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Original Article

Comparison of the effects of olsalazine and decitabine on the expression of CDH1 and uPA genes and cytotoxicity in MDA-MB-231 breast cancer cells

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Abstract

Background and purpose: Since DNA methyltransferase enzymes play a key role in DNA methylation, they can be used as a target to alter epigenetic changes and treat cancer. Recent studies have shown that olsalazine, through its potent inhibitory effect on the DNA methyltransferase enzyme, can be a good option. The aim of this study was to investigate the effects of olsalazine on cell viability and expression of CDH1 and uPA genes in MDA-MB-231 cells compared with decitabine.
Experimental approach: The cytotoxicity of the drugs was determined using a standard MTT assay. MDA-MB-231 cells were treated with olsalazine and decitabine with concentrations less than IC_{50} to evaluate the effect of drugs on the expression of genes. RNA was extracted from the cells after 24 and 48 h and CDH1and uPA gene expression were evaluated by quantitative real-time polymerase chain reaction method.
Findings/Results: The cytotoxicity of the two drugs was comparable. The IC_{50} values at 24 h were 4000 and 4500 μM for olsalazine and decitabine, respectively. The IC_{50} values of both drugs were about 300 μM at 48 h. Statistical analyzes showed a significant increase in CDH1 expression after 24-48 h treatment with olsalazine, and 48 h treatment with decitabine, without any significant increase in uPA expression.
Conclusion and implications: Our results showed that olsalazine has cellular toxicity comparable to decitabine in MDA-MB-231 cells. Also compared to decitabine, olsalazine causes a greater increase in expression of CDH1 without any significant increase in uPA expression. Therefore, it appears to be a good candidate for cancer treatment.

Keywords: Cancer; CDH1; Decitabine; Epigenetic; Olsalazine; uPA.

INTRODUCTION

DNA methylation is considered a key epigenetic mechanism that leads to the suppression of gene expression (1). This change plays an important role in the development of various diseases, including cancer. Cancer disease is generally due to changes in the expression and activity of the tumor suppressor genes and oncogenes. Studies have shown that hypermethylation of DNA in the promoter region of tumor suppressor genes has been associated with suppression of their expression in cancer and thus tumor progression and invasion (1-3). Also, the hypomethylation of oncogenes causes their re-expression and as a result, leads to cancer development (4).

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The cadherin1 gene (CDH1), which encodes a transmembrane glycoprotein called E-cadherin, plays a significant role in the intercellular attachment of epithelial tissue. It has been proved that promoter methylation but not mutation can suppress the expression of CDH1gene in cancer. Downregulation of E-cadherin is related to epithelial-to-mesenchymal transition and increases the invasive characteristics of cancerous cells; as it has been revealed in several breast cancer cell lines including MDA-MB-231 (5,6). Therefore, preventing the suppression of the expression of this gene or inducing its re-expression in cancer cells can be considered as a potential therapeutic target in cancer.

Owing to the reversible nature of epigenetic alterations, there is a great interest in correcting these defects *via* epigenetic therapy. At the present, researchers suggest the use of DNA methyltransferase (DNMT) inhibitors to resolve epigenetic defects, as it has been reported that aberrant DNA methylation usually results from changes in the expression or activity of DNMT enzymes (2,7). The DNMT inhibitors are structurally divided into three groups of nucleoside analogues, non-nucleoside analogues, and antisense oligonucleotides (2). Several potential DNMT inhibitors are in the clinical and pre-clinical trials. Decitabine (5-aza- 2’ - deoxycytidine) is one of the nucleoside analogues that has been approved by the FDA (2,8). Evidence has shown that treatment with decitabine leads to the re-expression of genes suppressed by methylation (4).

On the other hand, it has been observed that decitabine may also induce the expression of oncogenes and prometastatic genes, such as urokinase plasminogen activator (uPA) (9). The uPA gene expresses a protein from the serine protease family that catalyzes the conversion of plasminogen into the active form of plasmin. Plasmin activation leads to the digestion of many of the extracellular matrix components, such as laminin, fibronectin, and collagen. Studies have shown that uPA gene is commonly found to be methylated and inactivated in normal cells and the early stages of cancer, but in invasive forms of cancer, including breast cancer, prostate and other organs, could become demethylated and actively expressed (10-12).

Currently, studies are ongoing to identify compounds that can inhibit DNMT enzymes, with the aim of using them as helpful drugs in cancer treatment. Among these compounds, which have been investigated using *in silico* methods, olsalazine has been introduced as a potent DNMT inhibitor (13, 14). Olsalazine or azo-di-salicylate is a derivative of 5-amino-salicylic-acid and an anti-inflammatory drug that is orally used to treat inflammatory bowel disease or ulcerative colitis (15). Epidemiologic evidence has shown that treatment with 5-amino-salicylic acid prevents the development of colorectal cancer in patients with inflammatory bowel disease and significantly inhibits cell growth and induces apoptosis in 75% of cancer cells (16). Also, some studies have demonstrated the growth inhibitory, apoptosis-inducing, and finally antitumor effects of olsalazine in colorectal cancer (17,18). However, other studies have indicated the mitogenic effects of this drug in the intestine epithelial cells (19). According to studies, olsalazine is likely to inhibit more than one isoform of DNMT enzymes. This factor increases the probability of having more efficiency for olsalazine in inhibiting DNA methylation (20).

Considering the previous findings regarding the capability of olsalazine for inhibiting DNMTs and toward drug repurposing strategy in cancer therapy, the aim of this study was to investigate the effect of olsalazine as a novel epigenetic drug candidate on cell survival and also the expression of two cancer-related genes (CDH1 and uPA) which are regulated by DNA methylation in MDA-MB-231 breast cancer cells in comparison with the known epigenetic drug, decitabine.

MATERIALS AND METHODS

Cell line and reagents

Human breast adenocarcinoma epithelial-like cell line MDA-MB-231 (IBRC# C10684) was obtained from the Iranian Biological Resource Center (Tehran, Iran). Dulbecco's modified eagle medium 2 mm glutamine (DMEM-Glutamax), fetal bovine serum (FBS), penicillin-streptomycin solution, phosphate-buffered saline (PBS), 0.25% trypsin-ethylenediaminetetraacetic acid and 0.2%

trypan blue were purchased from Bioidea Company (Tehran, Iran) as sterile liquids. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder was obtained from Sigma-Aldrich (St. Louis, MO, USA). 5-Aza-2′-deoxycytidine (decitabine) and 3,3'-azobis 6-hydroxybenzoic acid (olsalazine) sodium powders were purchased from Santa-Cruz Biotechnology (Dallas, TX, USA).

in vitro cell viability analysis by MTT assay

In order to determine IC_{50} values of drugs, MDA-MB-231 cells were seeded in 96-well plates at 7.5×10^3 cells/well density in 200 µL complete DMEM-Glutamax medium. After 24 h incubation, cells were treated with decitabine or olsalazine at different concentrations (300-6000 µM) prepared in 100 µL DMEM-Glutamax medium supplemented with 10% FBS. Untreated cells were used as control. Cell viability was assessed 24 and 48 h after treatment using MTT assay. Briefly, 10 µL of MTT (12 mM) was added to each well and plates were incubated at 37 °C in darkness for 3 h. Then the medium on cells was replaced with 100 µL DMSO and plates were shaken for 20 min till the formazan crystals were solved. The absorbance was measured at 570 nm by a microplate reader (BioRad, USA) and used to calculate the percentage of viable cells. The results were expressed as the mean of three replicates.

Relative gene expression analysis by quantitative real-time polymerase chain reaction

To analyze the effect of olsalazine and decitabine on the expression of CDH1 and uPA genes, MDA-MB-231 cells were seeded in 24-well plates at 7.5×10^4 cells/well density. After 24 h incubation, cells were treated with sublethal concentrations of olsalazine or decitabine (300 µM), prepared in 500 µL DMEM-Glutamax medium supplemented with 10% FBS. Untreated cells were considered as control. After 24 and 48 h treatments, cells were harvested. RNA extraction and DNase I treatment was performed using Quick-RNA MicroPrep kit (Zymo Research, USA) as per the manufacturer's instructions. A total of 1 µg RNA was reverse transcribed by high capacity cDNA reverse transcription kit (Applied Biosystems, USA) according to the manufacturer's instructions using random hexamer.

All cDNA samples were diluted to ½ in nuclease-free water and 2 µL of each diluted cDNA was used for quantitative real-time polymerase chain reaction (qRT-PCR) to analyze the expression of CDH1 and uPA at the transcriptional level. The qRT-PCR reaction mixes for each gene were separately prepared in 20 µL final volume, containing 2 µL cDNA, 1X power SYBR green PCR master mix (Life Technologies, USA) and 0.125-0.25 µM of the related specific primer pairs. The primer sequences used in this study are provided in Table 1. The qRT-PCR reaction was performed in Rotor-Gene Q (Qiagen, Germany) using the following conditions: a first denaturation step for 10 min at 95 °C, followed by 45 cycles of; 15 s at 95 °C, 15 s at 53 °C and 20 s at 72 °C. A melt curve step was done ramping from 60 to 90 °C rising 0.5 °C/s. The relative expression ratio of the target genes was computed based on their RT-PCR efficiencies (E) and the crossing point values (C_P), using Pfaffl's equation (21). Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as the reference gene to normalize the data. No template control (NTC) was included in experiments. Each sample was analyzed in duplicate and all experiments were carried out thrice independently.

Table 1. List of real-time polymerase chain reaction primer sequences.

Genes	Primer sequences	Product length (bp)
CDH1	Forward: 5'-TCGCTTACACCATCCTCAGCCA-3'	113
CDH1	Reverse: 5'-ACTCTCTCGGTCCAGCCCAGT-3'	113
uPA	Forward: 5'-CCAAAGGCAGCAATGAACTT-3'	104
uPA	Reverse: 5'-GTTGCACCAGTGAATGTTGG-3'	104
GAPDH	Forward: 5'-CTCAACTACATGGTTTACA-3'	113
GAPDH	Reverse: 5'-AAGATGGTGATGGGATTT-3'	113

Statistical analysis

The results were expressed as mean ± SEM (standard error of the mean). Data analysis was performed using SPSS, version 16.0. One-way ANOVA followed by LSD post hoc test and Pearson correlation analysis was used to analyze the significance between different values. $P < 0.05$ was considered as significant.

RESULTS

Cytotoxicity of olsalazine and decitabine in MDA-MB-231 cells

The toxicity of two drugs was investigated on MDA-MB-231 cells at 24 and 48 h by the MTT method. As Fig. 1 shows, both drugs exhibit toxic effects at concentrations of more than 3000 μM in 24 h treatment and there is no significant difference in the level of toxicity between the two drugs at equal concentrations (Fig. 1A). Also, the toxicity of two drugs on MDA-MB-231 cells is approximately the same at 48 h, exhibiting toxic effects at concentrations greater than 300 μm and there is no statistically significant difference in the toxicity of the two drugs at equal concentrations (Fig. 2B). The IC_{50} values were obtained, about 4000 μM for olsalazine, and 4500 μM for decitabine at 24 h. Similarly, the IC_{50} was obtained approximately the same for both decitabine and olsalazine about 300 μM at 48 h.

Effect of olsalazine and decitabine on the expression of CDH1 and uPA genes

To evaluate the effect of drugs on CDH1 and uPA gene expression, MDA-MB-231 cells were treated with drugs for 24 and 48 h. Analysis of gene expression was performed by qRT-PCR according to the Pfaffl method. Regarding the expression of the CDH1 gene, a significant (5.96-fold) increase was observed in the samples treated with 300 μM olsalazine at 24 h. However, no significant change was observed in the samples treated with 300 μM decitabine (Fig. 2A). In addition, treatment with drugs did not cause any significant increase in uPA expression. Also according to 48 h treatment results, relating to CDH1 gene expression, a significant (7.7-fold) rise was observed in the 300 μM olsalazine treated sample, whereas in the sample treated with 300 μM decitabine, a 4.65-fold increase was observed, which was significantly less than olsalazine (Fig. 2B). Treatment with drugs did not have any significant effect on increasing the expression of uPA gene.

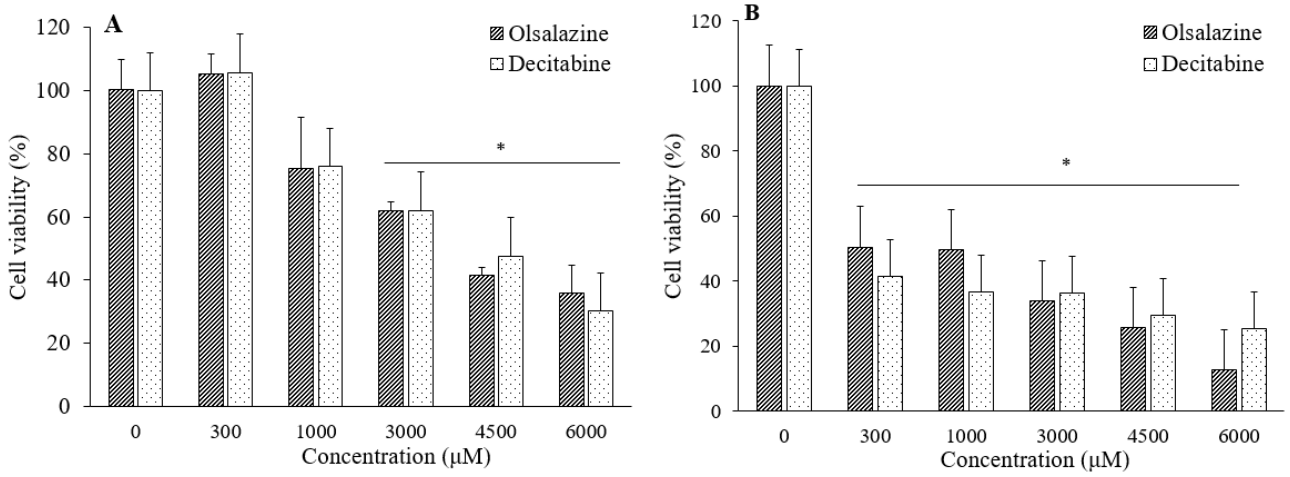

Fig. 1. Comparison of olsalazine and decitabine cytotoxicity on MDA-MB-231. The cytotoxicity of drugs on MDA-MB-231 cells was evaluated by MTT and the average percentage of live cells after (A) 24 h and (B) 48 h was plotted against different concentrations of the drugs. Data are presented as mean ± SEM, n = 3. *$P < 0.05$ Indicates the significant differences compared with the control group.

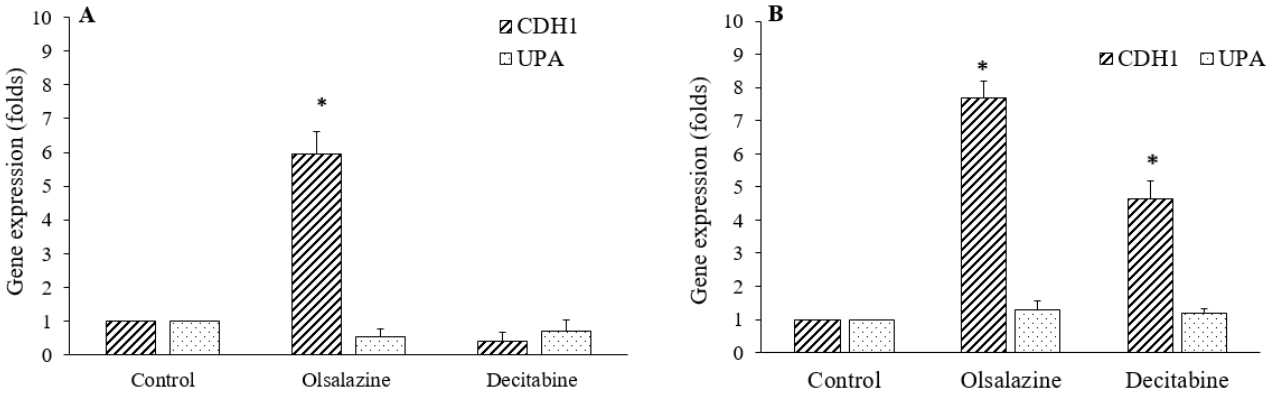

Fig. 2. Evaluation of the effect of olsalazine and decitabine on the expression of CDH1 and uPA genes. MDA-MB-231 cells were treated with olsalazine or decitabine at 300 μM, separately. The effect of drugs on the expression of CDH1 and uPA genes has been evaluated using quantitative polymerase chain reaction (A) 24 h and (B) 48 h after treatment. Untreated cells were used as the control group. Data are presented as mean ± SEM, n = 3. *P < 0.05 Indicates the significant differences compared with the control group.

DISCUSSION

Given the role of DNA methylation in regulating gene expression, playing an undoubted part in various diseases including cancer, researchers have proposed to use DNMT inhibitors to correct such epigenetic defects (1,2). Decitabine is one of the DNMT inhibitors which has been approved by the FDA for use in cancer and myelodysplastic syndrome (2,8). Meanwhile, toward drug repositioning strategy in cancer epigenetic therapy, olsalazine has been introduced as a novel potent DNA hypomethylating compound by *in silico* methods (13,14); however, *in vitro* studies need to be performed to determine its efficacy in this case. We previously studied the effects of olsalazine on cell toxicity and the expression of two cancer-related genes (CDH1 and uPA) in MCF-7 cells compared to decitabine and obtained some promising results (22). To complete our results, in the present study we aimed to assess these effects in the MDA-MB-231 cell line. Both cell lines are human breast carcinoma cells, yet they have many phenotypic and genotypic differences, especially respecting the expression of CDH1 and uPA genes. MCF-7 cells are non-invasive epithelial-like breast cancer cells expressing estrogen and progesterone receptors along with E-cadherin (CDH1) protein, while uPA gene is inactivated by promoter methylation. By contrast, MDA-MB-231 cells are triple-negative invasive breast adenocarcinoma cells, within which the CDH1 gene is not expressed due to hypermethylation of its promoter region, but uPA is actively expressed. This expression pattern of CDH1 and uPA in MDA-MB-231 cells corresponds to their mesenchymal morphology and highly metastatic phenotype. They have also been regarded as multidrug-resistant cancer cells (6,10,12,23).

Our results of cytotoxic assay indicated that neither of the drugs had an obvious cytotoxic effect on MDA-MB-231 cells at concentrations below 300 μm after 24 h (data not shown). The cytotoxicity of both drugs was equal up to 3000 μM at 24 h and slightly different at higher concentrations. The IC_{50} of olsalazine was obtained at approximately 4000 μM, and for decitabine at about 4500 μM at 24 h. Also, cytotoxic effects of both drugs increased after 48 h, with almost the same IC_{50} value (about 300 μM). Although the cytotoxicity seems not to be dose-dependent at 48 h treatment, a comparison of the cell responses at different time points might show a time-dependent effect. This delayed but strong cytotoxic effect for both drugs could be explained by the fact that these compounds could inhibit the cellular proliferation through interfering DNA synthesis, as discussed by Sharon *et al*. that one possible mechanism for inhibiting cellular proliferation by olsalazine might be the interaction with the synthesis of emerging thymidine, which is dependent on folate enzymes (24). Besides, according to Brown *et al*. olsalazine decreases the proliferation rate of tumor cells while increases the rate of apoptosis in rats with colorectal cancer (17).

Respecting the cytotoxic doses, Kastl *et al*. have previously shown that decitabine at 0.5-8 µM did not have any significant effect on the viability of MCF-7 and MDA-MB-231 cell lines after 24 h (25). Also, a study by Mendez *et al.* indicated that treating iHO1 cervical cancer cells with 1 µM decitabine or 0.1-10 µM olsalazine for 48 h did not have any significant effect on cell viability (14). These results are consistent with our primary results obtained from treatments with drugs at 0.1-100 µM (data not shown). In addition, according to our previous study performed on MCF-7 cells, the IC_{50} of olsalazine and decitabine were obtained about 1.75 mM and more than 3 mM at 24 h, respectively (22), which are in line with the present results in terms of concentration range; however, both drugs showed lower toxicity in MDA-MB-231 cells, which might be explained by their multidrug resistance property (23).

Nevertheless, Kar and colleagues reported the IC_{50} value of 15 µM for decitabine in MCF-7 and MDA-MB-231 cell lines at 24 h (26). Also, Ari *et al*. showed the IC_{50} value of about 10 µM for decitabine in MCF-7 cells after 48 h (27). These results are much lower than the IC_{50} value obtained in our study and in contrary to others' (14,22,25). Even, according to study olsalazine may have mitogenic effects in the intestinal epithelial cells (19). Although one possible explanation for minor variations in results might be different *in vitro* testing circumstances including the source of prepared materials or even the status of cells, it would not be acceptable for major differences. Finally, it seems necessary to indicate that hypomethylating agents act by reactivation of silenced genes and differentiation at low doses and cytotoxicity at high doses (28). This would be logical that epigenetic drugs are not expected to be potent cytotoxic agents, as they are used for correcting epigenetic defects.

Analyzing the effect of drugs on the expression of CDH1 in MDA-MB-231 cells showed an approximately 6-7.7 fold rise after 24-48 h treatment with 300 µM olsalazine, while 300 µM decitabine increased the CDH1 expression by 4.65-fold only after 48 h. These results indicated that olsalazine seems to act as a more potent and faster-acting epigenetic candidate. Also in our previous study olsalazine showed more ability to increase the expression of CDH1 in MCF-7 cells than decitabine; of course with a lower effect compared to MDA-MB-231 cells (22). According to Medina-Franco *et al*. olsalazine might be able to inhibit more than one isoform of DNMTs, suggesting its greater effectiveness (20). Nonetheless, in a study by Mendes *et al*. the effect of olsalazine on increasing the expression of the GFP gene suppressed by methylation in iHO1 cells was similar to that of decitabine (14).

In addition, our results showed that neither decitabine nor olsalazine did increase the uPA expression in MDA-MB-231 cells. This promising finding is in accordance with another study by Ari e*t al.* (27) although several studies have shown that treating MCF-7 cells with decitabine or DNMT inhibitors can activate the prometastatic uPA gene (9,22,27). In fact, DNMT inhibitors can induce the expression of methylation-inactivated genes (such as uPA in MCF-7 cells or CDH1 in MDA-MB-231 cells), but their usual dose and time may depend on the gene and cell type (4,27). Therefore, while using epigenetic drugs in cancer therapy, it seems critical to consider their effectiveness and off-target effects.

CONCLUSION

To summarize, our results showed that olsalazine has cellular toxicity comparable to decitabine in MDA-MB-231 cells. Also compared to decitabine, olsalazine significantly caused a greater increase in the expression of CDH1 without any significant increase in the expression of uPA. Therefore, it seems to be a good candidate for metastatic cancer treatment, although further studies are recommended in this regard.

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Conflict of interest statement

The authors declared no conflicts of interest in this study.

Authors' contribution

M. Naghitorabi contributed in concept and design of the study, acquisition of data, analysis and interpretation of data, drafting and editing of the manuscript and final approval of the version to be published; J. Mohammadi Asl and M. Mohammadi Asl contributed in acquisition of data, analysis and interpretation of data and drafting of the manuscript.

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