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BRIEF ARTICLE

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Novel 3,5-Bis(bromohydroxybenzylidene)piperidin-4-ones as Coactivator-Associated Arginine Methyltransferase 1 Inhibitors: Enzyme Selectivity and Cellular Activity

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Supporting Information

ABSTRACT: Coactivator-associated arginine methyltransferase 1 (CARM1) represents a valuable target for hormone-dependent tumors such as prostate and breast cancers. Here we report the enzyme and cellular characterization of the 1-benzyl-3,5-bis(3-bromo-4-hydroxybenzylidene)piperidin-4-one (**7g**) and its analogues **8a**−**l**. Among them, **7g**, **8e**, and **8l** displayed high and selective CARM1 inhibition, with lower or no activity against a panel of different PRMTs or HKMTs. In human LNCaP cells, **7g** showed a significant dose-dependent reduction of the PSA promoter activity.

Arginine methylation of mainly nuclear proteins is a reversible post-translational modification process involved in structural remodeling of chromatin.[1,2] Protein arginine methyltransferase (PRMT) enzymes remove the methyl group from the donor molecule *S*-adenosyl-L-methionine (AdoMet), generating the product *S*-adenosyl-L-homocystein (AdoHcy), and transfer this methyl residue to the terminal nitrogen atom(s) of the guanidinium side chain of an individual arginine residue in the target protein.[3] PRMTs are ubiquitously expressed in most cell types and tissues of the human body with the unique exception of PRMT8, which appears to be restricted to neurons in the brain.[4] Moreover, they differ in their substrate specificities and are therefore probably involved in different physiological processes. Among PRMTs, PRMT4/CARM1 (coactivator-associated arginine methyltransferase 1) was the first to be identified as a transcriptional regulator.[5] CARM1 methylates a number of proteins that are involved in transcription and RNA processing, including histone H3 (H3R17 and H3R26), amplified in breast cancer 1 (AIB1), p300/CBP (cAMP-responsive element binding protein [CREB] binding protein), poly(A)-binding protein 1 (PABP1), and coactivator of 150 kDa (CA150).[1] CARM1 requires its enzymatic activity for all its in vivo functions.[6] In cancer, CARM1 has been shown to regulate estrogen-stimulated MCF-7 breast cancer cell cycle progression through E2F1 up-regulation.[7] Moreover, CARM1 has been found up-regulated in castration-resistant prostate cancer[8] and in grade-3 breast tumors,[9] and CARM1 knockdown by siRNA completely inhibited prostate cancer LNCaP cell proliferation by induction of apoptosis.[10]

All these findings prompted researchers to develop molecules able to inhibit CARM1 activity, as potential anticancer agents. Some pyrazole-containing compounds (**1**−**4**) and the benzo[*d*]imidazole (**5**) have been reported as inhibitors of CARM1,[11−15] and the plant-derived ellagic acid (**6**)[16] has been recently shown to selectively block methylation at Arg17 of histone H3 (H3R17),[16] the CARM1 histone site for methylation (Chart S1 in Supporting Information).

Despite the fact that all of these compounds showed submicromolar inhibitory activity against CARM1, no inhibitor has been demonstrated to exhibit cellular effects to date.

Pursuing our searches on design, synthesis, and biological validation of small molecule modulators of epigenetic targets,[17] in 2008 we prepared and tested some bis(3-bromo-4-hydroxy- and 3,5-dibromo-4-hydroxyphenyl) compounds and their analogues against PRMT1,[18] CARM1,[18] SET7 (an histone lysine methyltransferase, HKMT),[18] p300/CBP (an HAT enzyme),[18,19] SIRT1, and SIRT2.[18] Depending on the extent of bromination of the molecule (presence of four bromine atoms) and on the nature of the linker connecting the two dibromophenol moieties (penta-1,4-dien-3-one, 2,6-dimethylene-(hetero)cycloalkanone, 1,1-(1,3-phenylene)diprop-2-en-1-one, and hepta-1,6-diene-3,5-dione), some of such compounds behaved as epigenetic multiple ligands (epi-MLs), they being active against all the tested enzymes.[18] In contrast, compounds carrying two or three bromine atoms in their structure or featuring a bis(3,5-dibromo-4-hydroxybenzamide) or bis(3,5-dibromo-4-hydroxyanilide) scaffold failed to be recognized as epi-MLs and showed some degree of selectivity against a particular epigenetic target.

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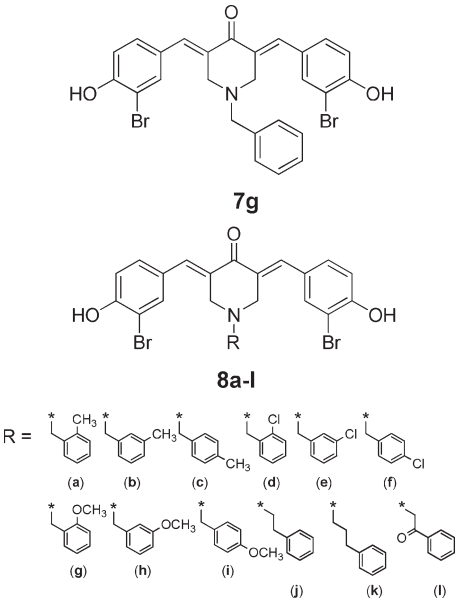

Figure 1. CARM1-selective inhibitors used in this study.

Thus, to identify CARM1-selective inhibitors among them and taking into account the fluorograph data previously reported, we determined the IC_{50} values for selected bis(bromo- and dibromophenol) compounds **7a**−**m** (see Figure S1 and Table S1 in Supporting Information) against PRMT1, CARM1, and the HKMT SET7. Among the tested compounds, **7b** showed high potency and selectivity in inhibiting PRMT1, whereas **7c**, **d**,**g**,**h**,**l**,**m** preferably inhibited CARM1, **7g** being the most potent (IC_{50} = 7.1 μM). With the exception of **7a**,**b**, all the tested compounds displayed very low (if any) inhibition against SET7.

Accordingly, we chose **7g** as our lead compound for selective CARM1 inhibition and prepared some 3,5-bis(3-bromo-4-hydroxybenzylidene)-1-benzylpiperidin-4-one analogues **8a**−**l** by insertion of a chlorine atom or a methyl or methoxy group at the ortho, meta, or para position of the *N*1-benzyl moiety or by replacing such benzyl group with a 2-phenylethyl, 3-phenylpropyl, or 2-oxo-2-phenylethyl moiety at N1 (Figure 1). These new compounds were tested as CARM1-selective inhibitors, and two of them together with **7g** were investigated in more detail in vitro and in vivo.

CHEMISTRY

3,5-Bis(3-bromo-4-(methoxymethoxy)benzylidene)piperidin-4-one **9**, the key intermediate of the title compounds, was prepared by condensation of 3-bromo-4-(methoxymethoxy)benzaldehyde[18] with 4-piperidone in alkaline medium (barium hydrate). Alkylation reactions of **9**, carried out at 60 °C with the appropriate alkyl bromide in the presence of dry potassium carbonate in acetonitrile, furnished the *N*-arylalkyl-3,5-bis(3-bromo-4-(methoxymethoxy)benzylidene)piperidin-4-ones **10a**−**l** that were subjected to acidic hydrolysis in methanolic 3 N HCl at 60 °C to afford the desired bis(3-bromo-4-hydroxybenzylidene) analogues **8a**−**l** (Scheme S1 in Supporting Information).

Experimental procedures for **9** and **10** and chemical and physical data (Tables S2−S4) for **8**−**10** are reported as Supporting Information.

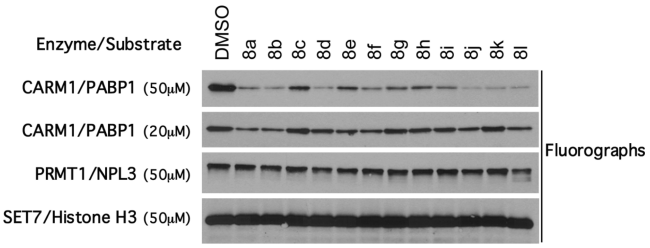

Figure 2. Inhibitory activities of **8a**−**l** against CARM1 using PABP1 as a substrate, PRMT1 using NPL3 as a substrate, and SET7 using histone H3 as a substrate. The concentrations of the compounds used in each in vitro methylation assay are shown.

Table 1. IC_{50} of 7g and 8a−f,j−l against CARM1, PRMT1, and SET7[a]

	IC_{50} (μM)		
compd	CARM1/PABP1	PRMT1/NPL3	SET7/H3
7g	8.6 ± 0.8	>667	>667
8a	10.3 ± 3.3	>667	>667
8b	15.2 ± 0.9	>667	>667
8c	11.9 ± 2.3	>667	>333
8d	12.5 ± 6.1	>667	>667
8e	8.1 ± 2.2	>667	174 ± 28
8f	12.2 ± 3.0	>667	>600
8j	14.8 ± 2.5	>667	>667
8k	16.0 ± 3.8	>667	>667
8l	14.4 ± 1.7	>667	149 ± 26

[a] Values were determined from at least two separate experiments. The mixture contained 0.1 μM GST-CARM1 and 0.5 μM GST-PABP1, 0.15 μM GST-PRMT1 and 0.5 μM GST-NPL3, or 0.15 μM GST-SET7 and 1.1 μM histone H3 with 0.22 μM [^{3}H]AdoMet and different concentrations of each compound for IC_{50} determinations. The software used for fitting curves and determining IC_{50} is SigmPlot. The equation used for fitting is $y = y_0 + a/1 + (x/x_0)b$.

RESULTS AND DISCUSSION

The new compounds were tested by fluorograph at 50 and 20 μM against CARM1 using PABP1 as a substrate[20] and at 50 μM against PRMT1 (substrate, the heterogeneous nuclear ribonucleoprotein NPL3)[21] and SET7 (substrate, histone H3) to assess their potency and selectivity (Figure 2). At 20 μM, the 4-methyl- and the 2-, 3-, and 4-methoxybenzyl analogues of **7g** (**8c** and **8g**−**i**) as well as the 3-phenylpropylpiperidone **8k** showed no effect against the PABP1 methylation; thus, the methoxy-containing compounds were excluded by IC_{50} calculation.

IC_{50} values for **7g** and **8a**−**f**,**j**−**l** were determined against CARM1 using PABP1 as a substrate and against PRMT1 and SET7 using NPL3 and histone H3 as substrates, respectively (Table 1). The corresponding IC_{50} curves are reported in Supporting Information. All the tested compounds displayed low micromolar activity against CARM1, the insertion of methyl and chloro substituents at the *N*1-benzyl moiety having only modulator effects on enzyme inhibition. The preferred position for introduction of a methyl group at the benzyl portion seems to be the ortho position (**8a**), while for chlorine insertion the benzyl meta position afforded the highest inhibitory activity (**8e**), similar to that of the lead **7g**.

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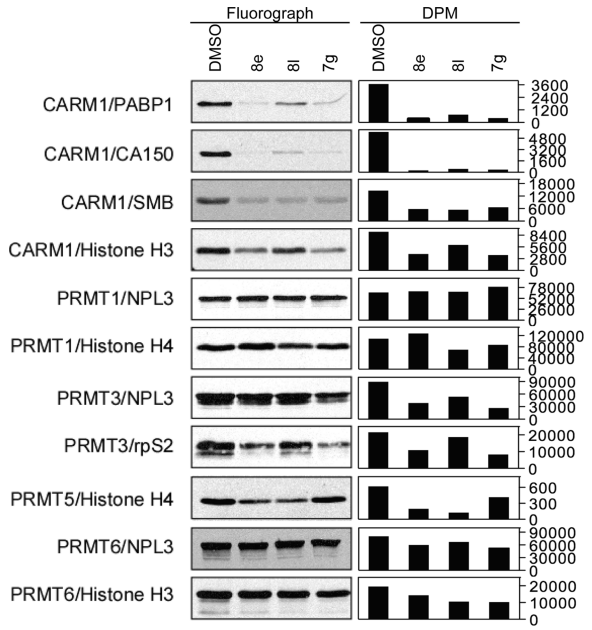

Figure 3. Inhibitory activity of **7g**, **8e**, and **8l** against CARM1 using PABP1, CA150, SMB, and histone H3 as substrates and against a panel of PRMTs (PRMT1, PRMT3, PRMT5, and PRMT6) using indicated histone and/or non-histone substrates. The fluorographs are shown in the left panels, and the tritium count for each band is depicted in the right panels.

All of the tested compounds were selective toward CARM1, showing very low (if any) activity against PRMT1 and SET7. Among them, we selected **7g**, **8e**, and **8l** for further experiments: **7g** and **8e** were the most potent inhibitors of CARM1 with PABP1 as a substrate (see Table 1), while **8l** was the only analogue carrying a structural diversity, the carbonyl group at the substituent at N1, that could influence its binding with the enzyme and its inhibitory behavior.

First, we repeated the CARM1 assay, testing **7g**, **8e**, and **8l** at 100 μM by fluorograph and using four different CARM1 substrates: PABP1, CA150,[22] the spliceosome protein SmB,[22] and histone H3 (Figure 3). All the three tested compounds strongly inhibited the CARM1 activity on the various substrates. Among these, CA150 was the most sensitive, whereas the use of histone H3 yielded the lowest CARM1 inhibition. To check the real selectivity of **7g**, **8e**, and **8l** against various PRMTs, we tested them at 100 μM against (i) PRMT1 using NPL3 and histone H4 as a non-histone and histone substrate, respectively, (ii) PRMT3 using NPL3 and the ribosome protein rpS2[23] as substrates, (iii) PRMT5 and histone H4 as a substrate, (iv) PRMT6 using NPL3 and histone H3 as substrates (Figure 3). In addition, **7g**, **8e**, and **8l** were tested at 100 μM against a panel of HKMTs, namely, SET7 (substrate, H3), DOTL1 (substrate, nucleosome), Suv39H1 (substrate, H3), and G9a (substrate, H3) (Figure 4). Against PRMTs, **7g** and **8e** were able to inhibit to some extent PRMT3, and **8e** and **8l** showed high inhibition of PRMT5 at 100 μM. Nevertheless in all cases the observed inhibition values were weaker than those observed with CARM1 when used at the same concentration (see Figure 3). No significant activity at 100 μM was registered for **7g**, **8e**, and **8l** against the tested HKMTs (see Figure 4).

Known CARM1 substrates such as PABP1 are hypermethylated in vivo, and this methylation is very stable. To test the

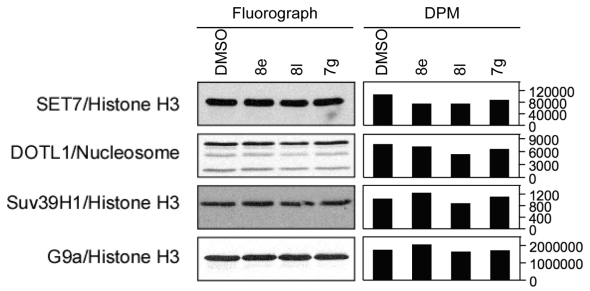

Figure 4. Inhibitory activities of **7g**, **8e**, and **8l** against a panel of HKMTs (SET7, DOTL1, Suv39H1, and G9a) using the indicated histone and/or non-histone substrates.

efficacy of potential PRMT inhibitors in cell may require days of treatment while waiting for the methylated substrates to turnover. Under these conditions, compounds with pleiotropic effects would be difficult to investigate in a cell-based assay. To reduce the exposure time of the compound to cells and bypass this problem, we developed a Flag-tagged PABP1 inducible cell line obtained by engineering a tetracycline-controlled transrepressor protein (TetR) in human embryonic kidney HEK293 cells.[24] The TetR protein binds to tet operator (tetO) sequences in the absence but not in the presence of tetracycline, silencing the transcriptional activities at the promoter.

We can thus easily distinguish between the endogenous PABP1 and the induced Flag-tagged PABP1 because of its slower migration by SDS−PAGE. We tested **7g**, **8e**, and **8l** in this reporter system. Upon addition of tetracycline, Flag-PABP1 is induced in HEK293 cells in the presence of the indicated compound, and its methylation status can be detected by the use of a methyl-specific PABP1 antibody generated in our lab.[24] In this reporter system, only **7g** was able to inhibit Flag-PABP1 methylation (Figure S4 in Supporting Information).

There is increasing evidence of the involvement of CARM1 in hormone responsive cancers such as prostate cancer. Thus, we determined the effect of **7g**, **8e**, and **8l** on prostate-specific antigen (PSA) promoter in human prostate adenocarcinoma LNCaP cells by using PSA luciferase assay, relative to a CMV-Renilla control (Figure 5, top). In particular, we transfected PSA reporter into LNCaP cells and then we treated the cells with increasing concentrations of **7g**, **8e**, or **8l** for 2 days.

As seen in Figure 5, a dose-dependent decrease of the reporter activity was observed with **7g** and **8e** up to 8−10 μM while **8l** was effective only at 30 μM. In parallel, we measured the cell viability through Cell Titer-Glo (CTG), based on quantitation of the ATP present (Figure 5, bottom). This was done to confirm that the observed PSA effects were the results of CARM1 inhibition and to rule out involvement of other targets and/or cell death. **7g** and **8l** displayed no or little effect on cell viability, at concentrations that impacted the luciferase assay.

In conclusion, we have reported on the ability of the 1-substituted-3,5-bis(3-bromo-4-hydroxybenzylidene)piperidin-4-ones **7g** and **8a**−**l** to selectively inhibit CARM1 activity. Compounds **7g**, **8e**, and **8l** were able to inhibit CARM1-mediated methylation of different substrates (PABP1, CA150, SmB, and H3) up to single-digit micromolar level, displaying low inhibitor activity (if any) against a panel of different PRMTs or HKMTs. In human prostate cancer LNCaP cells, **7g** showed a significant dose-dependent reduction of the PSA promoter activity at a concentration that did not affect cell viability.

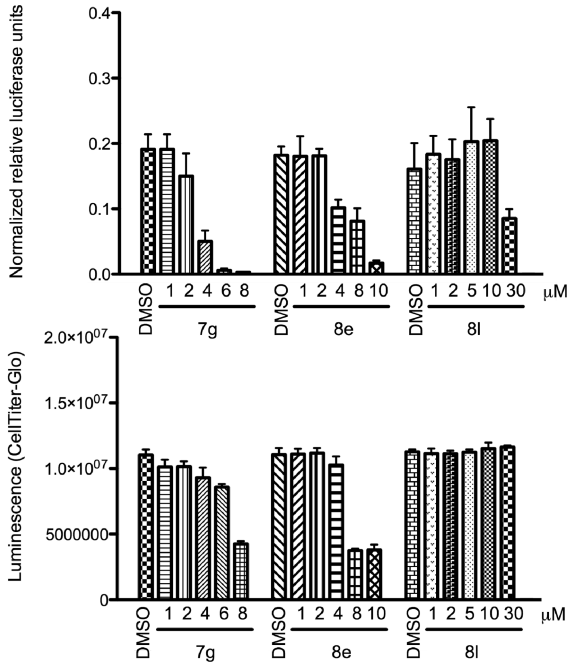

Figure 5. Effects of increasing concentrations of **7g**, **8e**, and **8l** on PSA promoter activity by luciferase assay in LNCaP cells relative to a CMV-Renilla control (top) and on cell viability based on quantitation of the ATP present, which is an indicator of metabolically active cells and is used to determine the viability of cells in culture (bottom). The results are presented as the mean ± SD calculated from triplicate luciferase assays.

EXPERIMENTAL SECTION

Chemistry. Melting points were determined on a Buchi 530 melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 MHz on a Bruker AC 400 spectrometer. Chemical shifts are reported in δ (ppm) units relative to the internal reference tetramethylsilane (Me_4Si). EIMS spectra were recorded with a Fisons Trio 1000 spectrometer; only molecular ions (M^+) and base peaks are given. All solvents were reagent grade and, when necessary, were purified and dried by standard methods. Organic solutions were dried over anhydrous sodium sulfate. Elemental analysis was used to determine purity of the described compounds, which is >95%. Analytical results are within ±0.40% of the theoretical values (see Table S3 in Supporting Information). All chemicals were purchased from Aldrich Chimica (Milan, Italy) or from Alfa Aesar (Milan, Italy) and were of the highest purity.

General Procedure for the Synthesis of N-Substituted 3,5-Bis(3-bromo-4-hydroxybenzylidene)piperidin-4-ones (8a−l). Example: 3,5-Bis(3-bromo-4-(hydroxybenzylidene)-1-(3-chlorobenzyl)piperidin-4-one (8e). A solution of **10e** (0.42 mmol, 0.3 g) in methanol (5 mL) and 3 N hydrochloric acid (5 mL) was stirred at 60 °C for 3 h. Then the suspension was neutralized with 1 N sodium hydrogen carbonate. The precipitated solid was filtered and washed with water (3 × 10 mL) and diethyl ether (3 × 10 mL) to give pure **8e** as a yellow powder. ^{1}H NMR (DMSO-d_6, 400 MHz, δ, ppm) δ 4.43−4.48 (s, 6H, $PhCH_2$ and CH_2 piperidone), 7.05−7.74 (m, 12H, PhCH and benzene protons), 11.13 (bs, 2H, OH) ppm; ^{13}C NMR (DMSO-d_6, 400 MHz, δ, ppm) δ 53.4 (2C), 63.9, 113.7 (2C), 118.0 (2C), 126.0, 126.9, 127.3, 128.7 (2C), 129.6 (2C), 131.3 (2C), 132.2, 134.0, 136.9, 140.6 (2C), 145.9 (2C), 155.8 (2C), 186.0 ppm. MS (EI) m/z: 588.95 $(M)^+$.

Experimental procedures for **9** and **10** and chemical and physical data (Tables S2−S4) for **8**−**10** are in Supporting Information.

Plasmids and Antibodies. See Supporting Information.

In Vitro Methylation Assay and IC_{50} Determination. The assays have been described in detail previously.[25] Briefly, all methylation reactions were performed in a final volume of 30 μL of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) and in the presence of *S*-adenosyl-l-[methyl-^{3}H]methionine ([^{3}H]AdoMet, 85 Ci/mmol from a 0.5 mCi/mL in dilute HCl/ethanol 9:1, pH 2.0−2.5, PerkinElmer Life Sciences). The mixture contained 0.5−1.5 μM substrate and 0.1−0.2 μM recombinant enzyme with 100 μM of each indicated compound for fluorograph (Figures 3 and 4) or different doses of each compound for IC_{50} determination (Table 1). The mixture 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 fluorography. After fluorography, the same PVDF membrane was stained by Ponceau S and the visualized bands of substrate were cut to count dpm by using a liquid scintillation analyzer (Tri-Carb, Packard) for graphic depiction or IC_{50} determination.

Cell Lines and Cultures. See Supporting Information.

Luciferase Assay. LNCaP cells were cultured in phenol-red-free RPMI 1640 supplemented with 10% charcoal-stripped fetal calf serum. Approximately 20 h before transfection, cells were seeded into each well of 24-well culture dishes. The cells in each well were transfected with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. For each transfection, 300 ng of PSA(ARE)-LUC and 2 ng of humanized CMV-Renilla internal control were used. After 12 h of transfection, cells were treated with 20 nM DHT to induce PSA-firefly and to indicate the amount of compound. After 42−44 h, the cells were washed twice with PBS and harvested. Five of six cells were used to perform luciferase assay using the dual luciferase assay system (Promega) (Figure 5 top), and one of six cells was used to determine cell viability using CellTiter-Glo luminescent reagent (Promega) according to the manufacturer's protocol (Figure 5 bottom).

ASSOCIATED CONTENT

Supporting Information. Chemistry and experimental details; IC_{50} curves for **7g** and **8a**−**l** against CARM1/PABP1, PRMT1/NPL3, and SET7/H3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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ABBREVIATIONS USED

AdoHcy, *S*-adenosyl-L-homocystein; AdoMet, *S*-adenosyl-L-methionine; AIB1, amplified in breast cancer 1; CA150, coactivator of

150 kDa; CREB, cAMP-responsive element binding protein; NPL3, heterogeneous nuclear ribonucleoprotein; PABP1, poly-(A)-binding protein 1; rpS2, ribosome protein; PSA, prostate-specific antigen; SmB, spliceosome protein; tetO, tet operator; TetR, tetracycline-controlled transrepressor protein

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