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Authors
Patra, Aditi
Deb, Moonmoon
Dahiya, Rajvir
et al.

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5-Aza-2′-deoxycytidine stress response and apoptosis in prostate cancer

Aditi Patra · Moonmoon Deb · Rajvir Dahiya · Samir Kumar Patra

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Abstract While studying on epigenetic regulatory mechanisms (DNA methylation at C-5 of –CpG– cytosine and demethylation of methylated DNA) of certain genes (FAS, CLU, E-cadh, CD44, and Cav-1) associated with prostate cancer development and its better management, we noticed that the used in vivo dose of 5-aza-2′-deoxycytidine (5.0 to 10.0 nM, sufficient to inhibit DNA methyltransferase activity in vitro) helped in the transcription of various genes with known (steroid receptors, AR and ER; ER variants, CD44, CDH1, BRCA1, TGFβR1, MMP3, MMP9, and UPA) and unknown (DAZ and Y-chromosome specific) proteins and the respective cells remained healthy in culture. At a moderate dose (20 to 200 nM) of the inhibitor, cells remain growth arrested. Upon subsequent challenge with increased dose (0.5 to 5.0 μM) of the inhibitor, we observed that the cellular morphology was changing and led to death of the cells with progress of time. Analyses of DNA and anti-, pro-, and apoptotic factors of the affected cells revealed that the molecular events that went on are characteristics of programmed cell death (apoptosis).

Keywords Epigenome · CpG hypermethylation · DNMT · 5-Aza-2′-deoxycytidine · Apoptosis · Prostate cancer

Abbreviations
DNMT(s) DNA methyltransferase(s)
HDAC(s) Histone deacetylase(s)
MBD(s) Methyl–CpG–DNA binding domain protein(s)
CaP Cancer of prostate
AzadC 5-Aza-2′-deoxycytidine

Introduction

Overexpression of DNA (cytosine-5-carbon) methyltransferases and hypermethylation of CpG islands at the regulatory regions of certain genes (for example, ER, CDH1, CD44, Casp3, GSTP1, MDR1, FAS, RASSF1A, P15INK4B, and P16INK4A) and global genome-wide hypomethylation are well known to be associated with prostate and other multiple cancer development and metastasis (Graff et al. 1995; Ferguson et al. 1997; Li et al. 2000; Szyf et al. 2000; Li et al. 2001; Nojima et al. 2001; Patra et al. 2001; Dasari et al. 2002; Jones and Baylin 2002; Patra et al. 2002; Sasaki et al. 2002; Chen et al. 2003; Jaenisch and Bird 2003; Patra et al. 2003; Patra 2008a; Patra et al. 2008a; Patra and Szyf 2008; Patra and Bettuzzi 2009). The CpG sites in these gene promoter regions are rarely methylated in normal cells except in, e.g., inactivated X-chromosome and imprinted genes. Some non-regulatory site CpG island methylation has no direct effect on gene
activity (Patra 2008a; Patra et al. 2008a; Patra and Szyf 2008). It is now revealed that abnormal methylation of CpG islands is not restricted to cancer cells but can also occur during aging and during early stages of tumor development (Patra et al. 2008a). While the co-existence of genome-wide hypomethylation and site-selected hypermethylation are well documented by chemical analyses of bases from total genomic DNA and gene-specific DNA segment, the specific mechanisms at the level of enzymes, co-substrates, and repressor proteins are subject to immense specific mechanisms at the level of enzymes, co-

...hypomethylation and site-selected hypermethylation are well documented by chemical analyses of bases from total genomic DNA and gene-specific DNA, perhaps due to adduct formation with DNA containing 5-azadC replacing cytosine. Gradual increase of 5-azadC in cell culture medium caused dose-dependent over-expression of apoptosis genes like Bax, Bax, and caspase-3, including many other genes in TSUPr1 and DuPro cell lines (Table 3).

### Experimental procedures

**Cell culture and drug(s) treatment**

Prostatic cancer cell lines of human origin PC3, TSUPr1, and DuPro were purchased from American Type Culture Collection (Manassas, VA, USA). Cell lines were cultured and maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamate, and 0.1 μM of penicillin and streptomycin. Media and supplements were obtained from the University of California at San Francisco Cell Culture Facility. For drug treatment, exponentially growing cells were seeded at a density of ∼10^6 cells/75 cm^2 flask. After 6 h, the cells were treated at different concentrations (nanomolar to micromolar) of 5-azadC (Sigma Chemical Co., St. Louis, MO, USA) and marked as zero “0” time. Trichostatin A was dissolved in PBS and diluted with the required volume of the medium, and cells were also treated at different concentrations (4, 10, and 50 nM) of MG-132 (Calbiochem, San Diego, CA, USA) inhibitor of protease-some, dissolved in DMSO and diluted with the required volume of medium for 8–12 h prior to harvest for further investigations. All cells were harvested after 96 h unless otherwise mentioned.
Assays of enzymes

The DNMT enzymatic activity was measured following a previous protocol as standardized in our laboratory (Patra et al. 2001, 2002, 2003). Poly•dI–dC duplex or poly•dG–dC duplex was used as substrate and S-adenosylmethionine (tritiated methyl) as methyl group donor. All assays were performed in duplicates in three sets of independent experiments. Background levels were determined in assays in which the template DNA was omitted. Statistical analyses were performed using Student’s t test.

Caspase-3-like activity was measured according to the kit supplied by Calbiochem. Caspase-3 assay kit employs the colorimetric substrate DEVD-pNA that upon cleavage exhibits increased absorption at 405 nm. A caspase-3 inhibitor (Ac-DEVD-CHO) is also used as a prototypic control inhibitor. The DEVD amino acid sequence is derived from the caspase-3 cleavage site in PARP. All the kit components were thawed and immersed in an ice bath until use. Briefly, caspase-3 inhibitor I was warmed to room temperature. In a separate microcentrifuge tube, it was diluted in assay buffer (1:200). Caspase-3 (15 μl, approximately 30 U) diluted in assay buffer (1:50) was used as control. Caspase-3 was not used in assay buffer to blanks. Caspase-3 inhibitor (20 μl) was added in triplicate tubes. Reaction was started by adding 200 μM (final concentration) caspase-3 substrate I and pre-equilibrated to assay temperature at 25°C and measured in a colorimeter/microplate reader.

Histone deacetylase (HDAC) enzymatic activity was measured using fluorescent amide, N-(4-methyl-7-coumarinyl)-N-α-(tert-butoxy-carbonyl)-N-Ω-acetyllysinamide (MAL) (Calbiochem) as a potential substrate following the published protocol of ours and other laboratories (Patra et al. 2001; Cervoni et al. 2002) as well as using a radioactive kit (Upstate Biotechnology). The background fluorescence was kept significantly low using filters, and nanomolar (nM) concentration of MAL was used to avoid interference (inner-filter effects). In the presence of trichostatin A (Calbiochem), a potent inhibitor of HDAC (Taunton et al. 1996; Hoffmann et al. 1999), no activity was observed (see also Patra et al. 2001, 2003). Statistical analyses were performed using Student’s t test.

Table 1 Comparison of % activity (average of three sets of independent experiments) of the enzymes without and with increasing concentration of drug

| Enzymes | Control | 5-AzadC (nM) |
|---------|---------|-------------|
|         |         | 10  | 20   | 35  | 50   | 75  | 100  | 250  | 500  |
| DNMT    | 100     | 71.23 | 50.36 | 5.14 | 2.32 | –   | –   | –    | –    |
| HDAC    | 100     | 90.62 | 81.07 | nd   | 73.34 | 61.58 | nd   | nd   | nd   |
| Caspase-3 | –     | –    | –    | –    | –    | 2.3±1.73 | 5.67±3.46 | 27.5±8.40 | 41.49±7.0 |

nd not detected

Nucleic acid extraction and reverse transcription–PCR

Total RNA was extracted using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). Cytosolic and nuclear RNA were prepared using TRI reagent after control lysing of cells and separation by centrifugations of those contents. RNA (1–2 µg) was reverse transcribed using random hexamer primers and Superscript II, reverse transcriptase (Life Technologies Inc., Gaithersburg, MD, USA) in a 25-µl reaction volume. cDNA was amplified by PCR using primers specific for the genes Bcl2, Bak, Bax, Bcl-XL, and caspase-3. β-Actin, glyceraldehyde-3-phosphatedehydrogenase (GAPDH), and histone 4 (H4) genes were also amplified as internal controls to ensure high quality. Primer sequences specific for β-actin gene (TCTACAATGAGCTGCGTGTG, sense; ATCTCCCTCTGCATCTGTC, antisense), GAPDH gene (GAAGGTTGAAAGTCCGGTGC, sense; GAAGATGCGCTGTC, antisense), and H4 gene (CAA CATTCAGGGCATCACCAA, sense; CCGAATCA TACTCCAAGAA, antisense) were used and resulted in 682-, 226-, and 131-bp products, respectively. The PCR sample mixtures, in a 10-µl volume, contained 1× PCR buffer (Sigma), 0.2 mM of each dNTP (Sigma), 4 ng of TaqStart antibody (Clonetech, Palo Alto, CA, USA), 0.2 µM primers, and 0.5 µl RedTaq DNA polymerase (Sigma). PCR reactions were performed in a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA) at 94°C for 1 min, 26 cycles at 94°C for 20 s, 57°C for 20 s, and 72°C for 30 s, followed by an extension step at 72°C for 5 min. The PCR products were electrophoresed through a 1.2% agarose gel containing ethidium bromide and were visualized by UV detection.

Immunocytochemistry

Prostatic cancer cell lines were grown in chamber slides and stained as our standardized protocol (Patra et al. 2001, 2002, 2003). In brief, sub-confluent cells were fixed by 10% formalin and were permeabilized by 0.1% triton X-100 in PBS. The endogenous peroxidase activity was blocked by incubation in 5% H2O2 in methanol for 20 min. The cells were pre-blocked using Ultra V block (Lab
Vision Corporation, Fremont, CA, USA) for 10 min and incubated for overnight at 4°C with goat polyclonal antibody against caspase-3 (Santa Cruz Biotech, CA, USA). New Zealand white rabbits polyclonal antibody against DNMT1 peptide (I0015801K; Research Genetics, Inc., Huntsville, AL, USA; or met-cat 2) and goat polyclonal antibody against Bcl2, Bak, Bax, BelXs, and MBD1 (Santa Cruz Biotech). The antibody for HDAC1 (1:100), DNMT1 (1:1,000), and others was diluted according to the manufacturer’s instructions (and several titration) with primary antibody dilution buffer (Bio-medea, Foster City, CA, USA). The cells were washed in PBS then incubated with anti-goat biotinylated secondary antibody (Santa Cruz Biotech) for 30 min followed by another wash and incubation with HRP-conjugated streptavidin. Finally, reactions were visualized by incubation with DAB (substrate and chromogen) and counterstaining with Harris hematoxylin. For negative control, cells were incubated overnight with dilution buffer (no primary antibody).

TUNEL assays

Cells were permeabilized with 0.1% Triton for 2 min, fixed for 10 min in 4% paraformaldehyde in PBS, and stored in 70% ethanol before assay.Slides were equilibrated in terminal transferase (Roche) and incubated for 60 min with TdT (200 U/ml) and biotin-16-dUTP (10 μM; Roche). Following two washes with 4× SSC, TUNEL-positive cells were detected with avidin–Texas Red (1:100; Jackson).

Results

In vitro DNMTase, HDACs, and caspase-3 activity: inhibition of DNMT activity is not sufficient for induction of caspase-3 activity

The measurement of DNMTase enzyme activity, using poly (dI–dC) and poly (dG–dC) duplex as substrates, in cancer cell lines (PC3, TSUPr1, and DUPro) and tissues (post-
operated tissues obtained from UCSF and VA Medical Center, San Francisco) showed the increased activity in cancer, and it is clear that in prostate cancer expression of DNMT is very high as compared to BPH-1 cell line and BPH tissues. Enzyme activity of HDAC in prostate cancer is also higher than normal or BPH (Patra et al. 2001, 2003). There was no expression of caspase-3 activity in prostate cancer cell lines. Treatment of cells with 10, 20, 35, 50, 75, 100, 250, and 500 nM 5-azadC respectively supplemented in culture medium abolished the in vitro DNMT activity.

**Fig. 2** Immunocytochemistry. Sub-confluent cells were fixed by 10% formalin and were permeabilized by 0.1% triton X-100 in PBS. The endogenous peroxidase activity was blocked by incubation in 5% H₂O₂ in methanol for 20 min. The cells were pre-blocked using Ultra V block (Lab Vision) for 10 min and incubated for overnight at 4°C with respective antibodies. The cells were washed in PBS and then incubated with anti-goat biotinylated secondary antibody (Santa Cruz Biotech) for 30 min followed by another wash and incubation with HRP-conjugated streptavidin. Finally, reactions were visualized by incubation with DAB (substrate and chromogen) and counterstaining with Harris hematoxylin. For negative control, cells were incubated overnight with dilution buffer (no primary antibody). Representative examples of expression of Bcl2 in untreated TSUPr1 (a). In 250 nM 5-azadC-treated TSUPr1 cells, the amount of expression of Bcl2 was very high (b). Caspase-3: there was no expression in control/untreated TSUPr1 (c). The amount of expression in 5-azadC 250 nM 5-azadC-treated TSUPr1 cells was very high (d). Bax: there was no expression in control/untreated TSUPr1 (e), reasonably in 5-azadC 250 nM 5-azadC-treated TSUPr1 cells (f).
around 75 nM and significantly decreased HDAC activity in BPH-1, DuPro, and other cancer cell lines. Although in the TSUPr1 cells the DNMT enzyme activity was blocked at 50/75 nM of 5-azadC, the significant caspase-3 activity was traced around 250 nM (see Table 1 for details).

Effect of drugs (5-azadC, DEVD-CHO, and MG-132) on cell growth

The hypomethylating agent 5-azadC is known to block DNMTs activity by forming covalent adducts with the daughter strand in replicating cells/organisms (Patra and Bettuzzi 2009). We found that in culture medium supplemented with 5-azadC at concentrations of 10, 20, 35, 50, 75, and 100 nM, the cells grow well. A gradual decrease in growth rate (i.e., increase in cell doubling time) was observed in all cases beyond 75 nM. All prostate cancer cell lines showed a significantly similar pattern of decrease in enzyme activity and growth rate, except PC3 cell line. The cancer cell line PC3 was found to be highly prone to death at low, 35-nM concentrations of 5-azadC compared to other cancer cell lines (for example, TSUPr1 and DuPro) that needed the drug concentration at 350 to 500 nM range (about 100 times higher) for growth arrest and very high concentrations (1.0–5.0 μM) of drug to undergo death. We found that the use of 50-nM concentration of 5-azadC in the culture medium of growing cells (thus incorporated into the genomic DNA) completely inactivated the enzyme DNMT activity to methylate either of the substrates poly(dI–dC) and poly(dG–dC) in vitro, but the cells grew well. At higher dose around 250 nM, cells were growth arrested and changes in morphology were observed. The morphology of the cells (Fig. 1a, b and inset) around 250 nM to 5 μM indicates that this change is due to apoptosis induced by the drug. At lower dose (below 50 nM) of MG-132 (proteasome inhibitor) and DEVD-CHO (caspase-3 inhibitor), no effective cell growth retardation or arrest and no change in morphology have been observed.

Inactivation of DNMT1 causes DNA hypomethylation and activation of caspase-3, Bak, Bax, and Bel-XL and over-expression of Bcl2 and DNMT1

The expression of Bak, Bax, Bcl-XL, caspase-3, DNMT1, and HDAC1 as visualized by immunocytochemistry method is represented in Fig. 2a–f and summarized in Table 2. It is clear that the expression of pro-apoptotic factors and caspase-3 was triggered by higher doses of the 5-azadC and

![Fig. 3](image_url)  
**Fig. 3** RT–PCR: expression of mRNA of Bak, Bax, Bel2, and caspase-3 at various concentration of 5-azadC. β-Actin gene was used as positive control for this experiment (see “Experimental procedures” for further details).

![Fig. 4](image_url)  
**Fig. 4** Caspase-3 activity of purified enzyme, and 100, 250, 500 nM, and 5.0 μM 5-azadC-treated TSUPr1 cells lysates, and of 250 nM 5-azadC-treated TSUPr1 cell lysates in presence of caspase-3 inhibitor (Ac-DEVD-CHO). Among different TSUPr1 cell lysates, activity is maximum at 500-nM concentration of 5-azadC. A control (without drug treatment) TSUPr1 cell lysate had no caspase-3 activity and not shown in the figure.
the anti-apoptotic factor Bcl2, and DNMT1 expressions were increased significantly. This is understandable and can be explained in the way that, as DNA methylation is a post-replicative process and DNMT1 is trapped by DNA at N-5 of 5-azadC, cells produce more and more DNMT1. On the other hand, Bcl2 overexpression can be explained in the way that anti-apoptotic factor is fighting to keep the cell to strive under the threat imposed by higher concentration of the drug. mRNA expression of DNMT1 and Bcl2 was not decreased due to 5-azadC treatment up to 500 nM. We also tested the HDAC activity of the 50-nM-drug-treated cell lines and found a 15–20% decrease in cancer cell lines. The decrease in HDAC1 mRNA expression in cancer cell lines by 50 nM 5-azadC was confirmed by RT–PCR analyses. The 5-azadC dose-dependent expression of Bak, Bax, Bcl-X, and caspase-3 mRNA in prostate cancer cell line TSUPr1 is represented in Fig. 3. DuPro cell line also showed similar pattern. The proteasome inhibitor MG-132 at considerably higher concentration (>250 nM) induced cell death by blocking the ubiquitin–proteasome pathway and thus by the accumulation of unwanted protein components with the cytosol which eventually disturbs intracellular trafficking. We found that at reasonably lower concentration up to 50 nM of MG-132, the cell growth was not arrested.

Caspase-3 activity

Caspase-3 was induced in PC3, TSUPr1, and DuPro cell lines using 250 nM 5-azadC in culture medium. For drug treatment, exponentially growing cells were seeded at a density of ~10^6 cells/75 cm² flask. After 6 h, the cells were treated at different concentrations (nanomolar to micromolar) of 5-azadC and cells were collected for biochemical investigation when they were 90% confluent. Induction of caspase-3 gene and expression of the protein are visualized by RT–PCR and immunochemistry, respectively. However, it did not confirm that caspase-3 was functionally active. To confirm that caspase-3 was active, we measured the enzyme activity of the cellular extracts of cultured TSUPr1 cells at different concentrations of 5-azadC (Fig. 4 and Table 1). DEVD-pNA was used as the substrate and purified caspase-3 (Calbiochem) used as reference/positive control. It is apparent that even complete inhibition of DNMTase activity using AzadC in between 50 and 75 nM in culture (as reflected by in vitro measurement of DNMTase activity) does not result in caspase-3 activity (Table 1). Higher dose (>75 nM) of the drug is necessary for significant expression and activity of caspase-3, and we observed that at 250 nM concentration of 5-azadC the caspase-3 activity is optimum for characterizing apoptotic factors and reaches a maximum around 500 nM of 5-azadC. Use of DEVD-CHO as inhibitor of caspase-3, simultaneously with 5-azadC, prevented the cell death and in vitro caspase activity partially. Hence, our result supports that death was due to caspase-3 activation. TUNEL assay also confirmed that the cells died due to apoptosis (figure not shown).

**Discussion**

Overexpression of active DNA methyltransferase and deregulation of cell proliferation in conjunction with the suppression of apoptosis constitutes a minimal common platform for the initiation and progression of most neoplastic lesions. Cells of the normal adult prostate or those constituting primary prostate cancer are dependent on androgen/estrogen for survival and proliferation. Upon withdrawal of androgen, the rate of apoptosis overtakes the rate of cell proliferation, thereby causing involution of the normal prostate and regression of the tumor. On the other hand, estrogen has a palliative effect on prostate cancer. The essential role for aberrant DNA methylation, especially repression of androgen, estrogen, and progesterone, and many Y-chromosome specific genes associated with CpG-island promoter hypermethylation in the development of prostate cancer, and E-cadherin and CD44 inactivation associated with metastasis, led us to address the requirement for demethylation/hypomethylation induced re-expression and reversal of prostate cancer (Li et al. 2000, 2001; Wijermans et al. 2000; Nojima et al. 2001; Patra et al. 2001, 2002, 2003, 2008a; Dasari et al. 2002; Sasaki et al. 2002; Sato et al. 2003; DeSimone 2004; Lavelle et al. 2006; Yoo and Jones 2006; Hellebrekers et al. 2007; Lavelle et al. 2008; Table 3). In view of this, we had revisited the effects of 5-azadC as potential therapeutic agent for the treatment of prostate cancer. Our results clearly indicate that the enhanced expression of DNA methyltransferase and hyperactivity of the enzyme is associated with the disease prostate cancer (Patra et al.)
2001, 2002, 2003). 5-AzadC cannot prevent expression of DNMT (Patra and Bettuzzi 2009) or anti-apoptotic factor Bcl2 but helps in the expression of various steroid receptors like AR, ER, PR and E-cadherin, CD44 tumor suppressor, and cell cycle regulatory and various Y-chromosome-specific genes with unknown protein function (Table 3). The re-expression can lead to remission of the cancer. A higher dose of the drug can induce the expression of apoptotic factors and hence can be used for retardation of tumor growth. Apoptosis is characterized by stereotypic morphological changes like chromatin condensation, cytoplasmic shrinkage, lipid like phosphatidylserine (PS) exposure on cell membrane, membrane rupture and segregation of lipid raft-associated cell surface marker proteins, ziosis, and formation of apoptotic bodies (Ferguson et al. 1997; Gurumurthy et al. 2001; Jackson-Grusby et al. 2001; Patra and Patra 2003). The biochemical and molecular pathways involved in apoptosis can be approximately grouped as “private” pathways and “common” pathways. The “private” pathways induced by diverse insults converge on a “common” pathway comprising of a set of molecular components collectively known as caspases that activate a cascade of proteolytic events compelling to DNA fragmentation. Among the various insults by chemical agents and UV radiation, induced damage or FasL–FADD induced activation of pro-caspase-8 or p53 → Bax → cytochrome c induced activation of Apaf-1/caspase 9 ultimately leads to activation of caspase 3 and leads to death (apoptosis) (Gurumurthy et al. 2001; Patra 2008b). We have demonstrated that treatment of prostate cancer cell lines with 5-azadC overexpressed Bcl2 and DNMT1 in prostate cancer cell lines TSUPr1, DuPro, and PC3 dose dependently. The Bcl-2 gene has been implicated in a number of cancers, including melanoma, breast, prostate, and lung carcinomas, as well as schizophrenia and autoimmunity. It is also thought to be involved in resistance to conventional cancer treatment. This supports a role for Bcl2 in decreased apoptosis in the pathogenesis of cancer, including prostate cancer. DNMT1 overexpression in prostate and other cancers has been implicated to repress various cell cycle regulatory genes by promoter methylation and inactivation of genes. However, these two proteins could not prevent apoptosis of the respective cells (TSUPr1, DuPro, and PC3) at higher concentrations of the drug. The morphology of the 5-azadC-treated TSUPr1 and other cells is characteristic for apoptotic cells, a biochemical process initiated by expression of Bak and Bax and finally completely destructed by activated caspase-3 activity. 5-Aza-2’-deoxycytidine, an analog of cytosine base (Wijermans et al. 2000; Christian 2002; Sato et al. 2003; DeSimone 2004; Claus et al. 2005; Lavelle et al. 2006; Yoo and Jones 2006; Hellebrekers et al. 2007; Lavelle et al. 2008; Patra and Bettuzzi 2009), has the potential to kill tumor cells. The genotoxic stress imposed to the surrounding normal cells and epigenetic reprogramming due to inclusion of 5-azadC in the genome of the cells has bedeviled and questioned its therapeutic use (Ferguson et al. 1997; Ng et al. 2000; Kress et al. 2001; Li et al. 2004; Pakneshan et al. 2004; Jones and Baylin 2007; Patra and Bettuzzi 2007; Zhu and Yao 2007; Patra et al. 2010). Potent inhibitors of DNA methylation with less side effects and toxicity originating from bioflavonoids, including tea catechins and other death-inducing membrane raft orchestrating drugs, are emerging (Fang et al. 2003; Miyaji et al. 2005; Mollinedo and Gajate 2006; Patra and Bettuzzi 2007; Patra 2008b; Patra et al. 2008b; Patra and Szyf 2008).

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Conflict of interest The authors declare no conflict of interest.

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