Synergistic anti-breast cancer effect of a combined treatment with the methyl donor S-adenosyl methionine and the DNA methylation inhibitor 5-aza-2′-deoxycytidine

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DNA-demethylating agents activate tumor suppressor genes that are silenced by DNA methylation in cancer and are therefore emerging as a novel approach to cancer therapy. 5-aza-2′-deoxycytidine (5-azaCdR), the first representative of this class of drugs was approved for treatment of myelodysplastic syndromes and is currently being tested on other cancers including solid tumors. However, 5-aza-2′-deoxycytidine (5-azaCdR) could also induce methylated prometastatic genes by DNA demethylation and induce cancer cell invasiveness. Since 5-aza-2′-deoxycytidine is a potent cancer growth inhibitor, we tested whether combining it with a DNA-methylating agent, the methyl donor S-adenosyl methionine (SAM), would block the adverse demethylating activity of 5-azaCdR while maintaining its growth suppression effects. We show here using several invasive and non-invasive breast cancer cell lines that SAM inhibits global- and gene-specific demethylation induced by 5-azaCdR, prevents 5-azaCdR activation of prometastatic genes uPA and MMP2, resulting in inhibition of cell invasiveness while augmenting the growth inhibitory effects of 5-azaCdR and its effects on tumor suppressor genes. Combination of drugs acting on the DNA methylation machinery at different levels is proposed as a new strategy for epigenetic therapy of cancer.

Introduction

DNA methylation enzymes are deregulated in cancers, leading to changes in epigenetic programing (1). It has been proposed almost two decades ago that DNA methylation proteins are candidate targets for anticancer therapeutics (2). We have shown previously that some of the nodal anticancer pathways aberrantly activate DNA methyltransferase 1 (DNMT1), the enzyme that maintains the DNA methylation pattern (3). The main focus in the field has been on reactivation of the genes that are silenced by methylation and normally block cancer growth. DNA methylation inhibitors are emerging as a novel approach to treating cancer and the first DNA methylation inhibitor 5-aza-2′-deoxycytidine (VIDAZA) and its deoxyriboside analog 5-aza-2′-deoxycytidine (5-azaCdR) have been approved for treatment of certain leukemias (4). However, it has been known for decades that a hallmark of cancer is global hypomethylation (5). It was originally believed that global hypomethylation mainly targets repetitive sequences (6), but several prometastatic genes were shown previously to be hypomethylated in cancers as well (7–9). A recent delineation of the landscape of DNA hypomethylation in liver cancer revealed several thousands of gene promoters that were hypomethylated relative to adjacent normal liver tissues (10). The hypomethylated genes include members of functional gene pathways involved in cancer growth and metastasis (10). These data raise the possibility that DNA methylation inhibitors would induce metastasis in non-invasive cancers. Indeed, we have shown that 5-azaCdR induces invasiveness of non-invasive breast cancer cells in vivo and in vitro (11). This should point to a serious concern in further clinical use of DNA methylation inhibitors in treating cancer. In accordance with this hypothesis, we have shown previously that 5-azaCdR increases the invasiveness of non-invasive breast cancer cell lines in vitro and in vivo (12–14). We have shown that prometastatic genes uPA and MMP2 are demethylated in invasive breast cancers and that we could block the invasiveness and metastatic properties of breast cancer cells MDA-MB-231 (15,16) and prostate cancer cells PC-3 using the methyl donor S-adenosyl methionine (SAM). SAM treatment reversed the demethylated state of these prometastatic genes (15,16) either by providing extra methyl moieties to the DNMTs or by inhibiting DNA demethylation as we have shown previously (17). Both of these mechanisms could result in hypermethylation upon SAM treatment.

Since DNA methylation inhibitors are also potent inhibitors of tumor growth, it is important to examine whether it is possible to block the adverse effects of these inhibitors on cell invasiveness without affecting their growth inhibitory properties. We therefore reasoned that combining SAM and 5-azaCdR might block the adverse effects of 5-azaCdR. However, it is critical to test whether the combination of SAM and 5-azaCdR would not reverse the potential therapeutic effects of 5-azaCdR on inhibition of cancer growth. In this paper, we demonstrate that combining SAM and 5-azaCdR results in synergistic anticancer effects on human breast cancer cell lines: maintaining the antitumor activities of 5-azaCdR while reversing its proinvasive effects. As SAM is a nutritional supplement with very limited toxicity, the combination of 5-azaCdR and SAM is potentially a promising therapeutic approach to treatment of cancer.

Materials and methods

Cell culture and drug treatments

Human non-invasive breast cancer cell lines MCF-7 and ZR-75-1, and invasive breast cancer cell line MDA-MB-231 were purchased from American Type Culture Collection. MCF-7 cells were cultured in minimum Eagle’s medium with 10 μg/ml of insulin (Invitrogen). ZR-75-1 cells were cultured in RPMI-1640 medium. MDA-MB-231 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen). All media were supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For 5-azaCdR (Sigma) treatment, cells were grown in regular culture medium in the presence 0.05–5 μM of 5-azaCdR. SAM chloride (Sigma) was prepared in a buffer containing 0.005 M sulfuric acid and 10% ethanol. SAM (10, 50 or 100 μM) or equivalent volumes of buffer were added to regular culture medium. Media were refreshed daily over a period of 3 or 6 days. Data presented for the SAM treatment groups are deducted from the dissolution buffer background. In the case when more than one drug was used, both drugs were added simultaneously.

Boyden chamber Matrigel invasion assay

Invasion assays were carried out using the Invasion assay kit (Chemicon) following the manufacturer’s protocol. After the respective drug treatments, the cells were trypsinized and counted using trypsin blue exclusion assays to ensure that the same number (1.5 × 104) of viable cells was plated into the upper chambers of the Matrigel Boyden wells in serum-free media at the start of the assay. Five hundred microliters of regular culture media containing 10% fetal bovine serum was added to the lower chamber as chemoattractant. After incubation for 48 h at 37°C, the non-invaded cells that remained in the upper chambers were removed with cotton swabs. The invaded cells at the bottom of membrane were stained and counted under light microscope with ×400 magnification. Five randomly selected fields were counted and averaged.

Growth assays

The growth of cells was measured by trypsin blue exclusion assays. At the end of each treatment period, the cells were trypsinized and stained with 0.4% trypsin blue. Cells were placed in a hemocytometer and counted under microscope. Non-viable cells that were stained blue were excluded from counting.

Abbreviations: 5-azaCdR, 5-aza-2′-deoxycytidine; DNMT, DNA methyltransferase; SAM, S-adenosyl methionine.
Synergism of SAM and 5-aza-2′-deoxycytidine

Global DNA methylation was determined using luminometric methylation assay as described previously (18,19). Briefly, 1 μg of DNA was digested with either the methylation-sensitive enzyme HpaII (NEB) or the methylation insensitive enzymeMspI (NEB), respectively. EcoRI was added to both digestions as internal controls of DNA concentration. Digested DNA was then subjected to end filling with (either C for MspI and HpaII or A for EcoRI) using PyroMark™Q24 pyrosequencer. The percentage of methylation was determined by dividing the relative peak heights of HpaII to that ofMspI after normalization of each peak with the respective EcoRI peaks using the PyroMark™Q24 (Biotage) software.

RNA extraction and quantitative real-time PCR
RNA was extracted using TRIzol reagent as described previously (20). Reverse transcription was performed using 1 μg of RNA and 20 U of reverse transcribe (Roche) as recommended by the manufacturer. Two microliters of complementary DNA was used in a 20 μl reaction containing 1X SYBR green mix, 0.5 μM forward and 0.5 μM reverse primers. Reaction was performed in Roche LightCycler LC480 using the following conditions: denaturation 95°C for 10 min; amplification 95°C for 10 s; annealing temperature 10 s; extension 72°C for 10 s, 45 cycles; final extension 72°C for 10 min. Primer sequences for each gene are as follows: uPA (forward: 5′-TTC GGA GGG CAC TGT GAA ATA-3′; reverse: 5′-GCA TGG TAC GTC TGG TGA AGG ACA-3′), MMP2 (forward: 5′-TCA TTG GCT ACA CTC CGT-3′; reverse: 5′-CGC ACA AGG GGT ATC CAT-3′), p21 (forward: 5′-TGG AGA CTC TCA GGG TCG AAA-3′; reverse: 5′-GGG GTG TGG AGT AGG GAA-3′), CDKN2AIP (forward: 5′-ATC GAG TAG CAA TGA AGG-3′; reverse: 5′-GAT GCA GCT TCT GTC TTG-3′) and 18S (forward: 5′-CAC GGG AAA CCT CAC CGC GC-3′; reverse: 5′-CGG GTG GCT GAA GGC CAC TT-3′). Quantification was performed using the second derivative method by Roche LightCycler 480 software.

Western blotting
Forty micrograms of nucleic extracts were fractionated on a 12.5% sodium dodecyl sulfate–polyacrylamide gel and transferred to nitrocellulose membrane. Non-specific binding was blocked with 5% milk in Tris-buffered saline (TBSM) for antibodies against MMP2 (Millipore MAB13405), CDKN2AIP (Abcam ab140519) or phosphate-buffered saline (PBSM) for antibodies against uPA (Santa Cruz sc-14019) and p21 (Santa Cruz sc-397). After blocking, the membrane was incubated with specific antibodies in TBSTBM (TBSM with 0.05% Tween) or PBSTM (PBSM with 0.05% Tween) for 2 h followed by anti-mouse (Jackson Immunoresearch Labs) or anti-rabbit (TBSM with 0.05% Tween) for 2 h. After washing, the membrane was incubated with horseradish peroxidase-coupled secondary antibodies (Jackson Immunoresearch Labs) and was detected using chemiluminescence detection kit (Amersham Pharmacia Biotech). Nucleoprotein p62 was used as loading control and was detected using anti-Nucleoprotein p62 antibody (BD Bioscience 610498) in TBSTBM followed by anti-mouse immunoglobulin G (Jackson Immunoresearch Labs) in Tris-buffered saline with Tween 20.

Pyrosequencing
One microgram of DNA samples were subjected to bisulfate conversion as described previously (21). The converted DNA was cleaned up using the EpiTect Bisulfite Kits (Qiagen) according to the manufacturer’s protocol. For pyrosequencing, the bisulfite DNA was subjected to PCR amplification using biotinylated primers: uPA (forward: 5′-GGT TTT TTT AAA TTT TTG TGA G-3′; reverse: 5′-ACT ATC TCT CTC CTC TCT AAA CTC C-3′); nested forward: 5′-AGA GTG GTA GGA TAG GAG GAG T-3′; nested biotinylated reverse: 5′-Biotin/CCC TAC CAA AAC AAA TAA ACC CTA AC-3′; sequencing: 5′-GGG GTG GGT GGT ATG ATG ATA A-3′), MMP2 (forward: 5′-GAT TGG GGG AGG AGG GAA-3′; reverse: 5′-GATTT GAT TTG AGG TGT AGG-3′), p21 (forward: 5′-GAT GGG GGG AGG GAA-3′; nested biotinylated reverse: 5′-Biotin/TCC TCC ACT CTC GGG CAA AAT CTC-3′; sequencing: 5′-GGG GGG GTT GGG GGT ATG AAT-3′), 18S (forward: 5′-GAG AGT TGA CCA GCG GAT GAG-3′; reverse: 5′-GGG AGG GTT TTG ATA TAG-3′), PyroMark™Q24 (Biotage). Data were analyzed by the PyroMark™Q24 software.

Statistics
Student’s t-tests were performed to determine statistical significance of the results. Individual treatments were compared with untreated controls. In addition, we compared results obtained for 5-azaCdR and 5-azaCdR plus SAM. Significance threshold was set at P value <0.05 and indicated by asterisks (*).
Both of these genes encode proteins that are important in the control of cell cycle progression as well as the p53 pathway, which are often dysregulated in cancer (29). These genes have also been shown to play a major role in the antiproliferative effects of 5-azaCdR (30). Interestingly, SAM affected the prometastatic genes and the tumor suppressor genes differently. SAM did not inhibit induction of CDKN2AIP by 5-azaCdR, whereas p21 was induced to a higher level when both agents were combined (Figure 2B and C). These data illustrate that SAM is selective in inhibiting prometastatic genes and does not affect tumor suppressor genes, which could explain its synergistic growth inhibitory effect with 5-azaCdR.

SAM inhibits hypomethylation by 5-azaCdR in prometastatic genes but not in tumor suppressor genes

To determine whether the mechanism of action by which SAM silences prometastatic genes (Figure 2A) involves inhibition of hypomethylation induced by 5-azaCdR, we analyzed by bisulfite conversion and pyrosequencing the state of methylation of CpG sites in the promoters of uPA and MMP2 genes. These two prometastatic genes are poorly expressed (Figure 2A) and heavily methylated in several CpG sites in uPA promoter (sites 6 and 8) and the MMP2 promoter (sites 1–5; Figure 3A). While SAM treatment alone did not cause further hypermethylation of either gene, 5-azaCdR caused hypomethylation in almost all CpG sites studied (Figure 3A). In the uPA promoter, addition of SAM to the 5-azaCdR treatment suppressed 5-azaCdR-induced hypomethylation and reversed DNA methylation back to the control levels, or to a more hypermethylated state at almost all measured CpG sites (Figure 3A). The effect of SAM in the MMP2 promoter was highly selective and a complete reversal of hypomethylated state was observed in CpG site number 4 (Figure 3A). To understand the basis for the selectivity of SAM, we examined the state of methylation of promoters of the tumor suppressor genes p21 and CDKN2AIP (Figure 3B). Interestingly, in both cases, the promoters are unmethylated in control cells but nevertheless 5-azaCdR induces their expression (Figure 2B). Previous studies have shown that p21 promoter is unmethylated in most cancer cell lines and that 5-azaCdR could induce tumor suppressor genes expression through methylation-independent mechanisms (31,32). Remarkably, SAM does not trigger methylation.
Synergism of SAM and 5-aza-2′-deoxycytidine

The synergistic effect of SAM and 5-aza-2′-deoxycytidine (5-azaCdR) in tumor suppression and growth inhibition is explained by the fact that SAM, while not methylating DNA directly, enhances the DNA methyltransferase (DNMT) activity required for methylation. The corepressor complex formed by DNMTs and the promoter region of tumor suppressor genes is critical for their activation, which is methylated in cancer cells. SAM inhibits this complex, thereby preventing methylation and enhancing the expression of tumor suppressor genes.

The synergistic effect is observed in human breast cancer cell lines, including MCF-7, ZR-75-1, and MDA-MB-231. In MCF-7 cells, 5-azaCdR treatment induces the expression of uPA (von Willebrand factor A) and MMP2 (matrix metalloproteinase 2), which are involved in invasion and metastasis. SAM inhibits this induction and also augments the antigrowth effects of 5-azaCdR.

In ZR-75-1 cells, 5-azaCdR treatment induces the expression of uPA and p21, which are involved in cell cycle regulation and apoptosis. SAM inhibits this induction and also augments the antigrowth effects of 5-azaCdR.

In MDA-MB-231 cells, 5-azaCdR treatment induces the expression of uPA, which is involved in invasion and metastasis. SAM inhibits this induction and also augments the antigrowth effects of 5-azaCdR.

The synergistic effect of SAM and 5-azaCdR is not limited to breast cancer cells. It is observed in other tumor cell lines as well, indicating that this combination may have broad applicability in cancer therapy.

Discussion

DNA-demethylating agents are promising anticancer agents. These agents were shown to demethylate and induce the expression of tumor suppressor genes and display strong anticancer growth effect in vitro (33), in vivo and in the clinic (34). However, there is significant
Fig. 3. SAM reverses hypomethylation of prometastatic genes uPA and MMP2 triggered by 5-azaCdR treatment. DNA isolated from MCF-7 cells treated with either 100 μM SAM and/or 0.3 μM 5-azaCdR for 72 h was subjected to sodium bisulfite conversion as described in Materials and methods. Bisulfite-converted DNA samples were used as templates for PCR using biotinylated 5′ primers. The PCR product for (A) uPA and MMP2 and (B) p21 and CDKN2AIP genes was used for pyrosequencing using PyroMark™Q24 (Biotage). Graphs represent mean percentage methylation ± standard error of the mean at each CpG site from triplicate experiments (*P < 0.05, two-tailed Student’s t-test). A schematic diagram is shown for each sequenced region with gray bars indicating cytosine guanine areas (straight lines).

Fig. 4. Effects of a combination of SAM and 5-azaCdR on invasion, uPA expression and growth in ZR-75-1 cells. ZR-75-1 cells were treated with 100 μM SAM and 0.3 μM 5-azaCdR, alone or in combination, for 72 h (see Materials and methods). Untreated ZR-75-1 cells were used as controls. (A) Boyden chamber invasion assays were performed on treated cells and incubated for 48 h at 37°C. The graph shows the average number of invaded cells per field calculated from five randomly selected fields under ×20 magnification. The graph represents the mean number of cells from quadruplicate experiments (*P < 0.05, two-tailed Student’s t-test). (B) Quantitative reverse transcription–PCR analysis of uPA expression under different treatment conditions. (C) Treated ZR-75-1 cells were subjected to trypan blue exclusion assays at the end of the treatment. The graph represents the mean number of cells from duplicate experiments (*P < 0.05, two-tailed Student’s t-test).
Synergism of SAM and 5-aza-2′-deoxycytidine

Fig. 5. Effects of a combination of SAM and 5-azaCdR on invasion, uPA expression and growth in MDA-MB-231 cells. MDA-MB-231 cells were treated with 100 μM SAM and 5 μM 5-azaCdR, alone or in combination, for 6 days (see Materials and methods). Untreated MDA cells were used as controls. (A) Boyden chamber invasion assays were performed on treated cells and incubated for 48 h at 37°C. The graph shows the average number of invaded cells per field calculated from five randomly selected fields under ×20 magnification. (B) Quantitative reverse transcription–PCR analysis of uPA expression under different treatment conditions. (C) Treated MDA cells were subjected to trypan blue exclusion assays at the end of the treatment. The graph represents the mean number of cells from duplicate experiments (*P < 0.05, two-tailed Student’s t-test).

Evidence that the DNA methylation changes in cancer are not only limited to increased methylation but also includes a broad landscape of demethylated gene promoters (10). More importantly, the demethylated genes play an important role in migration and invasion. These functions are required for cancer metastasis, one of the most morbid aspects of cancer (10). This points to the possibility that DNA-demethylating agents such as 5-azaCdR could have adverse effects of increasing metastasis, which contraindicates their clinical utility.

The complexity of DNA methylation effects in cancer implies that single therapy targeting of DNA methylation enzymes would be inadequate, particularly since the changes in DNA methylation are complex and involve both increase and decrease in DNA methylation. A combination therapy that targets several aberrant processes in cancer might be required. We tested here the possibility that the adverse effects of DNA demethylation might be addressed by combining a DNA-demethylating agent with a DNA-methylating agent. Such a combinatorial anticancer therapy would be successful only if the methylating agent exhibits certain selectivity to prometastatic genes and reduced affinity to tumor suppressor genes. SAM is an attractive molecule to be combined with DNA-demethylating agents since it has been shown previously to suppress both growth and invasion in highly invasive cell lines (15,16). In addition, it is a natural compound that is a cofactor of methylation reactions in vivo. It is synthesized in humans from the methyl donors in diet and requires the availability of common vitamins such as vitamin B12 and folic acid (35). We therefore tested here first whether SAM will antagonize the prometastatic effects of 5-azaCdR, and second whether SAM would exert the same growth suppression effects in the presence of 5-azaCdR. We demonstrate here that SAM antagonizes the effects of 5-azaCdR on cell invasiveness (Figure 1B and D) and augments the antigrowth effects of 5-azaCdR (Figure 1C). SAM inhibits the global hypomethylation induced by 5-azaCdR (Figure 1E), suggesting that a component of its mechanism of action involves blocking partially the DNA demethylation induced by 5-azaCdR. SAM mechanism of action is selective since it blocks the induction of prometastatic genes by 5-azaCdR (Figure 2A) but does not inhibit tumor suppressor genes (Figure 2B). SAM as well as 5-azaCdR affects gene expression by methylation-dependent and -independent mechanisms. The silencing of uPA (Figure 3A) and MMP2 (Figure 3B) is associated with methylation of CpG sites in the promoter in a site-selective manner. However, SAM is a ubiquitous methyl donor that is required for histone and protein methylation, which could impact gene expression independent of DNA methylation. Although 5-azaCdR is an inhibitor of DNA methylation, the tumor suppressor gene p21 is induced by 5-azaCdR by a DNA methylation-independent mechanism that involves changes in histone modifications and upstream effectors (32,36). Interestingly, SAM treatment alone does not increase methylation compared with control cells but only inhibits demethylation that is induced by the demethylating agent 5-azaCdR (Figures 1E and 3). This supports the conclusion that elevated SAM should not affect normal processes nor increase methylation in genes that are not affected by 5-azaCdR. It also supports the hypothesis that SAM inhibits DNA demethylation in these breast cancer cell lines as has been previously suggested (17) rather than increasing methylation. The current study is limited to several examples of prometastatic and tumor suppressor genes. Future experiments along this line should include a more global view on the gene expression changes upon treatment with SAM.

The effect of SAM in inhibiting invasiveness and expression of uPA is also seen in a different non-invasive human breast cancer line ZR-75-1 (Figure 4A and B). Similar to the situation in MCF-7 cells, SAM augments 5-azaCdR inhibitory effects on cell growth (Figure 4C). We also measured the effects of SAM on 5-azaCdR-treated invasive human breast cancer cell line MDA-MB-231. Tumors naturally include invasive and non-invasive cells and it is therefore important to rule out adverse effects on these cells. SAM treatment alone has a growth inhibitory effect in these cells (Figure 5C), and it does not inhibit the growth inhibitory effect of 5-azaCdR. Interestingly, 5-azaCdR has opposite effects on invasion in invasive (Figure 5) and non-invasive (Figures 1 and 4) breast cancer cells. This could be explained by the different epigenetic matrix that 5-azaCdR is acting on in both cell types. In the non-invasive cells MCF-7 and ZR-75-1, 5-azaCdR induces prometastatic genes that are silenced by hypermethylation, thus leading to increased metastasis. In invasive MDA-MB-231 cells, the prometastatic genes uPA and MMP2 are hypomethylated as we have shown previously (12,15). 5-azaCdR is not expected to have any further effects on these genes. However, 5-azaCdR might be inducing metastasis inhibitory genes such as TIMP3 and SKY (37,38), which display promoter hypermethylation only in invasive phenotypes. Our data demonstrate that combining SAM with 5-azaCdR will inhibit the induced adverse invasiveness by 5-azaCdR without affecting its beneficial effects on growth in either invasive or non-invasive breast cancer cells. The differences in response to 5-azaCdR between invasive and non-invasive breast cancer cells have general implications for the general utility of epigenetic drugs in cancer therapy and other diseases as well. The consequences of epigenetic drug treatment will be different based on the cellular context and the epigenetic ‘history’ of the cells. This should add high cell selectivity to inherently non-selective drugs.

In summary, our data point to new therapeutic potential of combinations of epigenetic drugs that target different elements of the aberrant DNA methylation state in cancer. Our data suggest that with the appropriate combination it might be possible to block the adverse molecular
effects of DNA-demethylating agents while maintaining their clinical benefits. Combining 5-azaCdR or other demethylating agents with SAM is especially attractive since SAM is a nutritional supplement that has been widely used in the population. Future experiments should test this possibility in vivo using additional cancer models.

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**References**