNMT) remarkably

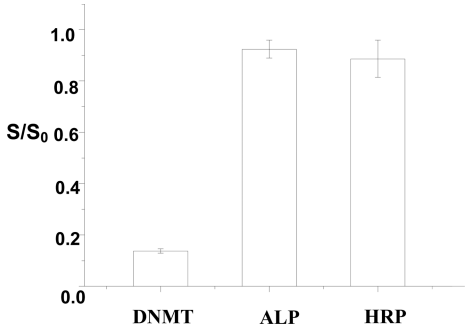

Figure 4. CL signal (S) for SAH test in the presence of DNA MTase (DNMT), ALP, or HRP (n = 3).

reduced the CL signal (S/S_0 = 13%) in the SAH assay, while in the presence of ALP and HRP, the CL intensity hardly decreased (<10%). Therefore, the proposed assay exhibited a specific response to DNA MTase.

Assay of Inhibition of DNA MTase Activity by Different Inhibitors. It is reported that DNA MTase inhibitors can reversibly alter the methyl level in DNA methylation associated diseases, especially in cancer. Therefore, the screening of DNA MTase inhibitors plays an important part in cancer treatment.

Nucleoside compounds of 5-Azacytidine (5-Aza), 5-Aza-2′-deoxycytidine (5-Aza-dC) and Lomeguatrib were selected as the model inhibitors to study the method application in screening of DNA MTase inhibitors.[22] Different concentration of the inhibitors was added in DNA MTase reaction solution, and the CL intensity of these samples was recorded as indicated above. The relative activity of DNA MTase against each inhibitor was calculated with the following equation:

$$\text{relative activity} = (I_1 - I_0)/(I_2 - I_0)$$

where I_0 is the CL intensity of sample without DNA MTase, I_1 is that with 4.0 U/mL DNA MTase, and I_2 is that with 4.0 U/mL DNA MTase mixed with DNA MTase inhibitors. The incubation time was still fixed at 3 h, because CL signal of DNA MTase upon these inhibitors did not reduced any more after 3 h (Figure S5).

The inhibition effects of 5-Aza, 5-Aza-dC and Lomeguatrib on DNA MTase are illustrated in parts A, B, and C of Figure 5, respectively. The relative activity of DNA MTase decreased dramatically as concentrations of the inhibitors increased. This result suggested that the inhibition of these inhibitors was in a dose-dependent process. It was reported that the inhibition of 5-Aza and 5-Aza-dC was in a competitive manner, since the methyl group from SAM could be simultaneously transferred to 5-Aza (or 5-Aza-dC) and cytosine residue in DNA by the

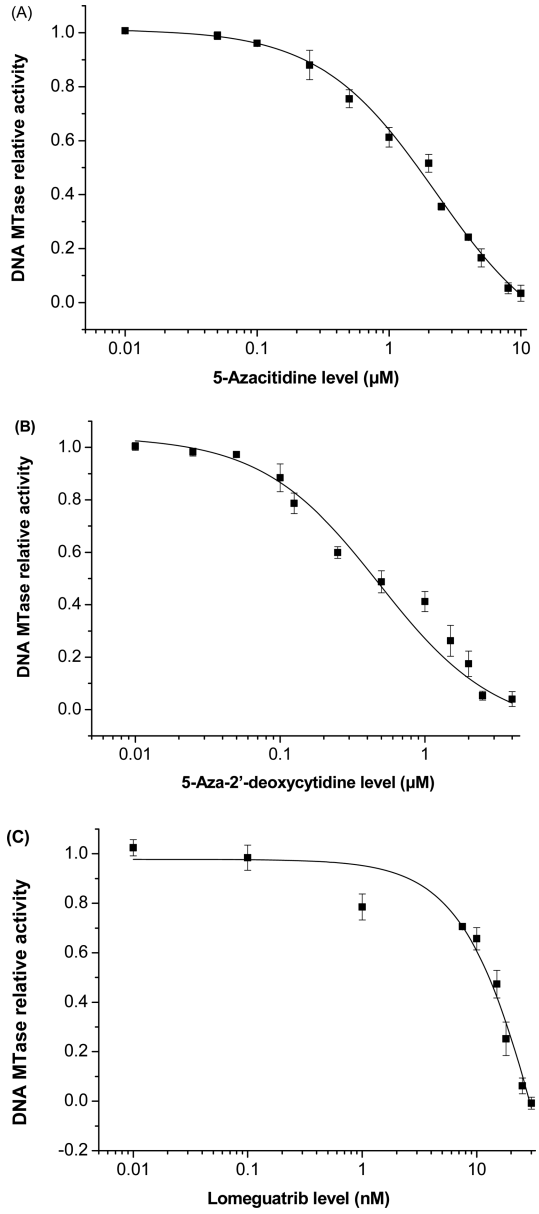

Figure 5. Inhibition effect of 5-Azacytidine (A), 5-Aza-2′-deoxycytidine (B), and Lomeguatrib (C) on DNA MTase activity (n = 3).

catalysis of DNA MTase. The IC_{50} values for 5-Aza and 5-Aza-dC were 2.26 μM and 0.48 μM, respectively. Lomeguatrib exhibited a higher inhibition efficiency (IC_{50} = 40.57 nM) than 5-Aza and 5-Aza-dC. This observation is the consistent with the reported study.[19,20] In addition, the inhibition efficiency for these inhibitors determined by CLIA is in good agreement with that in published work.[22] These results demonstrated that the proposed assay can be successfully applied in DNA MTase inhibitor screening and is a potentially useful tool for anticancer drug discovery.

CONCLUSION

In this work, we have prepared polyclonal antibodies against SAH and developed a CLIA to detect DNA MTase activity by quantification of SAH. The method could detect as low as 9.8 ng mL^{-1} SAH, and were not interfered by SAM or nucleoside analogs. The DNA MTase activity could be determined in the range of 0.1 to 8.0 U/mL. The presented CLIA method was

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applied in high-throughput screening of DNA MTase inhibitors. As DNA methylation and DNA MTase exhibit important roles in cancer detection and treatment, this rapid and cost-effective assay for DNA MTase activity assay is an attractive candidate as monitoring platform in clinical research. Furthermore, this is the first study to detect DNA MTase activity by measurement of SAH in a methylation solution (method comparison shown in Table S3). Therefore, the findings in this work may contribute to further application of SAH immunoassays in evaluation of DNA MTase activity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b01579.

Production of SAH polyclonal antibodies, UV and MS profiles of immunogens, optimization of SAM and SAH antibody concentration, kinetic results of DNA MTase methylation with and without inhibitor, and the comparison of the proposed method with reported studies (PDF)

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Notes

The authors declare no competing financial interest.

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