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6-Thioguanine Reactivates Epigenetically Silenced Genes in Acute Lymphoblastic Leukemia Cells by Facilitating Proteasome-mediated Degradation of DNMT1

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Abstract

Thiopurines including 6-thioguanine (^{S}G), 6-mercaptopurine and azathioprine are effective anticancer agents with remarkable success in clinical practice, especially in effective treatment of acute lymphoblastic leukemia (ALL). ^{S}G is understood to act as a DNA hypomethylating agent in ALL cells, however, the underlying mechanism leading to global cytosine demethylation remains unclear. Here we report that ^{S}G treatment results in reactivation of epigenetically silenced genes in T leukemia cells. Bisulfite genomic sequencing revealed that ^{S}G treatment universally elicited demethylation in the promoters and/or first exons of the genes that were reactivated. ^{S}G treatment also attenuated the expression of histone lysine-specific demethylase 1 (LSD1), thereby stimulating lysine methylation of the DNA methylase DNMT1 and triggering its degradation via the ubiquitin-proteasomal pathway. Taken together, our findings reveal a previously uncharacterized but vital mechanistic link between ^{S}G treatment and DNA hypomethylation.

Keywords

6-Thioguanine; Acute lymphoblastic leukemia; Gene expression; DNMT1; lysine-specific demethylase 1

Introduction

In mammalian cells, methylation of DNA at the C5 of cytosine at CpG dinucleotide is one of the major epigenetic modifications that play important roles in embryonic development,

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gene regulation, cell differentiation, and genomic imprinting (1–2). Aberrant methylation within CpG islands in the genome links to genomic instability and leads to the development of many diseases, including cancer (3–4). Promoter CpG methylation is generally correlated with gene silencing. Previous studies showed that tumor suppressor genes are silenced due to methylation of CpG islands in their promoter regions (5–6). Therefore, promoter cytosine demethylation and the resultant reactivation of silenced genes in cancer cells are feasible approaches to cancer therapy.

DNA methylation in mammalian cells is established and maintained by a family of DNA (cytosine-5)-methyltransferases (DNMTs) including DNMT1, DNMT3A, and DNMT3B (7). DNMT1 functions primarily as a maintenance DNA methyltransferase responsible for methylating hemimethylated CpG sites following DNA replication (8–9), whereas DNMT3A and DNMT3B exhibit *de novo* methyltransferase activity that establishes DNA methylation patterns (10–11). Dysregulation in DNMT1 was thought to play a critical role in cellular transformation (12). Along this line, constitutive over-expression of an exogenous mouse DNMT1 results in a significant increase in global DNA methylation which is accompanied by tumorigenic transformation in NIH 3T3 mouse fibroblasts (13). In contrast, DNMT1 knockouts are resistant to colorectal tumorigenesis (14), and knockdown of DNMT1 by either antisense or siRNA results in demethylation and activation of tumor suppressor genes (15–16). DNMT1 is up-regulated in multiple human cancers (17–18) and previous studies showed that the regulatory regions of tumor suppressor genes are hypermethylated in tumors (19). Therefore, DNMT1 has been proposed as a target for anticancer therapy (12). Indeed, preclinical studies using antisense to DNMT1 have shown inhibition of tumor growth both *in vitro* (16) and *in vivo* (20).

Thiopurine drugs, which were first synthesized and investigated by Elion and co-workers (21–22), are widely used as anticancer and immunosuppressive agents and they have achieved remarkable success in clinical practice, especially for acute lymphoblastic leukemia (ALL) treatment (23–27). ^{S}G is the ultimate active metabolite of all thiopurine prodrugs. It was proposed that ^{S}G exerts its cytotoxic effect via its incorporation into DNA, its subsequent methylation by *S*-adenosyl-L-methionine (*S*-AdoMet) to render S^6-methylthioguanine (S^6mG), which directs the misincorporation of dTMP during DNA replication (27). The resulting S^6mG:T mispair can trigger the post-replicative mismatch repair (MMR) pathway and futile cycles of repair synthesis may ultimately induce cell death (27). On the other hand, the very low level of conversion of DNA ^{S}G to S^6mG (<0.02%) in ^{S}G-treated leukemia cells and the relatively high mutagenic potential of ^{S}G itself suggest that DNA ^{S}G may trigger the MMR pathway without being converted to S^6mG (28–30). However, the proposed MMR-related mechanism may not be the only pathway for ^{S}G to exert its cytotoxic effect during ALL treatment viewing that MMR-deficient ALL cells were also sensitive toward ^{S}G (31).

Our recent study showed that the treatment of Jurkat-T cells with ^{S}G could lead to a significant decrease in global cytosine methylation (32). Likewise, the level of cytosine methylation in newly synthesized DNA decreased in MOLT-F4 human malignant lymphoblastic cells and HEK-293 T cells upon treatment with ^{S}G or 6-MP (33–34). In addition, treatment of HEK-293T cells with ^{S}G or 6-MP could elicit a decrease in the enzymatic activity of DNMT1 in the whole cell lysate and a drop in the level of DNMT1 protein (34). However, the mechanism through which the ^{S}G induces decreases in DNMT1 protein level and global cytosine methylation remains unclear.

Recent reports revealed that the stability of DNMT1 was regulated by the ubiquitin-proteasome pathway (35), which involves the methylation of lysine residues in DNMT1 through a dynamic interplay between histone lysine methyltransferase Set7 (36–37) and

histone lysine-specific demethylase 1 (LSD1) (37). These studies provided a mechanistic link between DNA and histone methylation systems. Additionally, DNMT1 was found to be rapidly and selectively degraded upon treatment with 5-azacytidine (5-aza-C) or 5-aza-2'-deoxycytidine (5-aza-CdR) through the ubiquitin-proteasomal pathway (35). Therefore, we reason that the ^{S}G-induced decrease in DNMT1 may occur through a similar mechanism. In the present study, we demonstrated that ^{S}G could reactivate epigenetically silenced genes in leukemic cells by facilitating proteasome-mediated degradation of DNMT1. In addition, this process involves the down-regulation of LSD1, which established the mechanism underlying the ^{S}G-induced hypomethylation in leukemic cells.

Materials and Methods

Cell culture

Jurkat-T, CEM, HEK-293T, and HL-60 cells (ATCC) were cultured under the ATCC-recommended conditions. Jurkat-T and HEK-293T cells were treated with 3 μM ^{S}G (Sigma) and/or 25 μM MG132 (Enzo Life Sciences International, Plymouth Meeting, PA), which is a proteasome inhibitor. The whole-cell extracts were prepared by suspending cells in CelLytic^{TM} M (Sigma) lysis buffer containing protease inhibitor cocktail (Sigma). Genomic DNA from the cultured cells was isolated by extraction with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and desalted by ethanol precipitation. Total RNA was extracted from the cultured cells by using RNAeasy mini kit (Qiagen, Valencia, CA).

HPLC quantification of global cytosine methylation

The level of global cytosine methylation was measured using our previously established method (32). Briefly, genomic DNA (~50 μg) was digested with 2 units of nuclease P1 and 0.008 unit of calf spleen phosphodiesterase in a buffer containing 30 mM sodium acetate (pH 5.5) and 1 mM zinc acetate at 37°C for 4 h. To the digestion mixture were then added 12.5 units of alkaline phosphatase and 0.05 unit of snake venom phosphodiesterase in a 50-mM Tris-HCl buffer (pH 8.6). The digestion was continued at 37°C for 3 h, and the enzymes were removed by chloroform extraction. The amount of nucleosides in the mixture was quantified by UV absorbance measurements. The mixtures were then separated by HPLC on an Agilent 1100 capillary pump (Agilent Technologies, Palo Alto, CA) with an Agilent 1100 UV detector monitoring at 260 nm. A 4.6×250 mm Polaris C18 column (5 μm in particle size, Varian Inc., Palo Alto, CA) was used. A solution of 10 mM ammonium formate (pH 4.0, solution A) and a mixture of 10 mM ammonium formate and acetonitrile (70:30, v/v, solution B) were employed as mobile phases. A gradient of 5 min 0–4% B, 45 min 4–30% B and 5 min 30–100% B was used, and the flow rate was 0.80 mL/min. Under these conditions, we were able to resolve 5-methyl-2′-deoxycytidine (5-mdC) from other nucleosides. The global cytosine methylation in cells was quantified based on the peak areas of 5-mdC and 2′-deoxycytidine (dC) with the consideration of the extinction coefficients of the two nucleosides at 260 nm (5020 and 7250 L mol ^{1} cm ^{1} for 5-mdC and dC, respectively).

Bisulfite genomic sequencing analysis

Genomic DNA was treated with sodium bisulfite by using EZ DNA Methylation Kit (Orange, CA). Amplified PCR products for RPIB9 (Rap2-binding protein 9), PCDHGA12 (protocadherin-γ subfamily A member 12), DCC (deleted in colorectal cancer) and asparaginase were subcloned using the pGEM-T cloning system (Promega). PCR primers were listed in Table S1. Approximately 15 colonies for each gene were sequenced using an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

Quantitative real-time RT-PCR

cDNA was synthesized by using iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's recommended procedures. Briefly, 1 μg of total RNA was reverse-transcribed with 1 μL iScript reverse transcriptase and 4 μL 5×iScript reaction mixture in a 20 μL reaction volume. The reaction was carried out at 25°C for 5 min and at 42°C for 30 min. The reverse transcriptase was then deactivated by heating at 85°C for 5 min.

Quantitative real-time qRT-PCR was performed using iQ™ SYBR Green Supermix kit (Bio-Rad) on a Bio-Rad iCycler system (Bio-Rad), and the running conditions were at 95°C for 3 min and 45 cycles at 95°C for 15 s, 56°C for 30 s and 72°C for 45 s. The comparative cycle threshold (Ct) method (ΔΔCt) was used for the relative quanti cation of gene expression (38), and GAPDH gene was used as the internal control. The mRNA level of each gene was normalized to that of the internal control. The primers for real-time PCR were list on Table S2.

RNA interference assay, quantitative real-time RT-PCR and Western blot analysis

LSD1 siRNA (5'-UGAAUUAGCUGAAACACAAUU-3') and siGENOME Non-Targeting siRNA (D-001210-02-05) were obtained from Dharmacon (Lafayette, CO). HEK-293T cells were transfected with 50 nM siRNA along with Lipofectamine 2000 (Invitrogen) following the manufacturer's recommended procedures. Briefly, cells were seeded in 6-well plates at 70% confluence (~3×10^5 cells per well) and transfected with 100 pmol synthetic duplex siRNAs using Lipofectamine 2000 reagent. Cells were incubated for 48 h, after which total RNA and cellular extracts were prepared. Quantitative real-time RT-PCR was conducted using the same procedures as described above. Primer sequences used for RT-PCR were shown in Table S2, and GAPDH was used as an internal control. Cell extracts were subjected to Western blot analysis; antibodies that specifically recognized human DNMT1 (New England Biolabs), DNMT1-K142me (36), LSD1 (Cell Signaling, Danvers, MA), β-actin (Abcam, Cambridge, MA) were used at 1:3000, 1:1000, 1:10,000 and 1:10,000 dilutions, respectively. Horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (Abcam) was used at a 1:10,000 dilution.

Results

^{S}G treatment leads to decrease in global cytosine methylation in cultured human cells

Thiopurine drugs have been successfully employed for treating acute lymphoblastic leukemia (ALL) (27) and a number of genes in bone marrow leukocytes from ALL patients and in two ALL cell lines (Jurkat-T and NALM-6) were found to be epigenetically silenced (39). Viewing that the mean peak concentration of ^{S}G in plasma of ALL patients was 0.52 ± 0.72 μM after oral ^{S}G administration (60 mg/m^2) and 2.7 ± 1.4 μM after continuous intravenous infusion (20 mg/m^2/h) (40), we treated Jurkat-T cells with 3 μM ^{S}G from 6 to 24 h and assessed the level of global cytosine methylation by using an HPLC method (Figure S1 shows a typical HPLC trace for monitoring the global cytosine methylation) (32). It turned out that treatment with 3 μM ^{S}G for 6 and 24 h led to appreciable decreases in global cytosine methylation in Jurkat-T cells, i.e., the percentage of cytosine methylation dropped from 3.74 ± 0.02% in untreated cells to 3.57 ± 0.12%, 3.52 ± 0.08% and 3.34 ± 0.03% in cells treated with 3 μM of ^{S}G for 6, 12 and 24 h, respectively (Figure 1A). Likewise, ^{S}G exposure could lead to loss in global cytosine methylation in other human cell lines. In this regard, treatment of HEK-293T, HL-60 and CEM cells with 3 μM of ^{S}G for 24 h resulted in decreases in cytosine methylation from 3.61 ± 0.16%, 3.63 ± 0.04% and 4.05 ± 0.14% to 3.46 ± 0.03%, 3.29 ± 0.07%, and 3.75 ± 0.11%, respectively (Figure 1B).

^{S}G treatment resulted in the reactivation of genes silenced in ALL cells

Previous large-scale CpG methylation analysis revealed that the promoter regions of 11 genes were aberrantly methylated in primary leukocytes from ALL patients and in cultured ALL cells (39). To determine whether the ^{S}G-induced global cytosine hypomethylation can reactivate the expression of these genes, we examined, by using quantitative real-time RT-PCR, the mRNA levels of these genes in Jurkat-T cells before and after ^{S}G treatment. It turned out that mRNA expression levels of all these genes increased in Jurkat-T cells after ^{S}G treatment (Figure 1C). For instance, there were more than 4-fold increases in mRNA levels of DCC, KCNK2, LRP1B, NKX6-1, NOPE, PCDHGA12 and RPIB9 genes after treatment with ^{S}G for 48 h.

^{S}G induced cytosine demethylation in promoter and/or the first exon of genes silenced in ALL cells

To gain insights into the mechanisms responsible for the reactivation of the silenced genes upon ^{S}G treatment, we performed bisulfite genomic sequencing of the first exon of PCDHGA12 gene, the predicted promoter and the first exon of RPIB9 gene, and the predicted promoters of the asparaginase and DCC genes in Jurkat-T cells (Figure 2). These regions were chosen because the methylation status of the promoter and the first exon of genes are important for the epigenetic regulation of gene expression (5,39) and the expression levels of these genes were substantially elevated after ^{S}G treatment (Figure 1C). Consistent with the global cytosine methylation and quantitative real-time RT-PCR results, the methylation level in the promoter and/or first exon of these examined genes dropped by 7–16% upon ^{S}G treatment, suggesting that ^{S}G could induce the demethylation in the predicted promoter and/or the first exon of genes thereby stimulating the expression of these genes.

^{S}G treatment led to the proteasomal degradation of DNMT1

To further explore the molecular mechanism underlying the ^{S}G-induced cytosine demethylation, we determined the protein levels of DNMT1 in the extracts of cells treated with ^{S}G for various time periods since DNMT1 is the major maintenance cytosine methyltransferase in mammalian cells. Immunoblot analysis of cell extract indeed revealed a time-dependent decrease in DNMT1 level following ^{S}G treatment (Figure 3A).

To determine whether the drop in DNMT1 level was due to the down-regulation of its transcription, we measured the mRNA level of DNMT1 by real-time RT-PCR (Figure 3B). Upon treatment with 3 μM ^{S}G, the DNMT1 transcript level was similar to that of the control cells (Figure 3B). Thus, the ^{S}G-induced decrease in DNMT1 protein level was not due to a decline in its mRNA level.

A previous report showed that DNMT1 could be rapidly degraded upon 5-aza-CdR treatment through the ubiquitin-proteasomal pathway (35). We reasoned that the ^{S}G-induced DNMT1 degradation might also occur through the same pathway. If this is the case, the drug-elicited DNMT1 degradation should be rescued by pre-treating the cells with a proteasome inhibitor. It turned out that treatment of cells with proteasome inhibitor MG132 indeed restored the percentage of global cytosine methylation (Figure 1A) and abolished the ^{S}G-mediated decrease in DNMT1 protein level (Figure 3A), supporting that the ^{S}G-induced degradation of DNMT1 involved the proteasomal pathway.

^{S}G could induce the down-regulation of LSD1 and the increase in lysine methylation in DNMT1

Recent studies revealed that the stability of DNMT1 protein was dynamically regulated by its methylation on lysine residues via histone lysine methyltransferase Set7 (36–37) and

histone lysine-specific demethylase 1 (LSD1) (37). Thus, we further investigated whether the ^{S}G-mediated degradation of DNMT1 involved the alteration in the protein level of Set7 and/or LSD1. It turned out that the treatment of Jurkat-T cells with ^{S}G led to a decrease in LSD1 at both the mRNA and protein levels (Figure 4A and 4C); the ^{S}G-induced down-regulation of LSD1 in Jurkat-T cells was not restored by MG132 at either mRNA or protein level (Figure 4B and 4D), which pointed to the transcriptional regulation of LSD1. However, there was no apparent alteration in Set7 level in Jurkat-T cells treated with ^{S}G (Figure S2). In addition, siRNA knockdown of LSD1 in HEK-293T cells (Figure S3) induced the degradation of DNMT1 as did ^{S}G (Figure S4 A and B), revealing that ^{S}G may trigger DNMT1 degradation via diminishing the LSD1 expression.

It was reported that K142 was a major site responsible for the regulation of DNMT1 stability (36). Next, we assessed whether ^{S}G treatment could result in the alteration of K142 methylation in DNMT1. Immunoblot results showed that there was no significant change in K142-methylated DNMT1 in Jurkat-T or HEK-293T cells upon treatment with ^{S}G (Figure S5 and Figure S4C). However, the total amount of DNMT1 protein decreased markedly upon ^{S}G treatment (Figure 3A), suggesting that the percentage of K142-methylated DNMT1 increased upon ^{S}G treatment. In addition, the methylated DNMT1 protein was rapidly degraded by the ubiquitin-proteasome pathway, which may account for the lack of accumulation of the methylated DNMT1 upon ^{S}G treatment. In this vein, it was found that the methylated DNMT1 had a much shorter half-life (2–6 h) than its unmethylated counterpart (12–24 h) (36). Taken together, our results demonstrated that ^{S}G could decrease the expression of LSD1, which led to enhanced lysine methylation in DNMT1 and its subsequent degradation via the proteasomal pathway.

Discussion

We investigated the epigenetic effect of ^{S}G and discovered a novel mechanism underlying the ^{S}G-induced global cytosine demethylation in ALL cells. Our results demonstrated that ^{S}G exerted its epigenetic effect by downregulating the expression of LSD1, thereby enhancing the lysine methylation level in DNMT1 and triggering its degradation via the proteasomal pathway. The diminished DNMT1 expression led to subsequent promoter demethylation and reactivation of epigenetically silenced genes in ALL cells.

Treatment of Jurkat-T cells with ^{S}G resulted in elevated expression of 12 genes, 11 of which were previously shown to be epigenetically silenced in primary ALL cells and two ALL cell lines (39). Although the explicit roles of these genes in the pathobiology of ALL remain unclear, the epigenetically silenced state of these genes in ALL cells and their reactivation upon ^{S}G treatment suggest that these genes might serve as important molecular targets for ALL treatment and act as biomarkers for monitoring the efficacy of ALL treatment. ABCB1, RPIB9 and PCDHGA12 have functions that may be associated with patient response to ALL chemotherapy (41–42). DCC, DLC-1 and LRP1B were identified as tumor suppressor genes and were aberrantly methylated in cancer cells (43–45). Bisulfite sequencing analysis revealed the drug-induced demethylation in the putative promoter and/or the first exon of DCC, asparaginase, RPIB9, and PCDHGA12 genes, which provided insights into the mechanisms accounting for the elevated mRNA expression of these genes in Jurkat-T cells after ^{S}G treatment.

An interesting observation is that the expression level of the asparaginase gene was increased upon ^{S}G treatment. Different from normal cells, ALL cells are unable to synthesize the non-essential amino acid asparagine (46); thus, the survival of leukemic cells depends on circulating asparagine. This forms the basis of using *E. coli* asparaginase in the clinical treatment of ALL (46–47), where the enzyme catalyzes the decomposition of L-

asparagine to L-aspartic acid and ammonia. In current protocols of ALL treatment, asparaginase, along with other drugs, are often used in the remission-induction phase, whereas methotrexate plus mercaptopurine are frequently used in consolidation treatment (48). Our real-time PCR results showed a 3-fold increase in asparaginase expression upon ^{S}G treatment at 24 h, which may further deprives the leukemic cells of asparagine and contributes to the killing of residual leukemic cells during the consolidation treatment. Thus, our results underscored a potentially new pathway contributing to the antileukemic effect of ^{S}G. It is important to investigate in the future whether the same finding can be made for ALL patients administered with the thiopurine drug.

It has been recently demonstrated that LSD1 was able to demethylate and stabilize DNMT1 protein from its degradation via the ubiquitin-proteasome pathway (37). By using metabolic labeling method, the authors found that the methylation level of DNMT1 was markedly increased in Aof2$^{1lox/1lox}$ (LSD1-deficient) cells when compared to Aof2$^{2lox/+}$ (LSD1-proficient) cells (37). These results underscored enhanced methylation of DNMT1 protein in the absence of LSD1, suggesting that DNMT1 is susceptible to LSD1-mediated lysine demethylation *in vivo*. The stability of DNMT1 was also regulated by the histone methyltransferase activity of Set7 through the methylation of DNMT1 at K142 (36). In the current study, we found that LSD1 was decreased at both the mRNA and protein level, but there was no significant change in Set7 level upon ^{S}G treatment, revealing that the diminished expression of LSD1 may lead to enhanced DNMT1 methylation. The methylated DNMT1 can then be subjected to degradation via the ubiquitin-proteasome pathway. Taken together, this study offers a rational explanation for the demethylation in DNA and reactivation of silenced genes by ^{S}G and underscores a new epigenetic effect of ^{S}G on leukemia treatment.

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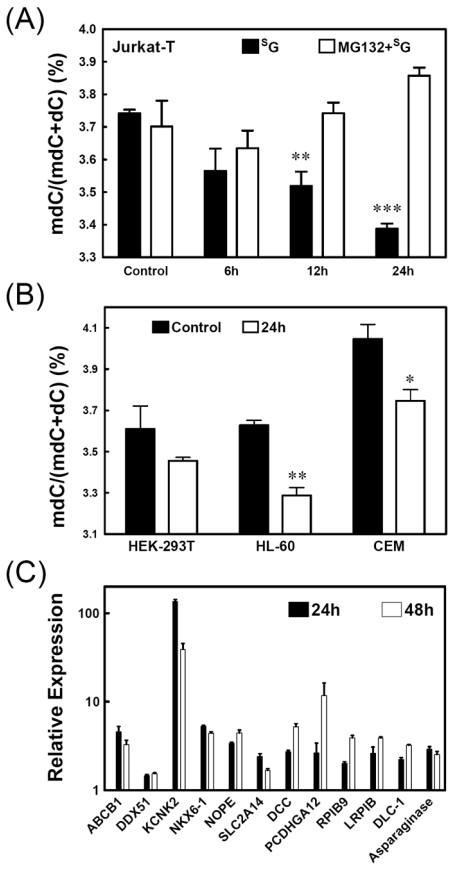

Figure 1. ^{S}G treatment results in decreased global cytosine methylation and increased expression of epigenetically silenced genes in human cells

(A) The percentages of global cytosine methylation in genomic DNA isolated from Jurkat-T cells that were either treated with ^{S}G alone or pretreated with MG132 (25 μM) for 2 h and then treated with ^{S}G (3 μM) for the indicated time periods. (B) The percentages of global cytosine methylation in genomic DNA isolated from HEK-293T, HL-60 and CEM cells that were untreated or treated with ^{S}G (3 μM) for the time periods indicated. (C) Change in mRNA expression in Jurkat-T cells following treatment with ^{S}G for 24 h (white bar) or 48 h (black bar). The data represent the means and standard deviations of results from three independent drug treatments. The paired *t*-test was performed to evaluate the difference between control samples and treated samples in (A) and (B) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

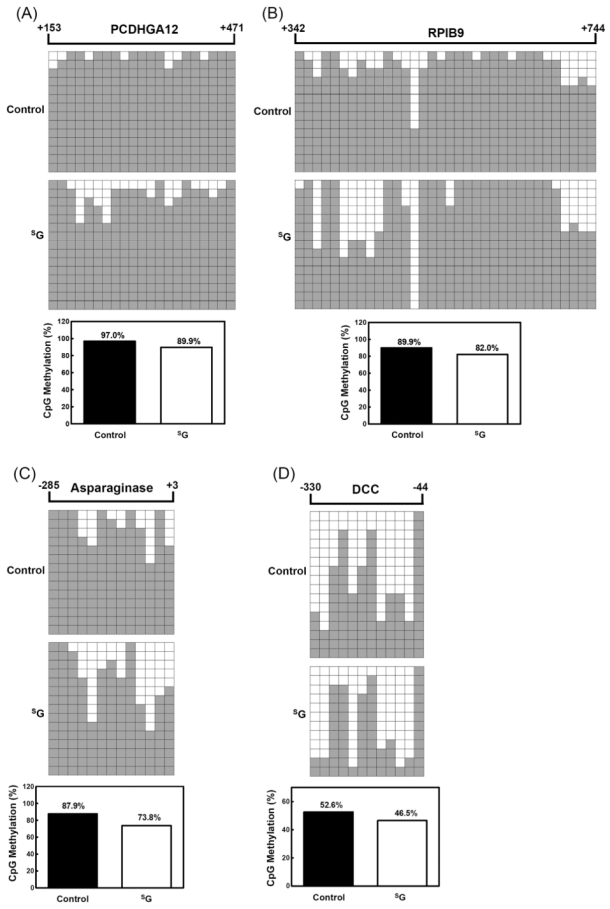

Figure 2. Bisulfite genomic sequencing shows that ^{S}G treatment leads to decreased methylation at CpG sites in the predicted promoter region and/or the first exon of genes

(A) PCDHGA12; (B) RPIB9; (C) Asparaginase; (D) DCC. Each square represents a single CpG site. The number of rows designates the number of the colonies sequenced. White and black squares represent unmethylated and methylated CpGs, respectively. The percentage of CpG methylation is listed underneath each figure.

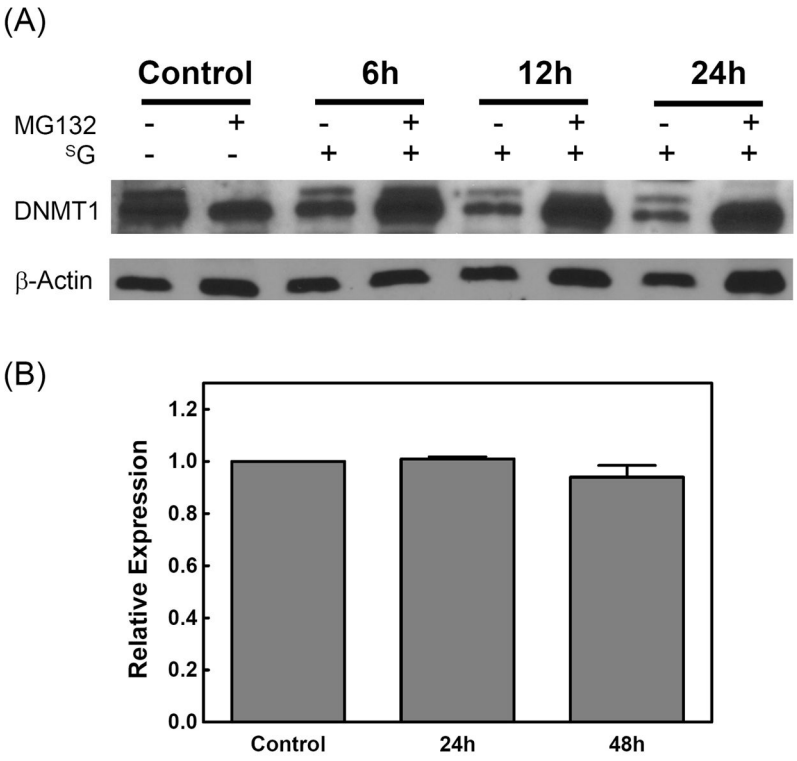

Figure 3. DNMT1 was degraded upon ^{S}G treatment and the degradation could be blocked by a proteasomal inhibitor

(A) Western blot analysis of DNMT1 with whole-cell extracts from Jurkat-T cells treated with ^{S}G (3 μM) alone or pretreated with MG132 (25 μM) for 2 h and then treated with ^{S}G for the indicated time periods. β-actin was used as a loading control. (B) Expression of DNMT1 mRNA is not reduced in cells treated with ^{S}G. The mRNA level of DNMT1 in Jurkat-T cells treated with ^{S}G (3 μM) for 24 or 48 h was analyzed by real-time RT-PCR. The bar diagram shows the fold change in the mRNA level of DNMT1. The results represent the means and standard deviations of data from three independent experiments.

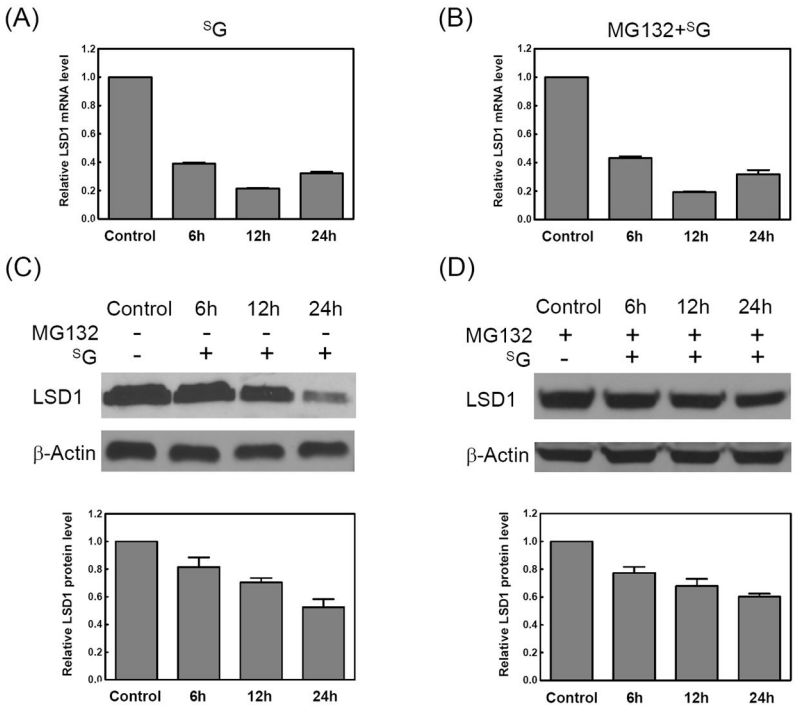

Figure 4. LSD1 was degraded upon ^{S}G treatment

(A) Real-time RT-PCR analysis of the expression of LSD1 mRNA in cells treated with ^{S}G for various time periods. (B) Real-time RT-PCR analysis of the expression of LSD1 mRNA in cells pretreated with MG132 (25 μM) for 2 h and then treated with ^{S}G (3 μM) for various time periods. The results represent the means and standard deviations of data from three independent experiments. (C) Western blot analysis of LSD1 with whole-cell extracts from Jurkat-T cells treated with ^{S}G (3 μM) for various time periods. (D) Western blot analysis of LSD1 with whole-cell extracts from Jurkat-T cells pretreated with MG132 (25 μM) for 2 h and then treated with ^{S}G for various time periods. The histograms shown under (C) and (D) are the LSD1 fold changes, which were obtained by normalizing the band intensity of LSD1 to that of the loading control, β-actin. The results represent the means and standard deviations of data from three independent drug treatment and Western blot experiments.