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Original article

Olsalazine inhibits cell proliferation and DNA methylation in canine lymphoid tumor cell lines

S. Itoh[1,2], J. Yamazaki[3,4], M. Iwahana[2], A. Tsukamoto[2]

[1] Laboratory of Biology, Azabu University,
School of Veterinary Medicine, 1-17-71 Fuchinobe, Chuou-ku, Sagamihara, Kanagawa 252-5201, Japan
[2] Laboratory of Laboratory Animal Science, Azabu University,
School of Veterinary Medicine, 1-17-71 Fuchinobe, Chuou-ku, Sagamihara, Kanagawa 252-5201, Japan
[3] Veterinary Teaching Hospital, Graduate School of Veterinary Medicine,
Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo, Hokkaido, 060-0808, Japan
[4] One Health Research Center, Hokkaido University.
Kita 18, Nishi 9, Kita-ku, Sapporo, Hokkaido, 060-0808, Japan

Abstract

Abnormal DNA methylation is involved in the initiation and progression of lymphoid tumors. Hence, DNA demethylating agents are promising candidate drugs for chemotherapy against these tumors. The salicylic acid derived anti-inflammatory agent, olsalazine, reportedly suppresses DNA methyltransferase in human cells and has the potential to be clinically applied as a DNA demethylating agent. In this study, we investigated the effects of olsalazine on cell proliferation and DNA methylation using canine lymphoid tumor cell lines (CLBL-1, GL-1, and UL-1). Treatment with olsalazine led to significant cell growth inhibition and increased the apoptotic rate in all three cell lines. Treatment with olsalazine reduced the total amount of 5-methylcytosine in genomic DNA, as assessed by enzyme-linked immunosorbent assay. Genome-wide analysis of DNA methylation revealed that 1,801 to 5,626 CpG sites showed decreased DNA methylation levels in three cell lines, including the promoter regions of ADAM23, FES, and CREB3L1 genes. The outcomes of the present study demonstrate that a DNA demethylating agent olsalazine, inhibits cell proliferation and DNA methylation in canine lymphoid tumor cells, suggesting that it can be a candidate drug for the treatment of lymphoid tumors in dogs.

Key words: DNA methylation, lymphoid tumors, olsalazine, canines

Introduction

Lymphoid tumors, such as lymphoma and acute lymphoid leukemia, are common tumors in dogs. The current standard treatment for canine lymphoma and acute lymphoid leukemia is CHOP-based chemotherapy (Moore 2016, Bennett et al. 2017). However, cases that do not respond to standard therapy have a poor prognosis, and even when remission is achieved, relapses occur due to the development of resistance. Additionally, serious adverse reactions may prevent the use of suitable chemotherapy. Although some CHOP variations and other chemotherapy protocols have been proposed, the overall clinical outcome has remained

Correspondence to: A. Tsukamoto, e-mail: a-tsukamoto@azabu-u.ac.jp. tel.: +81 42 754 7111
J. Yamazaki. e-mail: j.yamazaki@vetmed.hokudai.ac.jp. tel.: +81 11 706 5319
S. Itoh and A. Tsukamoto contributed equally to this work.

unchanged (Valerius et al. 1997, Hosoya et al. 2007). Therefore, new candidate drugs are needed for chemotherapy against lymphoid tumors in dogs.

DNA methylation plays a key role in disease mechanisms, such as tumorigenesis, where the de novo hypermethylation of tumor suppressor genes has been identified in neoplastic tissues (Herman et al. 1994, Merlo et al. 1995). Global hypomethylation also occurs frequently in tumors and may facilitate chromosome instability, leading to the formation of abnormal chromosomal structures (Eden et al. 2003). Recent advances in the genome-wide analysis of DNA methylation have revealed aberrant methylation in a variety of human diseases (Figueroa et al. 2009, Noushmehr et al. 2010). Aberrant DNA methylation has also been identified in dogs with tumors such as melanoma and lymphoma (Yamazaki et al. 2018, Ishizaki et al. 2020, Ohta et al. 2020). In human medicine, epigenetic therapies have emerged as promising anticancer approaches for various types of tumors. DNA demethylating agents inhibit DNA methyltransferase enzymes, reducing the overall levels of DNA methylation in cancer cells. Inhibition of DNA methylation causes the upregulation of apoptosis- and cell cycle-associated genes and induces cell death in tumor cells, demonstrating its antitumor effect (Mehdipour et al. 2020). Representative DNA demethylating agents clinically approved in human medicine are 5-azacitidine and decitabine. In humans, DNA demethylating agents are used for the treatment of myelodysplastic syndromes, acute myeloid leukemia, and diffuse large B-cell lymphoma (Pera et al. 2016, Mohammad et al. 2019, Ribeiro et al. 2019). With recent advances in the study of epigenetics in canine cancer, DNA demethylating agents are expected to be candidates for chemotherapies in veterinary medicine as well (Hahn et al. 2012, Fujiwara-Igarashi et al. 2014, Harman et al. 2016).

Olsalazine is a salicylic acid derived anti-inflammatory agent that is mainly used for the treatment of ulcerative colitis in humans (Murray et al. 2020). Olsalazine has also been used as an anti-inflammatory agent for the treatment of inflammatory bowel disease in dogs (Malewska et al. 2011). The advantage of salicylic acid-derived anti-inflammatory agents, including olsalazine, is that they have fewer known adverse reactions than other anti-inflammatory agents such as corticosteroids. Additionally, previous studies have also suggested the novel clinical efficacy of olsalazine for cancer therapy. Administration of olsalazine inhibited cell proliferation and induced apoptosis in a rodent model of colorectal cancer (Brown et al. 2000). Moreover, olsalazine has been suggested to be a DNA hypomethylating agent. A combination of computational and cell-based screening indicated that olsalazine has a structure similar to that of conventional DNA demethylating agents and inhibited DNA methyltransferase (DNMT) in HeLa cells (Mendez-Lucio et al. 2014).

In this study, we investigated the anti-tumor effects of olsalazine on canine lymphoid tumor cells. Furthermore, the role of olsalazine in DNA demethylation was evaluated, and its underlying mechanism for a potential anti-tumor effect was assessed using genome-wide analysis.

Materials and Methods

Cell cultures

Three canine lymphoid tumor cell lines (B cell type: CLBL-1 and GL-1; T cell type UL-1) were used in this study. All cell lines were grown in RPMI 1640 (Wako Pure Chemical Industries Ltd., Osaka, Japan) supplemented with 10 % fetal calf serum and 1% penicillin/streptomycin (Sigma Aldrich, St Louis, MO, USA), and cultured at 37°C in a humidified atmosphere of 5% CO_2. Blood was collected from healthy beagles, and peripheral blood mononuclear cells (PBMCs) were separated using Ficoll/Hypaque gradient centrifugation (Lymphoprep; Abbott Diagnostics Technologies AS, Norway). This study was approved by the Institutional Animal Care and Use Committee of Azabu University (Approval No. 200108-7).

Reagents

Olsalazine was purchased from Tokyo Chemical Industry (Japan) and dissolved in dimethyl sulfoxide (DMSO). In each assay, the final concentration of DMSO was adjusted to 0.1 %.

Cell viability assay

The cytotoxic effect of olsalazine was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo, Japan), as previously described (Tomiyasu et al. 2014). Each cell line was seeded at 2.5×10^5 cells/mL in 96-well plates. The cells were exposed to olsalazine for 48 h. CCK-8 reagent was added to each well, and the plates were incubated for 4 h. After incubation, the absorbance of each well was measured at 450 nm using a microplate reader (Power Scan HT, DS Pharma Biomedical, Japan). The 50% inhibitory concentration (IC_{50}) values for olsalazine were determined in each cell line.

Apoptosis assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to assess olsalazine-induced apoptosis. Each cell line

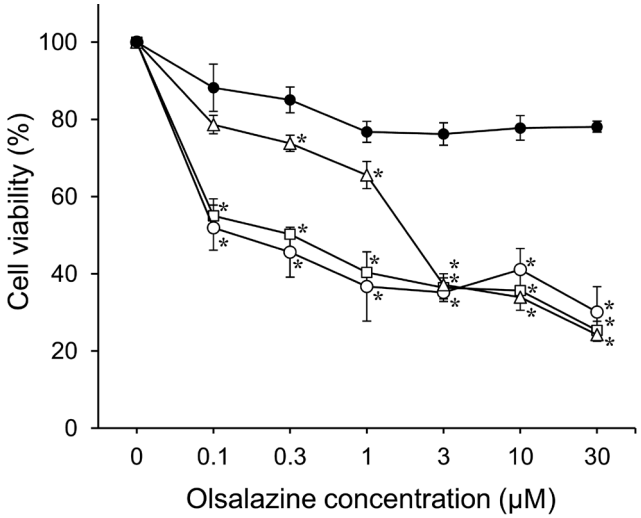

Fig. 1. Cell viability in canine lymphoid tumor cell lines treated with olsalazine and drug vehicle.
○: CLBL-1, □: GL-1, △: UL-1, ●: canine peripheral blood mononuclear cells (PBMCs). Each cell was treated with olsalazine (0-30μM) for 48 h. * Statistically significant differences ($p<0.05$) were observed between PBMC and each lymphoid tumor cell line.

was treated with olsalazine at an IC_{50} concentration and incubated for 24 h. After incubation, 2.5×10^5 cells were subjected to the cytospin process. The TUNEL assay was performed using the DeadEnd™ Fluorometric TUNEL System (Promega Corporation, WI, USA) according to the manufacturer's instructions. 4',6-diamidino-2-phenylindole (DAPI) staining was also performed for nuclear counterstaining. Positive cells were counted in five consecutive high-power fields (HPFs) on each slide, and the rate of apoptosis was calculated.

Quantitative analysis of 5-methylcytosine (5-mC) in genomic DNA

To evaluate DNA methylation levels after olsalazine treatment, the total amount of 5-mC in genomic DNA was measured by enzyme-linked immunosorbent assay (ELISA). 1.0×10^6 cells of each cell line were seeded and pre-cultured for 24 h. Olsalazine was then added at 0.03 μM. After 48 h, the pellet was precipitated by centrifugation (4 °C, 300 × g, 10 min). Genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Inc., Hilden, Germany) and stored at -20°C. Genomic DNA was diluted to 20 ng/μL with distilled water. Methylated DNA was measured using a 5-mC DNA ELISA kit (ZYMO RESEARCH Corp, CA, USA) with absorbance at 450 nm in a plate reader (POWERCAN HT, DS Pharma Biomedical Co., Ltd., Japan).

Digital restriction enzyme analysis of methylation (DREAM)

Genome-wide DNA methylation analysis using next-generation sequencing was performed as previously described (Yamazaki et al. 2018). We used the University of California, Santa Cruz (UCSC) definition of CpG islands (CGI): GC content of 50% or greater, length > 200 bp, a ratio greater than 0.6 of the observed number of CG dinucleotides to the expected number based on the number of Gs and Cs in segment (Gardiner-Garden and Frommer 1987). Sites at promoter regions were defined as being located within 1 kb upstream of transcription start sites (TSS) of given genes annotated by the Ensemble database (Flicek et al. 2014).

Statistical Analyses

Cell viability of cancer cell lines and PBMCs were compared using a two-way analysis of variance (ANOVA), followed by a Student's t-test with Bonferroni correction. Student's t-test was used for the analysis of TUNEL assay and 5mC DNA ELISA. $p<0.05$ was considered statistically significant. All analyses were performed using commercially available statistical software (Stat Mate IV; ATMS Co., Ltd., Tokyo, Japan).

Results

Cell viability assay

The results of the cell viability assay are shown in Fig. 1. Treatment with olsalazine significantly reduced the viability of all three lymphoid tumor cell lines. IC_{50} (μM) for olsalazine in CLBL-1, GL-1, and UL-1 were 0.09, 0.21, and 1.64, respectively. The viability of these cancer cell lines was markedly lower than that of PBMCs from healthy dogs.

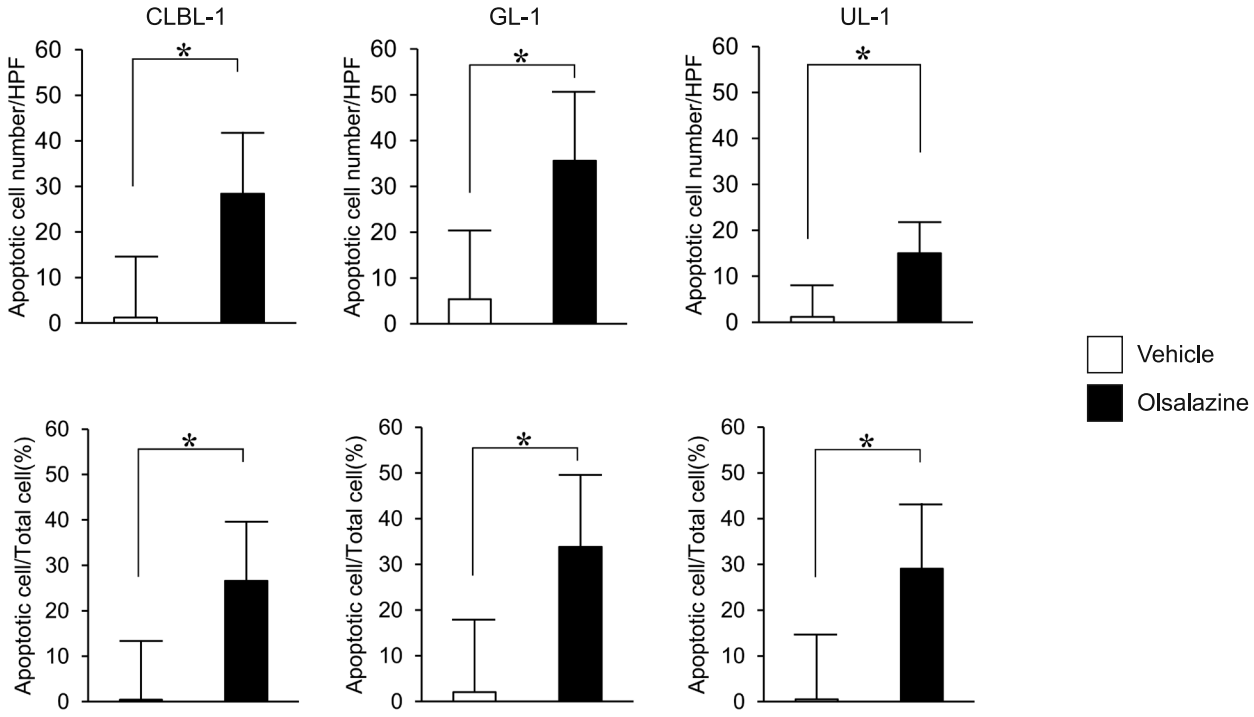

Fig. 2. The number and rate of apoptotic cells in canine lymphoid tumor cell lines treated with olsalazine. Cells were treated with olsalazine at IC_{50}, and the number and rate of apoptotic cells were compared with vehicle treated cells. * $p<0.05$

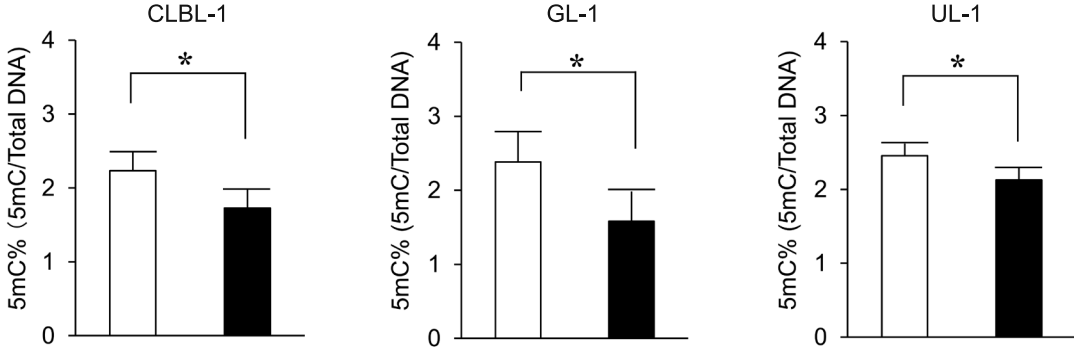

Fig. 3. Effect of olsalazine on 5-mC levels of genomic DNA in canine lymphoid tumor cell lines. * $p<0.05$

Apoptosis assay

The TUNEL assay was performed to assess the apoptosis induction of olsalazine at a concentration of IC_{50}, which was determined by the cell viability assay. In all cell lines, treatment with olsalazine significantly increased the number and rate of apoptosis compared to that of the vehicle (Fig. 2).

5-mC levels in genomic DNA

To gain insight into the link between the cytotoxic effect of olsalazine and DNA methylation, changes in the total amount of methylated cytosine (5-mC) in genomic DNA after olsalazine treatment were evaluated by ELISA (Fig. 3). The total amount of 5-mC was 2.2 %, 2.4 %, and 2.5 % in CLBL-1, GL-1, and UL-1, respectively, with DMSO treatment. Olsalazine treatment significantly reduced the methylated cytosine amounts to 1.7 %, 1.6 %, and 2.1 % in CLBL-1, GL-1, and UL-1, respectively.

DREAM

Finally, genome-wide analysis of DNA methylation by DREAM was performed to identify CpG sites demethylated by olsalazine treatment. A total of 66,656 CpG sites were analyzed in all three cell lines. Fig. 4 shows the effect of olsalazine treatment compared to the control DMSO, showing that the changes in DNA methylation status were greater in the order of CLBL-1, UL-1, and GL-1. Particularly, we found 1,801 to 5,626 CpG sites that decreased DNA methylation levels by more than 10 % in the three cell lines. The number of demethylated CpG sites was higher in CLBL-1 and UL-1 than in GL-1 (Table 1). In addition, the effect

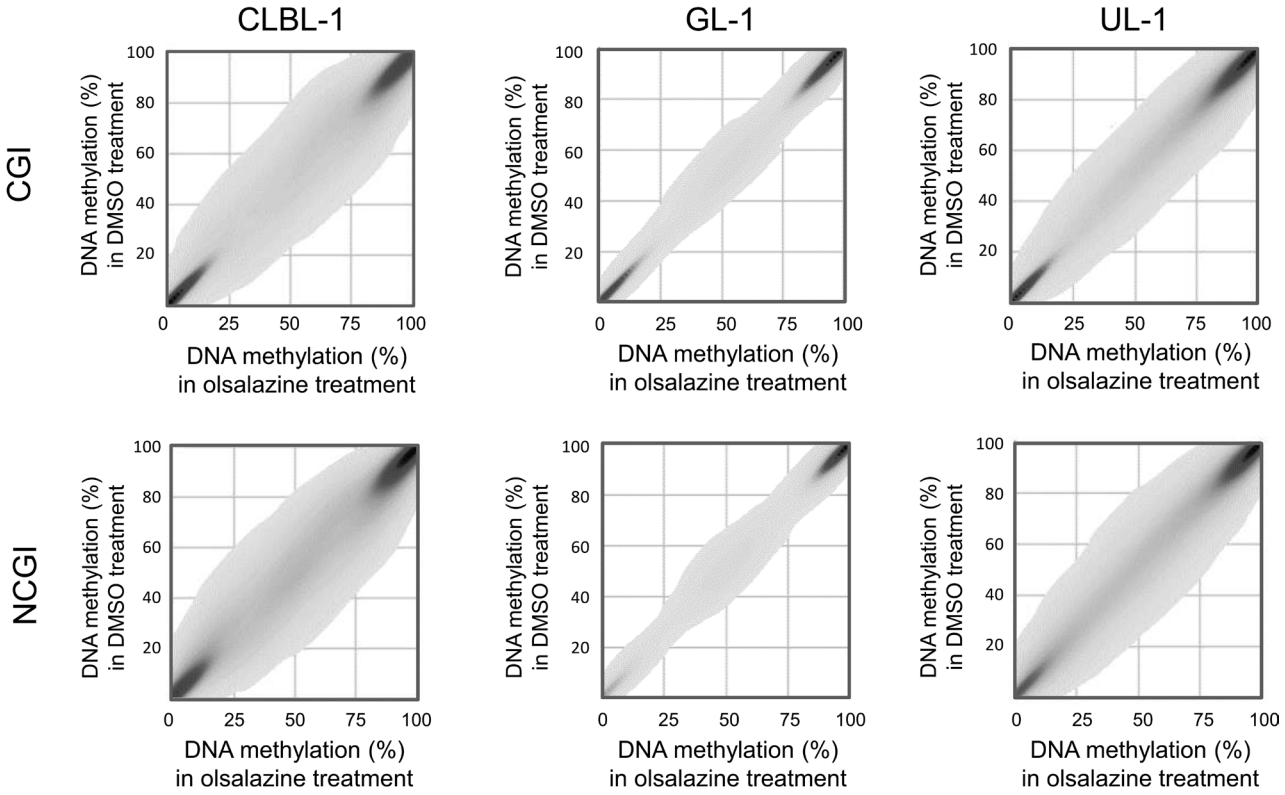

Fig. 4. Smooth scatter plots for DNA methylation levels.
26,840 CpG sites in CpG island (upper) and 39,813 non-CpG island (lower) are plotted separately. DNA methylation levels of GL-1, CLBL-1, and UL-1 treated with olsalazine are plotted on the x-axis; DNA methylation levels of GL-1, CLBL-1, and UL-1 without olsalazine are plotted on the y-axis.

Table 1. Nnumber of demethylated CpG sites after treatment with olsalazine in canine lymphoid tumor cell lines.

Cell		Number of demethylated CpG sites	Rate (%) of demethylated CpG sites
CLBL-1	CGI	1,878	7
	NCGI	3,748	9.4
	Total	5,626	8.4
GL-1	CGI	639	2.4
	NCGI	1,162	3
	Total	1,801	2.7
UL-1	CGI	1,416	5.3
	NCGI	3,344	8.4
	Total	7,760	7.1

of olsalazine was more prominent in NCGI (Non-CpG island) than in CGI in all three cell lines. We examined the overlap of CpG sites that were demethylated in the three cell lines and found that 17 sites (0.03 % of all CpG sites) were common to all cell lines and a total of 820 sites (1.2 % of all CpG sites) were common in both pairs of two cell lines (Fig. 5). Finally, we focused on promoter regions of genes where demethylation by olsalazine treatment may have had a direct effect on gene expression. Of the 837 CpG sites that were found to be demethylated in more than one cell line, 25 were in the promoter region of genes including ADAM23, FES, and CREB3L1 (Table 2).

Discussion

In this study, we demonstrated the anti-tumor action of olsalazine in canine lymphoid tumor cells. In addition, we also showed that olsalazine decreased DNA methylation in these cells. This study provides new insights into drug repurposing of olsalazine as an anti-cancer drug and DNA demethylating agent in dogs.

Initially, the anti-tumor action of olsalazine was assessed using a cell viability assay and TUNEL assay. Treatment with olsalazine inhibited cell viability and induced apoptosis in all three canine lymphoid tumor cell lines. In addition, the IC_{50} of olsalazine in PBMCs

Table 2. Genes with demethylated CpG sites in canine lymphoid tumor cell lines.

Gene	Chromosome	Coordinate	CLBL-1 DMSO	CLBL-1 Olsalazine	CLBL-1 differences	GL-1 DMSO	GL-1 Olsalazine	GL-1 differences	UL-1 DMSO	UL-1 Olsalazine	UL-1 differences
ACYP1	chr8	48169289	73.2	62.9	10.3	99.4	99.6	-0.2	93.8	65.2	28.6
ADAM12	chr28	35485580	56.4	38.8	17.7	98.7	98.4	0.3	53.9	39.1	14.8
ALPL	chr2	77615130	70.4	54.1	16.2	96.7	99.5	-2.9	97.3	71.3	26
cfa-mir 8797	chr36	1545897	77.3	66.7	10.6	93.7	96.1	-2.3	74.8	61.6	13.2
FES	chr3	53357449	91.3	81.2	10.1	13.5	18.6	-5.1	35.3	22	13.3
GPR182	chr10	1142711	84.6	62.5	22.1	99.2	98.8	0.4	79.8	69.1	10.7
GRB7	chr9	22748608	71.8	61	10.8	96.9	96.9	0	62.8	23.3	39.5
MISP	chr20	57844001	17.9	7.4	10.5	94.4	88.9	5.5	74.1	62.3	11.9
NKAIN3	chr29	12502952	89.8	66.1	23.7	98.5	98.5	0	83.5	68.7	14.8
PENK	chr29	7762563	73.9	59.1	14.8	43.3	48.2	-4.8	80.2	67.1	13.1
PLCXD2	chr33	16342857	56.7	24.8	32	99.3	99.2	0.1	88.3	75.7	12.6
PROKR1	chr10	67547219	41.2	28.2	13	35.8	45.1	-9.3	42	26.6	15.4
REN	chr38	755823	86	75	11	14.2	9.8	4.5	72.8	58.6	14.3
VAV2	chr9	50142265	61.6	39.1	22.5	13.5	9.6	3.9	88	75.4	12.6
XKR5	chr16	58853169	78.6	66.1	12.4	99.3	99.4	-0.1	77.1	64.4	12.7
ADAM23	chr37	14996397	79.5	68.7	10.8	52	36.9	15.1	68.1	67.6	0.5
CAV2	chr14	55437229	36	18.3	17.7	91	80.4	10.6	99.6	99.7	-0.1
PSD2	chr2	35026068	49.5	29.1	20.5	98.8	87.8	11	1.4	1.6	-0.2
SLCO4A1	chr24	46543907	61.4	40.4	21	98.3	87.7	10.6	76.9	90.7	-13.8
CACNG6	chr1	103244566	24.7	19.1	5.6	55.8	41.5	14.3	27.3	16.5	10.8
CREB3L1	chr18	43173905	0.7	1.7	-1	93.6	82.1	11.5	15.1	5	10.1
DYSF	chr17	51219300	21.2	12.1	9.1	96.8	86.5	10.3	91.9	75.5	16.3
MS4A13	chr18	55740541	87.2	85.5	1.7	58	47.8	10.2	87.9	66.7	21.2
PDGFA	chr6	16273718	74.1	72	2.1	94.8	83.7	11	80.2	67.6	12.6
REXO4	chr9	49837000	1	0.7	0.3	62.3	50.8	11.5	47.9	37.3	10.6

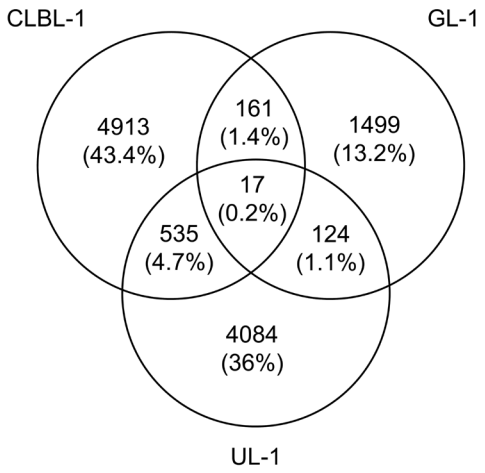

Fig. 5. Venn diagram showing the overlap of hypomethylated CpG sites at promoter regions of genes among GL-1, CLBL-1, and UL-1 after olsalazine treatment.

was markedly higher than that of lymphoid tumor cells, suggesting that this drug is less cytotoxic to normal cells. Based on the results of IC_{50}s in each cell line, CLBL-1 was found to be the most sensitive to olsalazine, and UL-1 was the least sensitive. This difference may be associated with the characteristics of each cell line. CLBL-1 is derived from diffuse large B-cell lymphoma and is more sensitive to common anti-cancer agents than GL-1, derived from B-cell leukemia (Pawlak et al. 2014). UL-1, which is derived from T-cell lymphoma, overexpressed ABCB1, and its expression level was higher than that in GL-1 and CLBL-1 (Tomiyasu et al. 2014). The IC_{50} of olsalazine in these lymphoid tumors was within the range of 0.09-1.64 μM. To date, pharmacokinetics and blood concentration after olsalazine administration have been demonstrated only in humans, and its effects in dogs are not fully understood. Olsalazine consists of two 5-aminosalicylic acid (5-ASA) molecules joined by an azo bridge. After oral administration, olsalazine is decomposed into 5-ASA in the large intestine, while only a part is absorbed from the intestine as olsalazine and is transferred to the bloodstream (Campbell and Berglindh 1988). In humans, the Cmax of olsalazine after oral administration was equivalent to 0.5 μM (Ryde and Ahnfelt 1988). One report demonstrates the antitumor action of olsalazine in vivo, using a rodent model of colorectal cancer. In that report, treatment with olsalazine increased the rate of tumor apoptosis, and reduced the rate of tumor cell proliferation. However, the exact mechanism of olsalazine action in cancer cell viability has not been clarified in vivo. Alteration of DNA methylation is associated with tumor invasion and anti-tumor immunity systems (Mohammad et al. 2014, Hogg et al. 2020). Following the results obtained from our in vitro study, further in vivo studies are needed to assess the efficacy of olsalazine in the chemotherapy of lymphoid tumors in dogs.

In this study, we analyzed the total amount of methylated cytosine in genomic DNA by ELISA and found a reduction in 5-mC in canine lymphoid cell lines treated with olsalazine. We also performed a genome-wide analysis to identify the individual CpG sites with DNA methylation changes following olsalazine treatment. The analysis revealed that 1,801 to 5,626 CpG sites (2.7% to 8.4% of all CpG sites analyzed) showed decreased DNA methylation levels of more than 10% compared to DMSO treatment. This frequency of demethylated CpG sites is consistent with a previous report of human myelodysplastic syndrome (MDS), where approximately 4% of CpG sites were demethylated (Grovdal et al. 2014). Intriguingly, DNA methylation analysis in a human colon cancer cell line at the single-cell level revealed that every cell had a different set of fully demethylated genes by azacitidine treatment, while overall methylation levels of these genes were very limited in bulk cells because of the averaging effect (Takeshima et al. 2020). Similar complex mechanisms of subcellular heterogeneity in demethylation events by olsalazine may also exist in canine lymphoma cell lines.

Finally, we addressed the promoter demethylation found in three cell lines using olsalazine. Although there were only 17 CpG sites that were commonly demethylated in all three cell lines, a total of 837 CpG sites (1.3 % of all CpG sites analyzed) were identified to be demethylated in at least two cell lines. Similarly, the overlap of demethylated genes between the two human ovarian tumor cell lines was found in only about 2.5 % of CpG sites (Tomar et al. 2016). Since even the baseline DNA methylation without olsalazine was different in the cell lines, the effects of demethylation may vary depending on individual tumor cells/patients. In addition, as IC_{50} was also found to be different among cell lines, the optimal concentration of olsalazine that

induced DNA demethylation could also be cell line dependent. It was previously reported that changes in DNA methylation were different between 24 h and 48 h after azacitidine treatment in human MDS patients (Tobiasson et al. 2017). Treatment conditions must be thoroughly examined to gain more insight into the presumably complex event of DNA demethylation by olsalazine.

Under the conditions used in this study, olsalazine treatment resulted in the demethylation of promoter regions of genes such as ADAM23, FES, and CREB3L1. ADAM23, a cell adhesion factor, is hypermethylated in human breast, pancreatic, brain, and colon cancers (Choi et al. 2009). FES, a tyrosine kinase, is normally expressed in myeloid cells and recently recognized as a tumor suppressor gene whose hypermethylation has been reported in human colon cancer (Shaffer and Smithgall 2009). CREB3L1 is a CREB/ /ATF transcription factor that is hypermethylated in bladder and breast cancers and also functions as a metastasis suppressor (Rose et al. 2014). Interestingly, all three genes were found to be downregulated by DNA hypermethylation and reactivated by DNA methylation inhibitors in tumor cell lines. It will be necessary to investigate whether the demethylation of these genes by olsalazine treatment in this study is associated with the reactivation of gene expression to understand the mechanism of the anti-tumor effect of this drug.

In summary, olsalazine shows anti-tumor action in canine lymphoid tumor cells by DNA demethylation, suggesting that olsalazine is a candidate drug for the chemotherapy of lymphoid tumors in dogs. Further studies are required to clarify the association between the cytotoxic effects and DNA demethylation induced by olsalazine.

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