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Xenoestrogens Regulate the Activity of Arginine Methyltransferases

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Abstract

Arginine methylation is a common posttranslational modification that has been strongly implicated in transcriptional regulation. The arginine methyltransferases (PRMTs) were first reported as transcriptional coactivators for the estrogen and androgen receptors. Compounds that inhibit these enzymes will provide us with valuable tools for dissecting the roles of these enzymes in cells, and will possibly also have therapeutic applications. In order to identify such inhibitors of the PRMTs, we performed a high throughput screen using a small molecule library a number of years ago. We termed these compounds AMIs (arginine methyltransferase inhibitors). The majority of these inhibitors were polyphenols, and one in particular (AMI-18) shared additional features with a group of known xenoestrogens. We thus tested a panel of xenoestrogens and found that a number of them possess the ability to inhibit PRMT activity *in vitro*. These inhibitors primarily target CARM1, and include licochalcone A, kepone, benzyl 4-hydroxybenzoate, and tamoxifen. We developed a cell-based reporter system for CARM1 activity, and showed that tamoxifen (IC₅₀=30 μM) inhibits this PRMT. The ability of these compounds to regulate the activity of transcriptional coactivators may be an unappreciated mechanism of action for xenoestrogens and may also explain the efficacy of high-dose tamoxifen treatment on estrogen receptor negative cancers.

Keywords

PRMT1; CARM1; Arginine methylation; Xenoestrogens

INTRODUCTION

The regulation of transcription by the estrogen receptor is coordinated by a host of coactivators and corepressors, which are recruited to chromatin by the receptor.^[1] These coregulators impact the local levels of lysine acetylation, lysine methylation and arginine methylation. Arginine methylation is catalyzed by the arginine methyltransferases (PRMTs). The link between PRMTs and transcriptional regulation was first realized with the finding that the coactivator associated arginine methyltransferase (CARM1/PRMT4) binds the p160 family of nuclear hormone receptor coactivators. The presence of CARM1 enhances transcriptional activation by nuclear receptors, possibly as a result of the specific methylation of histone H3 by CARM1.^[2] PRMT1, like CARM1, can also bind p160, and these two PRMTs have been shown to act in a synergistic manner to enhance reporter gene activation by nuclear receptors.^[3] The arginine methylation of histone H4 by PRMT1 facilitates the subsequent acetylation of H4 tails by p300.^[4] Thus, both the CARM1 deposited mark (H3R17me2a) and the PRMT1 deposited mark (H4R3me2a) are associated

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with transcriptional activation.^[5] Recently, PRMT6 was also demonstrated to be a coactivator of the estrogen receptor.^[6] Importantly, all three of the PRMTs (1, 4 & 6) that function as estrogen receptor coactivators, also display elevated expression levels in tumors.^[7, 8]

It has been proposed that the PRMTs could be therapeutic targets, and a number of efforts are underway to identify small molecule inhibitors that target this enzyme family.^[9–18] Some years ago, our group performed the first high-throughput screen for small molecule inhibitors of PRMT1.^[19] We initially characterized nine compounds from this screen, and these arginine methyltransferase inhibitors were termed AMIs. We recently started reevaluating compounds that were identified in this screen. One of these compounds (AMI-18) shares a number of structural features with chalcones.^[20] This prompted us to investigate the ability of chalcone itself, licochalcone A, and other xenoestrogens to inhibit PRMT activity.

A diverse collection of non-steroidal environmental chemicals interact with the estrogen receptor and either positively or negatively regulate transcriptional transactivation by this receptor. Most of these xenoestrogens have low affinity for the estrogen receptor (ER) and rather weak estrogenic activity.^[21, 22] These compounds interact with the binding pocket of the ER because they usually harbor phenolic rings and are, to varying degrees, structurally similar to estradiol.^[23] Xenoestrogens include estrogenic, anti-estrogenic and selective estrogen receptor modulator (SERM)-like compounds.^[24] The ability of different compounds to bind the ER and result in either activation or inactivation of this pathway has been explained by the selective receptor modulator (SRM) hypothesis.^[25, 26] The SRM hypothesis proposes that in the presence of agonists, nuclear receptors take on an active conformation and interact with coactivators.^[27] In the presence of antagonists, receptors fold into an inactive conformation that recruits corepressor activities. In the presence of SERMs, the receptor adopts an intermediate structure that has the ability to interact with either coactivators or corepressors, which may depend on the cellular environment including the local concentrations of these coregulators. Indeed, structural data of an ER/tamoxifen complex shows that this SERM causes a structural change that blocks the coactivator-docking site.^[28] It has been proposed that coactivators themselves may be good targets for drug development.^[29] The ability of xenoestrogens to bind the estrogen receptor is well documented. There is, however, a growing body of evidence suggesting that a subset of these compounds may possess additional biological targets.^[20, 30]

In this study, we find that a subset of xenoestrogens could indeed inhibit PRMT activity *in vitro*. We developed a methyl-specific antibody in conjunction with a cell-based reporter assay, and show that licochalcone A, benzyl 4-hydroxybenzoate, tamoxifen and 4-hydroxytamoxifen are able to attenuate CARM1 activity in cells. This raises the possibility that certain xenoestrogens may regulate the estrogen receptor pathway indirectly by targeting transcriptional coactivators.

RESULTS

Licochalcone A is a PRMT inhibitor

In a past study, we performed a small molecule screen for compounds that inhibit the activity of PRMT1.^[19] A reanalysis of this screen resulted in the identification of a chalcone-like compound (AMI-18) as a PRMT1 inhibitor (Figure 1A). This raises the possibility that other chalcone-like compounds may inhibit PRMT enzymatic activity. We thus tested the ability of chalcone and licochalcone A, a flavonoid isolated from liquorice root, to inhibit three different PRMTs (PRMT1, PRMT3 and CARM1) in an *in vitro* reaction. Chalcone does not inhibit PRMT activity, but licochalcone A does (Figure 1B).

Importantly, the ability of licochalcone A to inhibit the three PRMTs is relatively specific, because it displays no activity against SET7/9, which is a lysine methyltransferase. Chalcones have been reported to have an array of biological activities, including the ability to impact the estrogen receptor pathway, thus functioning as a xenoestrogen.^[31, 32]

An array of xenoestrogens possess PRMT inhibitor activity

To expand on the observation that chalcone-like compounds have PRMT inhibitor functions, we investigated the ability of different xenoestrogens to impact the activity of a panel of protein methyltransferases *in vitro*. The enzymes included in the screen were PRMT1, PRMT3, PRMT6, CARM1 and SET7/9. In addition, PRMT1, PRMT3 and CARM1 activity was assayed against two different substrates. Most of the tested compounds displayed minimal activity against the lysine methyltransferase, SET7/9. In contrast, the *in vitro* activity of a number of PRMTs was impacted by a subset of these compounds (Figure 2A). The results of this comparative activity screen are summarized in Figure 2B, and we see that the chalcone-like compounds, AMI-18 and licochalcone A (Lico) are rather broad-spectrum PRMT inhibitors. Whereas, tamoxifen (Tam), kepone (Kep), benzyl 4-hydroxybenzoate (B4H), raloxifene (Ral), and 4-hydroxytamoxifen (4OH) all displayed a higher degree of specificity. For further analysis of enzymatic specificity of these xenoestrogens, we focused on a set of six compounds (Lico, Tam, Kep, B4H, Ral & 4OH). The structures of all these compounds are shown in Figure S1.

A subset of xenostrogens inhibit CARM1 activity *in vitro*

Next, we investigated the ability of Lico, Tam, Kep, B4H, Ral, and 4OH to inhibit the activity of various PRMTs (PRMT1, RMT3, PRMT5, PRMT6 & CARM1) on an expanded set of substrates. In these experiments the inhibitor activity of each compound was accurately quantified by cutting out the radio-labeled band (Figure 3, left panel) and counting the tritium signal in each reaction (Figure 3, right panel). In this case, a fix concentration of 200 μ M of each compound was used in the *in vitro* methylation reaction. Again, we saw that licochalcone A is broad-spectrum PRMT inhibitors. We tested four different CARM1 substrates (PABP1, CA150, SMB and histone H3), two PRMT1 substrates (NPL3 and histone H4), two PRMT3 substrates (NPL3 and rpS2), two PRMT5 substrates (MBP and histone H4), and two PRMT6 substrates (NPL3 and histone H3). Under these conditions, CARM1 displayed the highest sensitivity to the compounds tested, with Lico, Tam, and 4OH showing inhibitor activation on all four substrates tested. Interestingly, the histone H3 methylation by CARM1 was most resistant to inhibition by these compounds. The *in vitro* methylation of the ribosomal protein rpS2 by PRMT3 was also blocked by this panel of compounds. Using the same approach, these six compounds display minimal inhibitor activity when tested against a panel of lysine methyltransferase, which includes Set7/9, DOTL1, Suv39H1 and G9a (Figure S2).

The IC₅₀ (the concentration of compound required to reduce the enzyme activity by 50%) value for the compound against CARM1 was established (Table 1). The three most potent CARM1 inhibitors in the *in vitro* assay (Figure 3 - top panel) - Lico, Tam, and 4OH - display IC₅₀s of roughly 30 μ M when tested against PABP1, and 60 μ M when tested against histone H3. Importantly, Lico, Tam, 4OH and Kep are all reversible inhibitors of CARM1 (Figure S3).

Developing a reporter for xenoestrogen-driven CARM1 inhibition in a cell line

We previously developed a cell-based reporter system for PRMT1 activity.^[19] This system cannot be used to gauge the activity of CARM1 in cells, because CARM1 does not methylate NPL3. In the past, we identified PABP1 as a major CARM1 substrate through a focused *in vitro* substrate screen, and we mapped the methylated arginine residues in

PABP1.^[33] Importantly, CARM1 is the only enzyme that methylates PABP1, and in CARM1 knockout cells PABP1 is hypomethylated.^[34] For this study, we raise methyl-specific antibodies to the PABP1 sequence - CGAIR*PAAPR*PPFS – where R* represents an asymmetrically dimethylated arginine residue. After affinity purification, the methyl-specific α PABP1 antibody (#162) displays a high degree of specificity. The methyl-specific α PABP1 antibody loses almost all its immuno reactivity in Westerns using CARM1 knockout extracts (Figure S4).

As yet, no arginine demethylases have been discovered.^[5] This methyl-mark is thus extremely stable. To test the efficacy of potential PRMT inhibitors in cell may require days of treatment, while waiting for the methylated substrates to turn-over. Under these conditions, compounds with pleiotropic effects would be difficult to investigate in a cell-based assay. To bypass this problem we developed a PABP1 inducible cell line. Upon treatment with tetracycline (Tet), these HEK293 cells express a flag-tagged form of full-length PABP1. This tag allows the induced form of PABP1 (fPABP1) to migrate more slowly by SDS-PAGE. We can thus easily distinguish between the endogenous PABP1 and the newly synthesized fPABP1. When fPABP1 expression is induced with Tet, in the presence of a potential CARM1 inhibitor, we can gauge the degree of fPABP1 methylation, using the methyl-specific PABP1 antibody that we developed (Figure 4A & B).

Using this approach, we can show that both Tam and 4OH can block the methylation of fPABP1 in cells. B4H is also effective in this assay, but requires high concentrations (160 μ M). Lico does not perform well in this cell-based assay, which suggests that it is not very cell permeable. As a control, we see that the global methyltransferase inhibitor, AdOx, also prevents fPABP1 methylation. The methyl-specific PABP1 antibody recognizes endogenous PABP1 in wt MEF extracts, but not in knockout or knockin (enzyme dead) MEF extracts, confirming the integrity of this methyl-specific antibody (Figure 4B).

We next used this approach to see if fPABP1 methylation was altered in a concentration-dependent fashion in cells, after treatment with Lico, Tam, 4OH and B4H (Figure 5). Indeed, we see that the IC₅₀s in cells (for Tam, 4OH and B4H), is very similar for that obtained in *in vitro* methylation reactions (Table 1 and Figure 5). Again, we see that Lico does not perform very well in this cell-based assay, although it is a good CARM1 inhibitor when assayed purely *in vitro*.

Discussion

CARM1 levels have been found to be elevated in castration-resistant prostate cancer,^[35, 36] as well as in aggressive breast tumors that also express high-levels of the oncogenic coactivator AIB1.^[7] AIB1 functions as a scaffolding molecule that bridges nuclear receptors and CARM1/PRMT1.^[3] Importantly, CARM1 methylates AIB1, thereby regulating its activity and stability.^[37, 38] The recruitment of CARM1 to ER α regulated promoters is dependent on the presence of AIB1, and CARM1 is essential for estrogen-induced proliferation of the MCF-7 breast cancer cell line.^[39] CARM1 is thus a good target for small molecule drug development.

The first screen for PRMT inhibitors was performed roughly six years ago.^[19] The identified small molecules (AMIs) did not block lysine methyltransferases, but they did inhibit all PRMTs and suppressed estrogen and androgen receptor-mediated transcriptional activation.^[19] Follow-up work shows that structural analogs of AMI1 and AMI5 display a certain degree of specificity, with certain compounds inhibiting PRMT1 better than CARM1.^[40, 41] However, other compounds in this analog series were less selective, inhibiting PRMTs, protein lysine methyltransferases, histone acetyltransferases, and sirtuins

with similar potency.^[42] A number of the AMIs are dye-like small molecules, and a set of these compounds has been studied using molecular modeling approaches to predict the docking modes of these compounds.^[43] Importantly, AMI1 may function primarily by targeting the substrate rather than the PRMT itself.^[11] The first target-based virtual screening approach to identify PRMT inhibitors was recently described.^[16] In this study, the docking program GOLD was applied to a homologue model of the rat PRMT3 crystal structure, and two compounds with cellular activity were discovered and characterized. The same group identified an additional lead structure using virtual screening, including a compound (RM65) that is a bisubstrate inhibitor, which blocks the AdoMet and arginine peptide binding pockets.^[17] The use of bisubstrate inhibitors of PRMTs has been developed further by Paul Thompson's group.^[14] They used an approach that transfers an AdoMet-like moiety (AAI) onto a peptide substrate in cells, which in turn functions as an inhibitor. This is the first example of a chemoenzymatically generated PRMT-target bisubstrate inhibitor. However, because this is a peptide-based inhibitor, it is unlikely to possess drug-like properties. The field of PRMT drug development for epigenetic therapy is thus still in its infancy, although there is commercial interest in developing PRMT-specific inhibitors, particularly against CARM1. Both Bristol-Myers Squibb and MethylGene have been optimizing pyrazole inhibitors of CARM1.^[44–47] These compounds seem to be selective for CARM1 (over PRMT1 & PRMT3), but they have not been shown to have activity in cells.

In this study, we found that PRMTs are inhibited by an array of xenoestrogens (Figure 2A). We focused on the ability of tamoxifen and 4-hydroxytamoxifen to inhibit CARM1 enzymatic activity in *in vitro* methylation assays (Figure 3), and demonstrated that these two related compounds inhibit CARM1 in a cellular setting as well (Figure 4B). Effects on a cellular reporter of CARM1 activity are readily detectable with treatment of 30 μ M tamoxifen.

There is precedent for xenoestrogens to target other proteins (tyrosine kinases, topoisomerase and PKC) in addition to the estrogen receptor. Clearly, tamoxifen primarily functions as an antiestrogen when used for breast cancer treatment. However, at doses five to ten-fold above those used for estrogen receptor antagonism, tamoxifen possesses therapeutic activity towards estrogen-negative cancers^[48] including malignant glioma,^[49, 50] desmoid tumors,^[51] renal cell carcinoma^[52] and ALS.^[53] It is believed that the primary mechanism of tamoxifen action in these cases, when high doses are used, is through inhibition of protein kinase C (PKC).^[30] Tamoxifen inhibits PKC with an IC_{50} of approximately 100 μ M, whereas it inhibits CARM1 with an IC_{50} of 30 μ M. Tamoxifen is a more potent inhibitor of CARM1 than it is of PKC. Thus, the mechanism involving the effects of high-dose tamoxifen treatments may need to be reevaluated.

Experimental Section

Plasmids, antibodies and chemicals

All GST fusion proteins were subcloned in pGEX6P-1 (Amersham Biosciences), which allows for the induced production of recombinant proteins fused to the C-terminal end of glutathione S-transferase. GST-PABP1^[33], GST-SMB^[54], GSTrPS2^[55] and GST-CA150^[56] have been described. GST-Npl3 was a gift from Pam Silver and has been described previously (39). GST-PRMT1, GST-PRMT3, GST-PRMT4, and GST-PRMT6 have been described^[57]. GST-Suv39H1 was a gift from Thomas Jenuwein^[58]. GST-SET7 and GST-DOT1 were gifts from Yi Zhang^[59]. GST-G9a was a gift from Yoichi Shinkai^[60]. Myc-PRMT5 was a gift from Stéphane Richard. Histones H3 and H4 (calf thymus) were purchased from Roche Applied Science. MBP was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). The methyl-specific PABP1 antibody was raised in rabbits against peptide sequence (CGAIR*PAAPR*PPFS) of PABP1. The anti-FLAG antibody

M2 and anti- β -actin antibodies were purchased from Sigma-Aldrich, and anti-c-Myc antibody from Covance. Licochalcone-A was purchased from ALEXIS Corporation, Raloxifene from Toronto Research Chemicals Inc., AIM-16 and 18 from Chembridge, all the other compounds from Sigma-Aldrich.

In Vitro Methylation Assay and IC₅₀ determination

All methylation reactions were carried out in the presence of S-adenosyl-1-[methyl-³H]methionine ([³H]AdoMet, 85 Ci/mmol from a 0.5 mCi/ml in dilute HCl/ethanol 9:1, pH 2.0–2.5, PerkinElmer Life Sciences) and PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4). Additional information pertaining to individual reaction conditions is described in each of the figure legends. The reaction contained 0.5–1.0 μ g of substrate and 0.1–0.2 μ g of recombinant enzyme with 200 μ M of each compound. The reaction was incubated at 30°C for 90 min and then separated by SDS/PAGE, transferred to a PVDF membrane, sprayed with Enhance™ (PerkinElmer Life Sciences), and exposed to film overnight for fluorograph. IC₅₀ determination has been described previously [41]. Briefly, after in vitro methylation reactions with different concentrations of each compound, the samples were separated by SDS-PAGE, transferred to a PVDF membrane stained by Ponceau S, and cut the visualized bands of substrate to count dpm by using liquid scintillation analyzer (Tri-Carb; Packard) to determine IC₅₀ value.

Cell Lines and Cultures

A stable/inducible T-Rex-3XFlag-PABP1 cell line was established by transfection with pcDNA/FRT/TO-3XFlag-PAPBP1 and pOG44 plasmids into Flp-in T-Rex HEK293 cells (Invitrogen). Transfected cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum supplemented with 10 μ g/ml blasticidin and 100 μ g/ml hygromycin. The resistant single colony was picked up to propagate. The generation of wild type and Carm1^{-/-} MEFs [34] from E13.5 embryos and enzyme-dead Carm1 knockin MEFs [61] from E14.5 embryo have been described. MEFs cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin, and streptomycin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Inhibitory effects of chalcones on a set of methyltransferases

A) The structural relationship between AMI-18 and chalcones is depicted. **B)** *In vitro* methylation reactions were performed with recombinant enzymes on different substrates (enzyme/substrate). Substrates (0.5 μ g) were incubated with recombinant enzymes (0.2 μ g) in the presence of 0.5 μ M [3 H] AdoMet and the indicated xenoestrogen (5 μ g) for 90 min at 30°C in the final volume of 30 μ l of PBS. Reactions were separated by SDS-PAGE, transferred to a PVDF membrane, sprayed with EnhanceTM (NEN) and exposed to film overnight. A control reaction was performed in the presence of DMSO (the xenoestrogens solvent) at 3.3% v/v. PRMT1, PRMT3 and CARM1 are arginine methyltransferases. SET7/9 is a lysine methyltransferase. NPL3, PABP1 and histone H3 are the enzyme substrates.

Figure 2. Inhibitory effects of xenoestrogens panel on a broad set of arginine methyltransferases

A) *In vitro* methylation reactions were performed with recombinant GST fused arginine methyltransferases and a set of different substrates, in the presence of indicated xenoestrogens. The lane number represents the compounds depicted in figure S1. GST of PRMT1, PRMT3 and PRMT6 were incubated with GST-Npl3 and GST-GAR as substrates. GST-CARM1 was incubated with GST-PABP1 and purified calf thymus histone H3 as substrates. GST-SET7/9 was incubated with purified calf thymus histone H3 as substrate. Substrates (0.5 μ g) were incubated with recombinant enzymes (0.2 μ g) in the presence of 0.5 μ M [3 H] AdoMet and the indicated xenoestrogen (5 μ g) for 90 min at 30°C in the final volume of 30 μ l of PBS. Reactions were separated by SDS-PAGE, transferred to a PVDF

membrane, sprayed with Enhance™ (NEN) and exposed to film overnight (top panel). A control reaction was performed in the presence of DMSO (the xenoestrogens solvent) at 3.3% v/v. **B**) The table shows the inhibitory ability of each xenoestrogen on the indicated enzymes. The number beside each xenoestrogen represents its lane in the fluorograph above. Strong (•••) & weak (•) inhibition.

Figure 3. Xenoestrogens are selective PRMT inhibitors

In vitro methylation reactions were performed with recombinant GST fused arginine methyltransferases and a set of different substrates, in the presence of indicated xenoestrogens. GST-CARM1 was incubated with GSTPABP, GST-SMB, GST-CA150 and calf thymus histone H3 as substrate, respectively. GST-PRMT1 was incubated with GST-NPL3 and histone H4 as substrates. GST-PRMT3 was incubated with GST-NPL3 and GST-rpS2 as substrates. GST-PRMT6 was incubated with GST-NPL3 and histone H3 as substrates. Myc-tag PRMT5 purified from CARM1 null cells was incubated with MBP and histone H4 as substrates. Substrates (0.5 μ g) were incubated with recombinant enzymes (0.2 μ g) in the presence of 0.5 μ M [3H] AdoMet and the indicated xenoestrogens (200 μ M) for

90 min at 30°C in the final volume of 30 μ l of PBS. Reactions were separated by SDS-PAGE, transferred to a PVDF membrane, sprayed with Enhance™ (NEN) and exposed to film overnight. The fluorographs are shown in the left panel, and the scintillation counts (dpm) of methylation levels are depicted in the right panel. A control reaction was performed in the presence of DMSO (the xenoestrogens solvent) at 3.3% v/v.

Figure 4. Effects of xenoestrogens on arginine methylation of induced fPABP1

A) The depiction of a cell-based reporter system for CARM1 activity. HEK293 cells were stably transfected with a Tet-inducible 3XFlag-tagged PABP1 (fPABP). Dots represent arginine methylation on both endogenous PABP1 and induced fPABP1. Less methylation will be reported on fPABP when induction takes place in the presence of an inhibitor. **B)** fPABP1 was induced by 0.5 μ g tetracycline in the presence of the indicated xenoestrogens and concentrations. The degree of methylated PABP1 was gauged using a methyl-specific PABP1 antibody. **C)** The graphic depiction of percentage effects of indicated concentrations of xenoestrogens on Flag-PABP1 methylation levels. Intensities of methylated fPABP1 and Flag in B were measured using image analysis software (ImageQuant TL 1D gel analysis),

respectively. Percentage obtained from quantifications of methylated fPABP1 levels (normalized to Flag levels) and represented as mean $\pm$ SD. Results are representative of five independent experiments.

Figure 5. Dose-dependent effect of xenoestrogens on the methylation state of fPABP1

The cells were treated with 0.5 μg tetracycline and indicated concentrations of xenoestrogens for 24 hrs, and the cell lysates were immunoblotted. Top panel represents western blot analysis of fPABP1 methylation status. Middle panel represents western blot analysis of Flag as a loading control. Bottom graphic panel shows percentage effects of indicated concentrations on fPABP1 methylation levels. Percentage obtained from normalized data (intensities of methylated fPABP1 to intensities of Flag) and each data point represents 5–8 repeats.

Table 1

Enz/Sub	Compound	IC ₅₀ (μ M)
CARM1/PABP1	Lico	28.35
	4OH	26.07
	Tam	30.41
	Kep	27.67
	B4H	473.6
CARM1/H3	Lico	68.95
	4OH	58.59
	Tam	43.31
	Kep	53.62
	B4H	309
PRMT1/NPL3	Lico	>333.3
	4OH	>333.3
	Tam	>333.3
	Kep	>333.3
	B4H	>333.3
SET7/H3	Lico	>333.3
	4OH	>333.3
	Tam	>333.3
	Kep	>333.3
	B4H	>333.3