Identification of Cyproheptadine as an Inhibitor of SET Domain Containing Lysine Methyltransferase 7/9 (Set7/9) That Regulates Estrogen-Dependent Transcription

Yasushi Takemoto,† Akihiro Ito,*†,‡ Hideaki Niwa,§ Mutsumi Okamura,‖ Takashi Fujiiwara,⊥ Tomoya Hirano,† Noriko Handa,§ Takashi Umehara,§ Takeshi Sonoda,‡ Kenji Ogawa,† Mohammad Tariq,†,†,‖ Norikazu Nishino,○ Shingo Dan,‖ Hiroyuki Kagechika,⊥ Takao Yamori,‖,∞ Shigeyuki Yokoyama,§ and Minoru Yoshida†,‡,#,∇,◆

†Chemical Genetics Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
‡Chemical Genomics Research Group, RIKEN Center for Sustainable Resource Science, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
§RIKEN Systems and Structural Biology Center, 1-7-22 Suehiro-cho, Tsurumi, Yokohama, Kanagawa 230-0045, Japan
‖Division of Molecular Pharmacology, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan
⊥Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University (TMDU), 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan
○Graduate School of Science and Engineering, Saitama University, 645 Shimo-Okubo, Sakura-ku, Saitama 338-8570, Japan
◆CREST Research Project, Japan Agency for Medical Research and Development, 1-7-1 Otemachi, Chiyoda-ku, Tokyo 100-0004, Japan

ABSTRACT: SET domain containing lysine methyltransferase 7/9 (Set7/9), a histone lysine methyltransferase (HMT), also methylates non-histone proteins including estrogen receptor (ER) α. ERα methylation by Set7/9 stabilizes ERα and activates its transcriptional activities, which are involved in the carcinogenesis of breast cancer. We identified cyproheptadine, a clinically approved antiallergy drug, as a Set7/9 inhibitor in a high-throughput screen using a fluorogenic substrate-based HMT assay. Kinetic and X-ray crystallographic analyses revealed that cyproheptadine binds in the substrate-binding pocket of Set7/9 and inhibits its enzymatic activity by competing with the methyl group acceptor. Treatment of human breast cancer cells (MCF7 cells) with cyproheptadine decreased the expression and transcriptional activity of ERα, thereby inhibiting estrogen-dependent cell growth. Our findings suggest that cyproheptadine can be repurposed for breast cancer treatment or used as a starting point for the discovery of an anti-hormone breast cancer drug through lead optimization.

INTRODUCTION

The role of histone lysine methylation in the regulation of transcription has been studied extensively. Reversible histone methylation is catalyzed by the opposing activities of two enzyme families, histone methyltransferases (HMTs) and histone demethylases (HDMs).1,2 HMTs methylate the ε-amino group of lysine residues by using S-(5′-adenosyl)-L-methionine (SAM) as the methyl donor. Recently, lysine methylation has also been found in a number of non-histone proteins, suggesting that reversible methylation regulates not only transcription but also a wide range of cellular processes.3,4 Non-histone protein methylation and demethylation are also catalyzed by HMTs and HDMs, respectively, indicating that these enzymes have broad substrate specificity. To date, several families of HMTs with different substrate specificities have been identified.

SET domain containing lysine methyltransferase 7/9 (Set7/9) was originally reported as a histone H3K4 methyltransferase.5,6

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This enzyme possesses a SET (Su(var)3-9, enhancer-of-zeste, trithorax) domain in its C-terminal region that is responsible for its catalytic activity. Set7/9 also methylates non-histone proteins, including estrogen receptor (ER) $\alpha$, suggesting it has a role in diverse biological processes.

ER$\alpha$ is a nuclear receptor and is one of the ligand-dependent transcriptional factors responsible for estrogen-responsive gene regulation. The transcriptional activity of ER$\alpha$ is regulated by posttranslational modifications such as methylation. Lys302 of ER$\alpha$ is methylated by Set7/9, which stabilizes ER$\alpha$ by blocking...
the ubiquitination of the same lysine residue. Approximately 70% of breast cancers express ERα, and they tend to rely on estrogen signaling for growth. Because the methylation of ERα by Set7/9 contributes to the stabilization and transactivation of ERα, a Set7/9 inhibitor would be a valuable tool not only for investigating the role of Set7/9 in estrogen signaling but also for assessing its potential as a therapeutic target.

Previously, we developed a high-throughput HMT assay system that uses fluorogenic substrates. We found that a peptide from ERα was the best substrate of Set7/9 among the substrates tested. In this study, we screened for inhibitors of Set7/9 using this assay system and found a promising compound that is already an approved drug for allergies.

Figure 2. Crystal structure analysis of the cyproheptadine-bound Set7/9 SET domain. (A) Ribbon diagram of the ternary complex. α-Helices are shown in red, β-strands are cyan, 310-helices are pink, and random coils are gray. The loop preceding the α3 helix is shown in pale cyan. Disordered regions in this loop are represented by dashed lines. The bound SAM and cyproheptadine molecules are represented by stick models, with C atoms in green and magenta, respectively. The O, N, and S atoms in the stick models are shown in red, blue, and orange, respectively. (B) Superimposition of Set7/9 from the Set7/9-SAM-cyproheptadine ternary complex, the Set7/9-SAM complex (PDB code 1N6A), and the Set7/9-SAH-ER peptide complex (PDB code 3CBM). SAH: S-(5′-adenosyl)-L-homocysteine. The three proteins and the bound ER peptide are represented by tube models in red, blue, gray, and yellow, respectively. The orientation is the same as in (A). In the ER peptide, the lysine targeted for methylation by Set7/9, Lys302, is represented by a stick model. The bound SAM or SAH molecules are represented by stick models, with the C atoms in the same color as the proteins. The cyproheptadine molecule is represented by a stick model, as in (A). (C) Simulated annealing omit χcalc(|Fo|−|Fc|) map (2.2σ level) for cyproheptadine. (D) Stereodiagram showing cyproheptadine recognition. The orientation is the same as in (A) and (B). The bound SAM and cyproheptadine molecules and the cyproheptadine-interacting residues are represented by stick models. Set7/9 is represented by a beige tube model. The loop preceding the α3 helix is shown in pale cyan. Hydrogen bonds are indicated by dashed red lines.

RESULTS

Rediscovery of Cyproheptadine as an Inhibitor of Set7/9. By using peptidyl-7-amino-4-methylcoumarin amide (MCA) with a sequence derived from ERα as a fluorogenic substrate, we performed a high-throughput screen for Set7/9 inhibitors in the RIKEN natural products depository (NPDepo) chemical library. We identified cyproheptadine as an inhibitor for Set7/9 (Figure 1A and Supporting Information Figure S1A) but not other HMTs including two histone H3 lysine 9 (H3K9) methyltransferases, euchromatic histone lysine N-methyltransferase 2 (EHMT2), also known as G9a, and suppressor of variegation 3−9 homologue 1 (Suv39H1), the histone H4 lysine 20 (H4K20) methyltransferase, SET domain containing lysine methyltransferase 2.
8 (Set8), also known as PR-Set7, or the histone H3 lysine 79 (H3K79) methyltransferase, DOT1 like histone H3K79 methyltransferase (Dot1L) (Supporting Information Figure S1A, Figure S1B, and Table S1). Cyproheptadine was originally developed as an antagonist of the histamine receptor (H1) and serotonin receptor (5-HT2A), and has been clinically used as an antiallergy drug. We confirmed that cyproheptadine inhibits the enzymatic activity of Set7/9 with a half-maximum inhibitory (IC50) value of 1.0 μM using an amplified luminescent proximity homogeneous assays-linked immunosorbent assay (AlphaLISA)-based assay12 (Figure 1B and Supporting Information Table S1). We also confirmed the in vitro inhibitory activity of cyproheptadine through a Western blot of a reaction in which glutathione S-transferase (GST)-fused histone H3 was used as substrate (Figure 1C).

To gain insight into the mechanism of Set7/9 inhibition by cyproheptadine, we first performed kinetic analyses using the fluorogenic substrate-based HMT assay. The Lineweaver–Burk plot shows that cyproheptadine was noncompetitive with SAM (Figure 1D) but competitive against the peptide substrate (Figure 1E). This suggests that cyproheptadine binds to the substrate-binding site of Set7/9. The Dixon plot revealed that the Ks value of cyproheptadine against Set7/9 was 15 μM (Figure 1F).

Crystal Structure of the Cyproheptadine-Set7/9 Complex. We determined the crystal structure of Set7/9 in complex with SAM and cyproheptadine at 2.0 Å resolution (Figure 2A and Table 1). Except for the C-terminal segment, the overall structure of the Set7/9-cyproheptadine complex is similar to the structure of the Set7/9-SAM complex and other Set7/9-encezyme-peptide ternary complexes (Figure 2B). The bound cyproheptadine molecule was unambiguously identified in the electron density map (Figure 2C). Cyproheptadine binds to the peptide-binding site of Set7/9, which is on the opposite side of the protein as the coenzyme-binding site (Figure 2D).7,14

Superimposition of this structure on the Set7/9-SAM-ER peptide structure revealed that the residues composing the lysine access channel adopt approximately the same conformations except for Tyr337 (Supporting Information Figure S2A and Figure S2B). The methylpiperidine ring of cyproheptadine is accommodated within the lysine access channel by the formation of hydrogen bonds and several hydrophobic and van der Waals interactions (Figure 2D). The main-chain carbonyl oxygen of Thr266 forms a hydrogen bond with the methylpiperidine ring nitrogen (2.7 Å). The hydroxyl groups of Tyr245 and Tyr305 form weak hydrogen bonding (O−H−C) with the methyl group of the methylpiperidine ring (3.5 and 3.1 Å, respectively). The aromatic ring of Tyr335 and the main chain of Asn263 and Gly264 contact the methylpiperidine ring. These interactions mimic some of the recognition mechanisms of the lysine targeted for methylation in the peptide-bound structures (Supporting Information Figure S2A and Figure S2B).

Tyr337, a component of the lysine access channel, is absent in the electron density map of the cyproheptadine complex, suggesting that it is flexible. The cyproheptadine dibenzocycloheptene ring is located at the equivalent position of the Tyr337 aromatic ring in the peptide-bound structures (Supporting Information Figure S2A and Figure S2B). The electron density of residues 338−349, located just before the C-terminal α helix (α3), is weak, and only the main chains of six residues are visible (Figure 2A). This region forms a loop structure and contacts the tricyclic dibenzocycloheptene ring of cyproheptadine (Figure 2D). The side chain of Trp260 is rotated by approximately 180° as compared to the peptide-bound structures, within which it packs against Arg or Lys at the (−2) position of the peptide via hydrophobic interactions, creating space for the loop and dibenzocycloheptene ring (Supporting Information Figure S2A and Figure S2B). The movement of Tyr337 and Trp260 results in the formation of a relatively large hole adjacent to the narrow lysine access channel (Supporting Information Figure S2C and Figure S2D).

The conformational change of residues 337−349 may explain the half-turn shift of the main chain of the C-terminal helix α3 toward the C-terminus (Figure 2B); it prevents Pro350 from sterically hindering cyproheptadine binding (Supporting Information Figure S2A and Figure S2B). Furthermore, it causes the partial occlusion of the peptide-binding groove, in that the main chain of the loop passes through the equivalent position of Arg or Lys at the (−2) position of the bound peptide (Supporting Information Figure S2C).

| Table 1. Crystallographic Data Collection and Refinement Statistics |
|-------------------------|-------------------------|
|                         | Set7/9-SAM-cyproheptadine |
| Data Collection         |                         |
| space group             | P2₁,2,2                  |
| cell dimensions         |                         |
| a, b, c (Å)             | 102.46, 39.37, 67.28     |
| resolution (Å)          | 50−2.0 (2.07−2.0)        |
| Rsym                    | 0.115 (0.487)            |
| I/σ(I)                  | 17.6 (4.2)               |
| completeness (%)         | 99.9 (100.0)             |
| redundancy               | 7.2 (7.3)                |
|                         |                         |
| Refinement              |                         |
| resolution (Å)          | 2.01                    |
| no. reflections         | 18,856                  |
| Rwork/Rfree             | 0.194/0.258             |
| no. atoms               |                         |
| protein                 | 1899                    |
| SAM                     | 27                      |
| cyproheptadine          | 23                      |
| Tris                    | 7                       |
| water                   | 179                     |
| B-factors               |                         |
| protein                 | 33.1                    |
| SAM                     | 36.0                    |
| cyproheptadine          | 42.3                    |
| Tris                    | 34.2                    |
| water                   | 36.6                    |
| rms deviations          |                         |
| bond lengths (Å)        | 0.008                   |
| bond angles (deg)       | 1.085                   |
| Ramachandran plot (%)   |                         |
| favored                 | 97.1                    |
| allowed                 | 2.9                     |
| disallowed              | 0                       |

*Values in parentheses are for highest resolution shell.*
the N-substituted group, that is, from a methyl to ethyl group. As shown in the cocrystal structure (Figure 2D), the space around the N-methyl group was relatively small, so this would be due to steric hindrance. These results suggest that the structural requirement of N-methyl group on the piperidine moiety of cyproheptadine would be relatively high for the inhibition of the enzymatic activity of Set7/9.

Destabilization of ERα in Cyproheptadine-Treated MCF7 Cells. Set7/9-mediated methylation of Lys302 of ERα is involved in the stabilization of ERα. We therefore examined the effect of cyproheptadine on the expression of ERα in human breast cancer cells expressing endogenous ERα (MCF7 cells). As shown in Figure 3A−C, cyproheptadine, as well as Set7/9 knockdown, reduced the amount of ERα in a dose- and time-dependent manner. The level of ERα mRNA expression was almost identical between control and cyproheptadine-treated MCF7 cells (Figure 3D). In agreement with a previous report, these results suggest that the inhibition of Set7/9 by cyproheptadine destabilizes ERα.

Next, we determined the half-life of ERα in cyproheptadine-treated MCF7 cells by using cycloheximide (CHX), an inhibitor of protein synthesis. The half-life of ERα in the absence of cyproheptadine was 15.6 ± 1.2 h, whereas it was 10.3 ± 1.0 h in drug-treated cells (Figure 3E), indicating that treatment with cyproheptadine accelerated the degradation of ERα.

The reduced ERα expression in the presence of cyproheptadine was restored by treatment with Z-Leu-Leu-Leu-al (MG132), a proteasome inhibitor (Figure 3F). Taken together, we conclude that cyproheptadine induces the destabilization of ERα through enhanced degradation of ERα by the proteasome.

Cyproheptadine is an antagonist to both H1 and 5-HT2A. To rule out the possibility that the destabilization of ERα was due to cyproheptadine’s other antagonistic activities, we examined the effect of structurally unrelated antagonists on ERα expression. Treatment with triprolidine, a histamine antagonist (Supporting Information Figure S3A), or ketanserin, a serotonin antagonist (Supporting Information Figure S3B), did not affect the expression of ERα in MCF7 cells (Supporting Information Figure S3C and Figure S3D). Consistent with this, both antagonists did not inhibit Set7/9 (Supporting Information Figure S3E). These results demonstrate that cyproheptadine promotes ERα degradation through a mechanism distinct from its activity against H1 and 5-HT2A.

Suppression of Estrogen-Induced Transcriptional Activation and Cell Viability by Cyproheptadine in MCF7 Cells. ERα regulates the transcription of specific target genes, such as pS2, by binding to the cognate estrogen responsive element (ERE) in response to estrogen. Because the expression of ERα was decreased in cyproheptadine-treated MCF7 cells (Figure 3), we examined whether cyproheptadine affected estrogen-induced transcriptional activation. Treatment with cyproheptadine for 48 h, as well as tamoxifen, an antagonist of ERα, inhibited ERE-dependent promoter activation and the increase of pS2 mRNA expression normally induced by β-estradiol (Figure 4A and Figure 4B). Moreover, cyproheptadine inhibited estrogen-dependent cell viability in MCF7 cells (Figure 5A), having a dose dependency similar to its inhibition of ERα protein expression and ERα-dependent transcription (Figures 3A and 4A). In conclusion, the ability of cyproheptadine to inhibit in vitro enzymatic activity coincided with its ability to suppress estrogen signaling in cells. Finally, we tested the in vivo antitumor activity of cyproheptadine by mouse xenograft model using fluorescent ubiquitination-based cell cycle indicator (Fucci) introduced human breast cancer MCF7 cells, as Fucci-MCF7 cell lines xenografted in nude mice can grow.19 Both single and continuous intraperitoneal administrations of cyproheptadine significantly inhibited the growth of Fucci-MCF7 tumors in nude mice (Figure 5B). Although continuous intraperitoneal administration of cyproheptadine slightly but significantly affected the body weights of xenografted mice, this decrease was recovered after continuous treatment with

### Table 2. SAR Analysis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cyproheptadine</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (µM)</td>
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</tr>
<tr>
<td>2</td>
<td>7.2</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
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<td>4</td>
<td>&gt;100</td>
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cyproheptadine for 8 days (Figure 5C). In addition, single intra-peritoneal administration of cyproheptadine decreased tumor volumes without affecting the body weights of xenografted mice. These observations suggest that the effect of cyproheptadine on inhibition of tumor growth in xenografted mice is distinct from its potential toxicity.

**DISCUSSION**

Recent proteomic studies have revealed a large number of non-histone substrates for Set7/9. Many of them, including ERα, are involved in carcinogenesis, suggesting that the modulation of Set7/9 activity could be a new strategy for breast cancer treatment. However, pharmacological evidence for the adequacy of Set7/9 as a target for therapy remains limited. A potent and specific Set7/9 inhibitor that is active in cells and tissues is necessary for initial proof-of-concept studies.

To date, several compounds that inhibit Set7/9 activity have been reported. The first small-molecule Set7/9 inhibitors were synthesized as SAM analogues, and their inhibitor-bound structures were subsequently elucidated. Recent high-throughput or virtual screening has identified several more inhibitory compounds, and the X-ray structure of Set7/9 in complex with an inhibitor has been reported. However, the cellular effectiveness or selectivity of most of these existing inhibitors is not well understood.

In this study, we found that cyproheptadine strongly inhibited in vitro Set7/9 activity, whereas four other HTMs did not (Figure 1 and Supporting Information Figure S1). We elucidated its unique mechanism of Set7/9 inhibition through structural analyses at the atomic level (Figure 2). Cyproheptadine generates a hole adjacent to the lysine access channel by inducing conformational changes in the amino acid residues important for

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**Figure 3.** Destabilization of ERα in cyproheptadine-treated MCF7 cells. (A, B) Effect of cyproheptadine on the expression of ERα protein in MCF7 cells. MCF7 cells were treated with various concentrations of cyproheptadine for 48 h (A) or 43 μM cyproheptadine for the indicated times (B). The lysates were immunoblotted with the indicated antibodies. (C) Effect of Set7/9 knockdown on the expression of ERα in MCF7 cells. The lysates from cells treated with various concentrations of siRNA oligos targeting Set7/9 mRNA were immunoblotted with the indicated antibodies. (D) Effect of cyproheptadine on the mRNA expression of ERα in MCF7 cells. The levels of ERα and GAPDH mRNA from cells treated with and without cyproheptadine for 48 h were measured by semiquantitative reverse transcription (RT)-PCR. (E) Effect of cyproheptadine on the protein stability of ERα in MCF7 cells. MCF7 cells were treated with and without 43 μM cyproheptadine in the presence of 1 μg/mL CHX for the indicated times. The lysates were immunoblotted as described in (A), and band intensity was measured. The error bars represent the standard deviation of three independent assays. The Student’s t test was applied to determine significance of differences between two groups: (+) P < 0.05. (F) Effect of MG132 on the cyproheptadine-induced destabilization of ERα in MCF7 cells. MCF7 cells were treated with and without 43 μM cyproheptadine for 36 h, followed by incubation with MG132 for 12 h. The lysates were immunoblotted as described in (A).
channel formation. In addition, cyproheptadine binding occludes the substrate-binding groove by inducing conformational changes in the C-terminal region. Cyproheptadine appears to access the catalytic center of Set7/9 from the substrate-binding side and interacted with the catalytic residues through a unique binding mechanism. We also demonstrated that cyproheptadine was active in cells and that it reduced the expression of ERα due to enhanced degradation of ERα by the proteasome, similar to Set7/9 knockdown (Figure 3). Although cyproheptadine is a known antagonist of H1 and 5-HT2A, the effect of cyproheptadine on ERα activity was independent of these receptors (Supporting Information Figure S3). Thus, cyproheptadine could be a valuable small-molecule tool for exploring Set7/9 activity.

Hormone therapy is the treatment most often used for ERα-positive breast cancer. Although tamoxifen is the most widely used anti-estrogen medication, recent data indicate that the efficacy of tamoxifen treatment depends on cytochrome P450 2D6 (CYP2D6) polymorphisms. Moreover, ERα-positive breast cancers can become resistant to anti-estrogens during treatment, though they retain ERα expression. An anti-estrogen treatment with a mechanism different from tamoxifen could be clinically useful.

In this regard, Set7/9 is a promising target because it is necessary for the estrogen-dependent transactivation of ER target genes. Indeed, we found that cyproheptadine blocked both estrogen signaling and estrogen-dependent breast cancer cell growth (Figures 4 and 5). We demonstrated for the first time that cyproheptadine is a Set7/9 inhibitor that is active in cells, and it will be useful for investigating the physiological role of Set7/9 in non-histone protein lysine methylation and the pathogenesis of breast cancer. Because cyproheptadine is an approved drug, it could be repurposed for breast cancer treatment. Indeed, the mouse xenograft model revealed that cyproheptadine inhibited in vivo breast cancer growth (Figure 5B). In addition, the derivatezitation of cyproheptadine coupled with structure-based analysis of enzyme inhibition including our SAR analyses (Table 2) may provide valuable information for lead optimization with cyproheptadine as a starting point.

**EXPERIMENTAL SECTION**

**Materials, Plasmids, Cell Lines.** Commercially available test compound, cyproheptadine, was purchased from Sigma-Aldrich (St. Louis, MO, USA). Commercially available test compound, tamoxifen, was purchased from.sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal ERα antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Rab[t polyclonal Set7/9 antibody and rabbit polyclonal GST antibody were purchased from Cell Signaling Technology (Danvers, MA, USA).

**Bacterial Protein Expression and Purification.** Bacterial Protein Expression and Purification. pGEX4T-3-histone H3 (1–57 a.a.), pET-28a(+)-Set7/9, pGEX4T-1-mG9a (706–stop a.a.), pGEX4T-3-mSuv39h1-H320R (74–412 a.a.), and pGEX4T-3-histone H3 (1–57 a.a.) were previously described. MCF7 cells and Fucci-MCF7 cells were kindly gifted by Dr. S. Saji and Dr. A. Miyawaki, respectively.

**In Vitro HMT Assays.** An AlphaLisa enzymatic assay was performed as described elsewhere. Briefly, recombinant Set7/9 proteins were incubated with a biotinylated histone H3-derived peptide (final concentration 50 nM) and SAM (final concentration 400 nM) in 10 μL of assay buffer (50 mM Tris-HCl [pH 8.8], 0.01% Tween-20, 5 mM MgCl2, 1 mM DTT). After 60 min at room temperature, anti-H3K4me1-2 acceptor beads (final concentration 20 μg/mL) and streptavidin donor beads (final concentration 20 μg/mL) were added and incubated for an additional 30 min at room temperature before detecting the signal with an EnSpirre Alpha plate reader (PerkinElmer, Waltham, MA, USA). For measuring in vitro HMT activities using Western blotting, His-Set7/9 (0.2 μg/μL) was pretreated in the presence or absence of cyproheptadine in HMT buffer for 1 h. Next, 1 mM SAM and 0.01 μg/μL GST-fused histone H3 (1–57 a.a.) were added to the reaction mixture and further incubated for 1 h at 37 °C. Loading buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 0.1% bromophenol blue) was added to the reaction mixtures before SDS-PAGE. Western blotting was performed with rabbit polyclonal His-Tag antibody (Medical & Biological Laboratories, Nagoya, Japan), rabbit polyclonal Set7/9 antibody (Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal His-Tag antibody (Medical & Biological Laboratories, Nagoya, Japan), rabbit polyclonal His-Tag antibody (Cell Signaling Technology, Danvers, MA, USA), and rabbit polyclonal GST antibody (Active Motif, Carlsbad, CA, USA).

**Article**

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Figure 4. Suppression of estrogen-induced transcriptional activation by cyproheptadine in MCF7 cells. (A) Effect of cyproheptadine on estrogen-induced ERE promoter activity in MCF7 cells. The error bars represent the standard deviation of four independent assays. (B) Effect of cyproheptadine on estrogen-induced pS2 mRNA expression in MCF7 cells. MCF7 cells grown in EFM were treated with cyproheptadine or tamoxifen for 24 h and further incubated with 10 nM β-estradiol in the presence of cyproheptadine or tamoxifen for 24 h. The levels of pS2 and GAPDH mRNA were measured by semiquantitative RT-PCR.
blue, 10% glycerol, and 5% 2-mercaptoethanol) was added to each sample, which was subsequently boiled for 3 min. The level of histone H3K4 methylation was detected as described below. The fluorogenic substrate-based HMT assay and the ELISA-based HMT assay were described previously.10,28,29

Crystal Structure Analysis. The human Set7/9 (residues 111−366) with an N-terminal artificial Gly-Ser-Ser-Gly-Ser-Ser-Gly sequence used for crystal structure analysis was synthesized and purified as described previously.22 The Set7/9-SAM complex was crystallized under the conditions described previously.22 Specifically, the crystals were grown by the hanging-drop method at 277 K with a reservoir solution containing 0.1 M Tris-HCl (pH 8.5) and 31% PEG 6000. The Set7/9-SAM-cyproheptadine ternary complex was formed by soaking the crystals with 3 mM cyproheptadine and 2.5% dimethyl sulfoxide (DMSO) (final concentration) for 24 h at 4 °C. The crystals were cryoprotected in well solution containing 20% glycerol and flash-cooled in liquid nitrogen. A 2.0 Å resolution data set was collected at the AR-NW12A beamline at the Photon Factory (Tsukuba, Japan). The crystals belonged to space group \(P_2_12_12\) with unit cell parameters \(a = 102.46 \text{ Å}, b = 3937 \text{ Å}, c = 67.28 \text{ Å}\). The data were processed using the HKL-2000 program.30 Molecular replacement was performed with Phaser, using the Set7/9-SAM complex (PDB code 1N6A)13 as the search model. Models were built with Coot and refined with PHENIX.33 The data-processing and refinement statistics are summarized in Table 1. The models in the figures were drawn using PyMOL (DeLano Scientific, Palo Alto, CA, USA).

The crystalline structure factors of the Set7/9-SAM-cyproheptadine complex have been deposited in Protein Data Bank, with the accession code 5AYF.

Organic Chemistry. General. All reagents were purchased from Sigma-Aldrich Chemical, Tokyo Kasei Kogyo, Wako Pure Chemical Industries, and Kanto Kagaku. Silica gel for column chromatography was purchased from Kanto Kagaku. NMR spectra were recorded on Bruker AVANCE 400 or Bruker AVANCE 500 spectrometers. Mass spectral data were obtained on Bruker Daltonics microTOF-2focus in the positive and negative ion detection modes. Melting points were taken on a Yanagimoto micro melting point apparatus and are uncorrected. The purity of newly synthesized compound was determined to be >95%, as determined by HPLC (reversed-phase column (Mightysil RP-18 GP 250-4.6) on JASCO UV-2077 and PU-2089) analysis.

Synthesis of 2. Ethyl bromide (10 μL, 0.13 mmol) was added to solution of 1 (30 mg, 0.11 mmol) and potassium carbonate (77 mg, 0.55 mmol) in acetonitrile (3 mL); then the reaction mixture was stirred for 24 h at room temperature. The mixture was poured into water and extracted with dichloromethane. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography. The product was treated with 6 M hydrochloric acid,
and 2 was obtained as hydrochloride salt (31 mg, 83%). 1H NMR (CDCl3, 400 MHz) δ 7.26–7.38 (m, 6H), δ 7.16–7.18 (m, 2H), δ 6.91 (s, 2H), 3.47 (br, 2H), 3.13 (br, 2H), 2.78 (br, 2H), 2.64 (br, 2H), 2.29 (br, 2H), 1.80 (br, 2H), 1.27 (br, 6H), 0.87 (br, 3H); 13C NMR (CDCl3, 125 MHz) δ 137.5, 134.5, 131.0, 128.8, 126.9, 52.5, 51.9, 26.4, 9.1; mp (CHCl3, hexane) 69.9–71.7°C; HRMS (ESI) calcld for C23H22N2O3 [M + H]+ 320.1500, found 320.1499.

Synthesis of 3. 3 was similarly prepared from 1 (33 mg, 0.12 mmol) and n-hexyl bromide (20 μL, 0.14 mmol). 3 was obtained as hydrochloride salt (47 mg, 91%). 1H NMR (CDCl3, 400 MHz) δ 7.26–7.37 (m, 6H), 7.16–7.18 (m, 2H), 6.91 (s, 2H), 3.47 (br, 2H), 3.13 (br, 2H), 2.78 (br, 2H), 2.64 (br, 2H), 2.29 (br, 2H), 1.80 (br, 2H), 1.27 (br, 6H), 0.87 (br, 3H); 13C NMR (CDCl3, 125 MHz) δ 137.5, 134.5, 131.1, 129.1, 128.5, 126.9, 57.3, 53.4, 31.0, 26.5, 26.2, 22.8, 13.8; mp (AcOEt, disopropyl ether) 169.7–171.2°C; HRMS (ESI) calcld for C22H21NO3N [M + Na]+ 358.1535, found 358.1528.

Synthesis of 4. Acetyl chloride (15 μL, 0.21 mmol) was added to a solution of 1 (42 mg, 0.14 mmol) and triethylamine (a few drops) in acetonitrile (3 mL). The reaction mixture was stirred for 6 h. The mixture was poured into water and extracted with dichloromethane. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography. 4 (44 mg, quant) was obtained as brown solid. 1H NMR (CDCl3, 400 MHz) δ 7.17–7.34 (m, 4H), 7.16–7.18 (m, 2H), 6.92 (s, 2H), 3.95 (m, 1H), 3.53–3.49 (m, 1H), 3.13–2.98 (m, 2H), 2.33–2.11 (m, 2H), 2.06 (s, 3H); 13C NMR (CDCl3, 125 MHz) δ 168.8, 138.6, 138.5, 135.2, 134.7, 134.6, 133.8, 131.1, 130.8, 128.3, 128.1, 128.0, 126.5, 116.1, 47.6, 42.9, 30.5, 29.6, 21.5; HRMS (ESI) calcld for C25H21NO6 [M + H]+ 392.1618, found 392.1618.

Cell Culture and siRNA Treatment. MCF7 cells were maintained in Roswell Park Memorial Institute (RPMI) supplemented with 10% fetal bovine serum (FBS). For estrogen-stimulation experiments, cells were incubated in estrogen-free medium (FSM). Medium was changed daily. Immobiloblotting was performed as described. Cells were lysed by sonication in RIPA buffer (25 mM HEPES [pH 7.8], 500 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 50 mM sodium fluoride (NaF), 0.1 mM sodium vanadate, 1.5% TritonX-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)) containing a protease inhibitor mixture. The lyses were centrifuged at 15 000 g for 15 min, and the concentration of the protein in each lystate was determined. Lyses were mixed with loading buffer and boiled for 3 min. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) by electroblotting. After the membranes were incubated with primary and secondary antibodies, proteins were detected using an Immobilon Western kit (Millipore, Billerica, MA, USA), and the luminescence was analyzed with a LAS-4000 image analyzer (Fujifilm, Tokyo, Japan).

RT-PCR. Total RNA was isolated from cells with TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Total RNA (2 μg) was reverse-transcribed with 0.6 μg of oligo(dT) primer using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA) at 42°C for 1 h. The synthesized first-strand cDNA was analyzed by PCR. The sequences of primers amplifying ERα, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and pS2 are as follows: ERα, 5′-ATGGACCCATGACCCTCCACG-3′ (forward) and 5′-TCGACC-GTGCCCAGGGGAAAAC-3′ (reverse); GAPDH, 5′-ATGGGGA-AAGTGAAGGTCGG-3′ (forward) and 5′-TTATCCTTTGGAGGCCATGT-3′ (reverse); pS2, 5′-ATGGCCACCATGGGAAAAC-3′ (forward) and 5′-CTAAAAATTCACACTCCTTCT-3′ (reverse).

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**Reporter Assay.** MCF7 cells (1.5 × 10⁵) were seeded onto a 24-well plate and incubated for 24 h. Then, the cells were washed with phosphate buffered saline (PBS), and the medium was replaced with EFM. After 3 days, the cells were transfected with pGL4.3×ERE-TA and hRuc-TK using the Lipofectamine 2000 reagent. Following incubation for 6 h, tamoxifen or cyproheptadine was added. After 18 h, the cells were treated with or without 10 nM β-estradiol for 24 h. Luciferase activity was measured using the dual-luciferase assay. The fold increase in the firefly luciferase activity over that of unstimulated cells was determined after normalization to Renilla luciferase activity.

**Estrogen-Dependent Cell Growth Assay.** MCF7 cells (1.5 × 10⁴) were seeded onto a 24-well plate. After 24 h, the cells were washed with PBS, and the medium was replaced with EFM. Then, the cells were treated with tamoxifen or cyproheptadine in the presence or the absence of 10 nM β-estradiol and incubated for 4 days. The cell number was counted using a hematocytometer.

**Xenograft Model.** Animal care and treatment were performed in accordance with the guidelines of the Animal Use and Care Committee of the Japanese Foundation for Cancer Research and conformed to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Female nude mice (Balb/C-nu/nu) aged 7 weeks were purchased from Charles River Laboratories (Wilmington, MA, USA). Approximately 2 × 10⁴ Fucci-MCF7 cells were inoculated in the mammary gland of each mouse. When tumor volume reached approximately 100 mm³ 13 days after inoculation, cyproheptadine dissolved in saline containing 10% ethanol was intraperitoneally administered once a day for 8 days from day 1 to day 8 at 20 mg/kg to one group, whereas the vehicle was delivered to the other group at same duration and route. In addition, cyproheptadine was intraperitoneally administered once at day 1 at 20 mg/kg to the third group. Tumor diameters were measured with a caliper, and tumor volume (mm³) was calculated as (a × b × b)/2, where a is the width (smallest diameter) and b is the length (largest diameter). Body weight and tumor volume were recorded every 3 or 4 days until day 15 after drug administration.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b01732.

Additional figures and table illustrating the inhibition data of compounds, and crystal structure analysis (PDF)

**Accession Codes**

PDB code for Set7/9-SAM-cyproheptadine complex is 5AVF.

**AUTHOR INFORMATION**

**Corresponding Author**

*Phone: 81-48-467-9518. E-mail: akihiro-i@iken.jp.*

**Present Address**

*T.: Center for Product Evaluation, Pharmaceuticals and Medical Device Agency, Shin-Kasumigaseki Building, 3-3-2 Kasumigaseki, Chiyodaku-ku, Tokyo 100-0013, Japan.*

**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS USED**

S-HT2A, serotonin receptor 2A receptor; AlphaLISA, amplified luminescent proximity homogeneous assays-linked immunosorbent assay; CHX, cycloheximide; CYP2D6, cytochrome P450 2D6; Dot1L, DOT1 like histone H3K79 methyltransferase; EDTA, ethylenediaminetetraacetic acid; EFM, estrogen-free medium; EHMT2, euchromatic histone lysine N-methyltransferase 2; ER, estrogen receptor; ERE, estrogen responsive element; EDTA, ethylenediaminetetraacetic acid; EFM, estrogen-free medium; 5-HT2A, serotonin receptor 2A receptor; AlphaLISA, amplification-based assay; ADP-ribosylation factor 1 (ARF1), GTPase; GTP, guanosine triphosphate; Ub, ubiquitin; MCA, 7-amino-4-methylcoumarin amide; Ni²⁺, nickel; PD, phosphate buffer; PBS, phosphate buffered saline; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; Fucci, fluorescent ubiquitination-based cell cycle indicator; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione-S-transferase; H4K20, histone H4 lysine 20; HDM, histone H1 receptor; H3K9, histone H3 lysine 9; H3K79, histone H3 lysine 79; H4K2, histone H4 lysine 20; DIM, dimethyl sulfoxide; DNA, deoxyribonucleic acid; doxorubicin, 1,4-dihydroxy-2-naphthoic acid; ER, estrogen receptor; ERE, estrogen responsive element; EDTA, ethylenediaminetetraacetic acid; EFM, estrogen-free medium; 5-HT2A, serotonin receptor 2A receptor; AlphaLISA, amplification-based assay; ADP-ribosylation factor 1 (ARF1), GTPase; GTP, guanosine triphosphate; Ub, ubiquitin; MCA, 7-amino-4-methylcoumarin amide; Ni²⁺, nickel; PD, phosphate buffer; PBS, phosphate buffered saline; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; Fucci, fluorescent ubiquitination-based cell cycle indicator; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione-S-transferase; H4K20, histone H4 lysine 20; HDM, histone H1 receptor; H3K9, histone H3 lysine 9; H3K79, histone H3 lysine 79; H4K20, histone H4 lysine 20; HDM, histone demethylase; HMT, histone lysine methyltransferase; IC₅₀, half-maximum inhibitory concentration; IPTG, isopropyl-β-D-galactopyranoside; MCA, 7-aminomethylcoumarin amide; M-MLV, Moloney murine leukemia virus; NaF, sodium fluoride; Ni²⁺, nickel; NPDepo, natural products depository; PBS, phosphate buffered saline; RPMI, Roswell Park Memorial Institute; PVDF, polyvinylidene difluoride; RNAi, RNA interference; SAM, S-adenosylmethionine; SAR, structure-activity relationship; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SET, Su(var)-3-9, enhancer-of-zeste, trithorax; SET7/9, SET domain containing lysine methyltransferase 7/9; SET8, SET domain containing lysine methyltransferase 8; siRNA, small interfering RNA; Suv39H1, suppressor of variegation 3–9 homologue 1

**REFERENCES**


