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# Combinatorial Antitumor Activity of Oxaliplatin with Epigenetic Modifying Agents, 5-Aza-CdR and FK228, in Human Gastric Cancer Cells

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## Abstract

Epigenetic silencing is considered to be a major mechanism for loss of activity in tumor suppressors. Reversal of epigenetic silencing by using inhibitors of DNA methyltransferase (DNMT) or histone deacetylases (HDACs) such as 5-Aza-CdR and FK228 has shown to enhance cytotoxic activities of several anticancer agents. This study aims to assess the combinatorial effects of gene-silencing reversal agents (5-Aza-CdR and FK228) and oxaliplatin in gastric cancer cells, i.e., Epstein-Barr virus (EBV)-negative SNU-638 and EBV-positive SNU-719 cells. The doublet combinatorial treatment of 5-Aza-CdR and FK228 exhibited synergistic effects in both cell lines, and this was further corroborated by Zta expression induction in SNU-719 cells. Three drug combinations as 5-Aza-CdR/FK228 followed by oxaliplatin, however, resulted in antagonistic effects in both cell lines. Simultaneous treatment with FK228 and oxaliplatin induced synergistic and additive effects in SNU-638 and SNU-719 cells, respectively. Three drug combinations as 5-Aza-CdR prior to FK228/oxaliplatin, however, again resulted in antagonistic effects in both cell lines. This work demonstrated that efficacy of doublet synergistic combination using DNMT or HDACs inhibitors can be compromised by adding the third drug in pre- or post-treatment approach in gastric cancer cells. This implies that the development of clinical trial protocols for triplet combinations using gene-silencing reversal agents should be carefully evaluated in light of their potential antagonistic effects.

**Key Words:** Combinatorial index, EBV, Zta, Synergism, Antagonism

## INTRODUCTION

Gastric cancer is one of the leading causes of cancer-related death worldwide, and Epstein-Barr virus (EBV)-associated gastric cancer comprises 1.3 to 20.1% of all cases of gastric cancer worldwide, making EBV one of the important causative agents for gastric cancer (Lee *et al*., 2009; Stanfield and Luftig, 2017). Combinatorial chemotherapy using cisplatin/5-fluorouracil (5-FU), docetaxel/cisplatin/5-FU (DCF), and epirubicin/cisplatin/5-FU (ECF) has been recommended as a standard treatment for gastric cancer (Morabito *et al*., 2009; Takayama *et al*., 2010). However, insufficient clinical efficacy of chemotherapy has been reported and attributed to drug resistance in gastric cancers (Shah and Ajani, 2010). EBV has also been known to induce chemoresistance in lymphoma and gastric cancer cells, as latent EBV gene products were related to impaired function of cell cycle check point and apoptosis (Leao *et al*., 2007; Banerjee *et al*., 2013; Kim *et al*., 2015; Yoon and Ko, 2017).

Oxaliplatin (Eloxatin™, LOHP) has been shown to possess potent cytotoxicity *in vitro*, and so is a promising candidate for treating advanced gastric cancer (Eriguchi *et al*., 2003; Chao *et al*., 2004; Bang *et al*., 2012). In fact, oxaliplatin showed a reduced toxicity and a decreased likelihood of developing resistance compared to another anticancer drug, cisplatin (Montagnani *et al*., 2011). Several previous studies have shown that combination treatment regimens that combine oxaliplatin with other anticancer agents can produce synergistic effects

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**Table 1.** Antiproliferative activity of 5-Aza-CdR, FK228 and oxaliplatin in SNU-638 and SNU-719 human gastric cancer cells

| SNU-638 | | 24 h | 48 h | 72 h |
|---|---|---|---|---|
| 5-Aza-CdR | $IC_{50}$ | 4.52 ± 0.27 | 1.01 ± 0.13 | 0.89 ± 0.21 |
| | R | 36.1 ± 9.9 | 33.9 ± 4.2 | 30.3 ± 5.8 |
| FK228 | $IC_{50}$ | 16.4 ± 8.9 | 3.72 ± 0.03 | 2.68 ± 0.13 |
| | R | 31.7 ± 1.9 | 4.11 ± 4.00 | 2.56 ± 2.24 |
| Oxaliplatin | $IC_{50}$ | 35.4 ± 7.3 | 6.63 ± 1.24 | 0.68 ± 0.15 |
| | R | 39.6 ± 6.6 | 15.2 ± 18.2 | 13.0 ± 6.15 |
| SNU-719 | | 24 h | 48 h | 72 h |
| 5-Aza-CdR | $IC_{50}$ | ND | 7.72 ± 0.01 | 5.76 ± 3.44 |
| | R | 52.7 ± 1.9 | 48.1 ± 3.1 | 47.8 ± 1.9 |
| FK228 | $IC_{50}$ | ND | 5.61 ± 0.08 | 3.06 ± 0.004 |
| | R | 55.3 ± 4.2 | 25.5 ± 2.05 | 2.67 ± 2.03 |
| Oxaliplatin | $IC_{50}$ | ND | 328 ± 3.9 | 1.24 ± 0.07 |
| | R | 72.7 ± 5.07 | 47.8 ± 2.0 | 36.6 ± 0.76 |

Cell viability was determined using the MTS assay. $IC_{50}$, the drug concentration that produces a 50% of the drug's maximum effect following 48 or 72 h of continuous exposure; expressed in nM for FK228 and μM for 5-Aza-CdR and oxaliplatin. R, the residual unaffected fraction (resistance fraction); $IC_{50}$ and R values were determined from dose-response curves analyzed using Eq. 1 and Eq. 2 (see Materials and Methods). Data are presented as the mean ± SD from at least three independent experiments. ND, not determined.

in treating human gastric cancer (Tanaka *et al*., 2005; Gu *et al*., 2006; Luo *et al*., 2010; Shi *et al*., 2013). New and effective treatment regimens for advanced gastric cancer, however, continue to lack in terms of treatment strategy, which is an important in improving treatment efficacy.

The loss of cellular tumor suppressing activity is now broadly accepted as an important step towards tumor initiation and growth. Furthermore, epigenetic silencing has also been shown to be a major mechanism causing the loss of tumor suppressor activity. Previous work has demonstrated the significance of altered gene expression by epigenetic silencing as many tumor suppressor genes are inactivated by hypermethylation and/or hypoacetylation in several cancers (Cameron *et al*., 1999; Fuks, 2005; Palii and Robertson, 2007). Epigenetic silencing is caused by integration of promoter hypermethylation with histone deacetylation, which is controlled by regulatory proteins such as DNA methyltransferase (DNMT), histone acetyltransferases (HATs), and histone deacetylases (HDACs) (Cameron *et al*., 1999; Baylin and Ohm, 2006; Jones and Baylin, 2007).

Several inhibitors, targeting DNMT and histone deacetylase, have been effectively utilized as anticancer drugs. For example, 5-aza-2'-deoxycytidine (5-Aza-CdR, Dacogen™, decitabine) is a DNMT inhibitor that successfully ameliorates cancer growth at a non-cytotoxic concentration in promyelocytic leukemia cells (Christman *et al*., 1983). Previous work has also shown that the cytotoxic activities of cisplatin and paclitaxel could be enhanced by co-treatment with 5-Aza-CdR, which acts to upregulate caspase-9 in lung cancer (Gomyo *et al*., 2004). It has also previously shown that 5-Aza-CdR induces apoptosis by both demethylating several apoptosis-related genes and through an unknown DNA methylation-independent mechanism, leading it to be approved by the FDA for myelodysplastic syndrome (MDS) treatment (Kuendgen and Lubbert, 2008; Li *et al*., 2014; Momparler *et al*., 2017). Other recent work has reported that low doses of DNMT inhibitors, such as Azacitidine and 5-Aza-CdR, cause antitumor effects for hematological neoplasms (Tsai *et al*., 2012). Among the class of histone deacetylase inhibitors (HDACIs), FK228 (Romidepsin, depsipeptide) has also previously been shown to possess potent anticancer activity by arresting the cell cycle as well as directly inducing cell death through various mechanisms (Mizutani *et al*., 2010; Ierano *et al*., 2013; Sun *et al*., 2017). Recently, combining bortezomib and 5-azacytidine provides a novel therapeutic method for treating EBV-associated gastric cancer (Fukayama, 2010).

This study aims to assess the combinatorial effects of gene silencing reversal agents, 5-Aza-CdR and FK228, and oxaliplatin in gastric cancer cells. We evaluated the combinatorial effect of these agents in an EBV-negative gastric cancer cell line (SNU-638) and an EBV-positive gastric cancer cell line (SNU-719). We combined these agents in doublet combination prior to or followed by the third agent. This approach was based on the recent studies in which doublet chemotherapy was suggested as a favored first-line therapy, and a sequential treatment (adding a third drug to a doublet chemotherapy) was recommended in terms of comparable overall survival and favorable toxicity profile in advanced gastric cancer patients (Bittoni *et al*., 2015; Laterza *et al*., 2017). We report varying degrees of synergistic or antagonistic combinational effects of 5-Aza-CdR, FK228, and oxaliplatin, which depends on the combinatorial regimen such as the dose and treatment schedule. These findings suggest that development of clinical trial protocols for triplet combinations of these three agents should be carefully designed and evaluated although combination of gene silencing reversal agents with oxaliplatin may offer therapeutic potential for treating gastric cancer.

## MATERIALS AND METHODS

### Chemicals and reagents

5-Aza-2'-deoxycytidine (5-Aza-CdR, Dacogen™, decitabine) was purchased from Sigma-Aldrich (St. Louis, MO, USA). FK228 (Romidepsin, depsipeptide) and oxaliplatin (Eloxatin™, LOHP) was kindly provided by Sanofi-Aventis (Malvern, PA, USA). The tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazoli-

um, inner salt) (MTS) was purchased from Promega (Madison, WI, USA). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA), while normal goat serum and the Zta antibody were purchased from Jackson ImmunoResearch Laboratory, Inc (West Grove, PA, USA) and Dako (Kyoto, Japan), respectively. Cy3-conjugated secondary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents including phenazine methosulfate (PMS) and trichostatin A (TSA) were purchased from Sigma-Aldrich unless otherwise noted.

### Cell culture conditions

Both human gastric cancer cell lines, SNU-638 (the EBV-negative) and SNU-719 (the EBV-positive), were obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were maintained in RPMI-1640 media supplemented with 10% FBS in a humidified 5% (v/v) $CO_2$ atmosphere at 37°C.

### Cytotoxicity measurements

Cell viability was assessed using the MTS assay according to the manufacturer's instructions. Briefly, this involves growing cells exponentially before seeding in 96-well plates at either 500 cells/well (SNU-638) or 2000 cells/well (SNU-719). Cells were then incubated overnight and exposed to various concentrations and combinations of drugs for the time indicated. After drug exposure, 20 μL of MTS/PMS solution was added to each well. After an additional 2-3 h of incubation, we measured absorbance at 490 nm.

### Immunofluorescence assay (IFA)

EBV lytic gene induction was assessed after single or combined drug exposure. TSA-treated cells were used as a reference for lytic induction. Briefly, SNU-719 cells were placed on eight-well microscope slides ($4\times10^5$ cells/well) where they were treated with the drug/drug combination indicated. After 24 or 48 h, cells were harvested, fixed with 100% ice-cold methanol, and blocked with 20% normal goat serum for 20 min at room temperature. After incubating with Zta antibody (1:40) for 1 h at room temperature, cells were washed with PBS and then incubated with the Cy3-conjugated secondary antibody in the dark for 1 h at room temperature. The protein expression level of fluorescent-labeled Zta was determined using a confocal microscope (Bio-Rad MRC-1024, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

### Data analysis

The $IC_{50}$ was defined as the drug concentration at which cell viability is equal to 50% of the no drug control, and calculated using Eq. 1 and Eq. 2:

$$\%\ \text{Cell viability} = \left(\frac{\text{mean absorbance of treated cells}}{\text{mean absorbance of control cells}}\right)\times 100 \quad \text{(Eq. 1)}$$

$$\%\ \text{Cell viability} = (100-\text{R})\times\left(1-\frac{[\text{D}]^m}{\text{K}_\text{d}{}^m+[\text{D}]^m}\right)\times 100 \quad \text{(Eq. 2)}$$

where D is the drug concentration, *m* is the Hill-type coefficient, R is the residual unaffected fraction (the resistance fraction), and $K_d$ is the concentration of drug that produces a 50% of the drug's maximum effect (Emax, 100-R) (Roell *et al*., 2017).

Drug interactions were characterized by using a combination index (CI) for various levels of cell death (Eq. 3). CI was calculated for an effect level of 50-80 %, i.e., $CI_{50}$-$CI_{80}$.

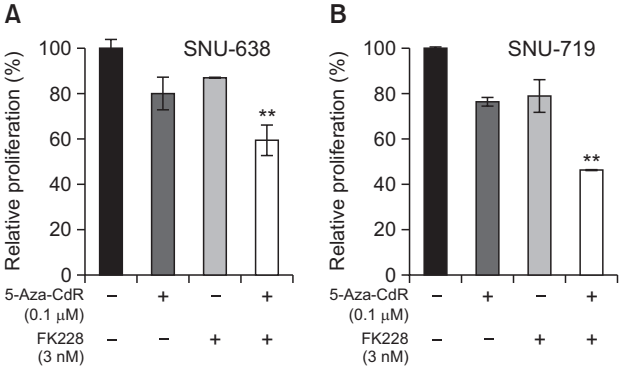


**Fig. 1.** Effects of combined treatment with 5-Aza-CdR and FK228. SNU-638 (A) and SNU-719 cells (B) were treated with single agent of 5-Aza-CdR (0.1 μM) and FK228 (3 nM), or with doublet combination of 5-Aza-CdR/FK228 for 24 h. After removal of drug-containing media, cells were further incubated in drug-free media for an additional 72 h and were then subjected to MTS assay. Data are presented as the survival rate relative to the control, which was assigned a value of "100%". Data are presented as the mean ± SD from three independent experiments. ** indicates synergistic effects.

$$\text{CI}_\text{X} = \frac{(\text{D})_\text{A}}{(\text{D}_\text{X})_\text{A}} + \frac{(\text{D})_\text{B}}{(\text{D}_\text{X})_\text{B}} + \alpha\frac{(\text{D})_\text{A}(\text{D})_\text{B}}{(\text{D}_\text{X})_\text{A}(\text{D}_\text{X})_\text{B}} \quad \text{(Eq. 3)}$$

where $CI_x$ is CI for a fixed effect level x identified for a combination of drug A and drug B. $(D_X)_A$ and $(D_X)_B$ are the concentrations of drug A or B necessary to produce effect x when applied in isolation. $(D)_A$ and $(D)_B$ are the concentrations of drug A or B required to produce effect x when applied in combination. $\alpha$ is 0 when A and B are mutually exclusive, and 1 when A and B are mutually non-exclusive (Chou and Talalay, 1984; Chung *et al*., 2009). CIx between 0.8 and 1.2 was defined as additive, ≤0.8 as synergistic, and ≥1.2 as antagonistic (Roell *et al*., 2017). The doublet or triplet combinations at fixed concentrations were analyzed by comparing experimental data to the reference additivity values calculated using Bliss independence model (Roell *et al*., 2017). The ratio of experimental survival rate to reference value between 0.8 and 1.2 was defined as additive, ≤0.8 as synergistic, and ≥1.2 as antagonistic.

## RESULTS

### Antiproliferative activity of 5-Aza-CdR, FK228, and oxaliplatin

We evaluated the antiproliferative activity of 5-Aza-CdR, FK228 and oxaliplatin treatment on both SNU-638 and SNU-719 gastric cell lines. We used model-fitting procedures to obtain the relevant pharmacodynamic parameters (Table 1). Cells were exposed to 5-Aza-CdR up to 25 μM, and high resistance fractions were shown ranging from 30.3% to 52.7% in both cell lines (Table 1). FK228 treatment produced strong antiproliferative effects with relatively low $IC_{50}$ values in both cell lines (Table 1). It is noted, however, that EBV-positive SNU-719 cells were more resistant than SNU-638 cells as shown by significantly higher R values until 48 h exposure; R

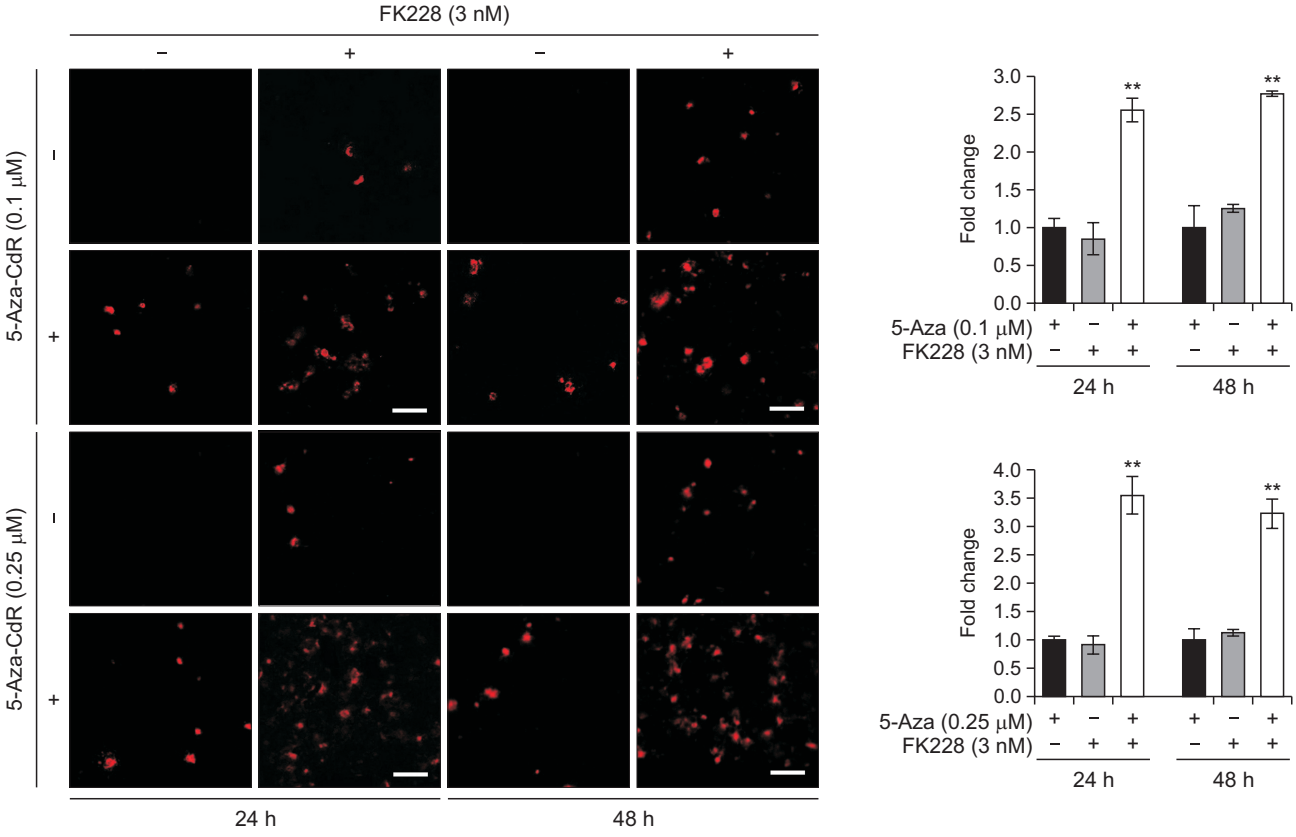


**Fig. 2.** Zta induction after treatment with 5-Aza-CdR and/or FK228 in SNU-719 cells. Immunofluorescence was used to measure Zta. Representative fluorescence images are shown using anti-Zta primary antibody (1:40) after 24 h and 48 h of drug treatment. Bars, 100 μm. The data are expressed as relative fold change to control, 5-Aza-CdR single treatment. Data are presented as the mean ± SD from three independent experiments. ** indicates synergistic effects.

fraction was 55.3% and 25.5% in SNU-719 cells as compared to 31.7% and 4.11% in SNU-638 cells after 24 h and 48 h, respectively ($p$<0.01). Nonetheless, we found that the resistance was plummeted by prolonging the exposure time to 72 h (Table 1). Treatment with oxaliplatin also required long exposures to obtain significant antiproliferative activity. For example, $IC_{50}$ values of oxaliplatin at 72 h exposure were lower by 10- and 300-folds compared to those of 48 h in SNU-638 and SNU-719 cells, respectively (Table 1). Despite the decreased $IC_{50}$ at 72 h exposure, sustained resistance to oxaliplatin was observed in EBV-positive SNU-719 cells as shown by particularly high resistance fraction (36.6%) (Table 1).

## Combinatorial effects of 5-Aza-CdR and FK228

Doublet synergy of 5-Aza-CdR and FK228 was evaluated after 72 h post-exposure incubation following 24 h drug exposure (Fig. 1). Post-exposure period of 72 h was included in order to synchronize treatment schedule with the triplet combination where exposure to 5-Aza-CdR and FK228 doublet was followed by 72 h exposure to oxaliplatin. Simultaneous exposure to 0.1 μM of 5-Aza-CdR and 3 nM of FK228 produced additive to synergistic antiproliferative effects in both SNU-638 and SNU-719 cells (Fig. 1). Combination using a higher concentration of 5-Aza-CdR (0.25 μM) also showed synergistic effects in both cell lines (data not shown). However, the synergism was not obtained when 5-Aza-CdR was combined with FK228 at concentrations below 3 nM concentration (data not shown).

## Induction of Zta expression in EBV-positive gastric cancer cells

Previous study has reported that the expression of viral immediate-early antigens such as Zta (EB1, BZLF1, ZEBRA) can be inhibited by CpG methylation (Feng *et al*., 2002). Furthermore, it has been shown that the expression of these genes can induce the lytic reactivation and death of tumor cells (Cayrol and Flemington, 1996; Hui *et al*., 2012; Wildeman *et al*., 2012). As such, we next sought to investigate whether the antiproliferative effect of gene silencing reversal agents is associated with the induction of lytic EBV genes in EBV-positive cells (SNU-719 cells). We found that Zta protein expression was dramatically elevated up to 37.5% and 85.6% above the expected value for additive interaction between 5-Aza-CdR and FK228 for 24 h and 48 h, respectively (Fig. 2). Additionally, the combinatorial treatment of 5-Aza-CdR with another HDAC inhibitor, trichostatin A (TSA), showed similar synergistic effects in terms of Zta expression (data not shown). These data suggest that lytic reactivation may be one of underlying mechanisms for synergistic cytotoxicity induced by 5-Aza-CdR and FK228 in EBV-positive SNU-719 cells.

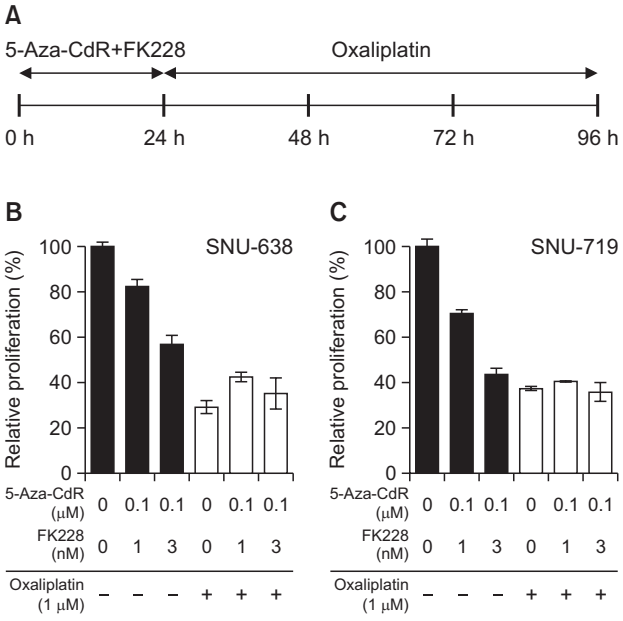


**Fig. 3.** Pre-treatment with 5-Aza-CdR/FK228 antagonized the anti-proliferative effect of oxaliplatin. Cells were pre-treated with 5-Aza-CdR (0.1 μM)/FK228 (1 nM or 3 nM) for 24 h, washed and then exposed to 1 μM of oxaliplatin for subsequent 72 h (A). Relative rate of proliferation was determined in SNU-638 (B) and SNU-719 (C) cells using the MTS assay. Data are presented as the mean ± SD from three independent experiments. All triplet combinations showed antagonism in both cell lines.

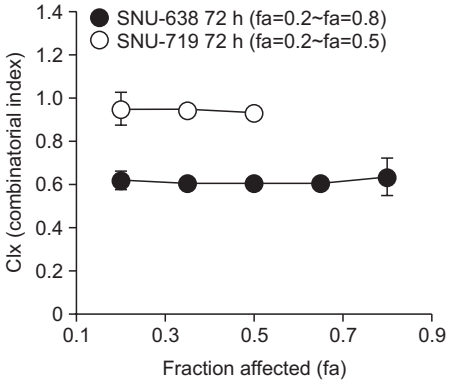


**Fig. 4.** Combination indexes ($CI_x$) versus affected fraction (fa) determined for combination of FK228 and oxaliplatin. Cells were treated with FK228/oxaliplatin for 72 h at fixed equitoxic ratios. $CI_x \leq 0.8$, $CI_x \geq 1.2$, and $0.8 < CI_x < 1.2$ indicate synergism, antagonism, and additivity, respectively (See methods for details).

### Effects of sequential treatment with 5-Aza-CdR/FK228 and oxaliplatin

Having identified synergistic effects between 5-Aza-CdR and FK228, we next sought to investigate the combination effect when oxaliplatin was added in a sequential manner. Cells were exposed to a doublet pre-treatment using 0.1 μM of 5-Aza-CdR and 1 nM or 3 nM of FK228 for 24 h and then subsequently to 1 μM oxaliplatin for an additional 72 h (Fig. 3A). Survival rates were determined using the MTS assay at 96 h. Oxaliplatin (1 μM) alone resulted in about 29.1% survival in SNU-638 cells (Fig. 3B). When combined with pre-treatment of 5-Aza-CdR/FK228, severe antagonistic effects were induced as shown by 2 folds higher proliferation rates compared to the expected values at two different concentration levels of 5-Aza-CdR/FK228 in SNU-638 cells (Fig. 3B). Similar level of antagonism occurred in SNU-719 cells (Fig. 3C). Antagonistic effect was also observed when either using higher concentrations of oxaliplatin (5 μM and 25 μM) or by prolonging the pre-treatment doublet for 48 h followed by 48 h exposure to oxaliplatin in both cell lines (data not shown).

### Combinatorial effects of 5-Aza-CdR pre-treatment followed by simultaneous FK228/oxaliplatin treatment

After unexpectedly identifying antagonistic interactions between gene silencing reversal agents (5-Aza-CdR/FK228) and oxaliplatin, we sought to modify the combination schedule. We first determined the combinatorial effect of FK228 and oxaliplatin given at the $IC_{50}$ molar ratio (1:170 at 72 h in both cell lines). The combination indexes (CI) were between 0.6 and 0.73 in SNU-638 cells and close to 1 in SNU-719 cells, indicating synergistic and additive interaction, respectively (Fig. 4). To that end, we tested the combination effect of 0.25 μM of 5-Aza-CdR treatment for 24 h prior to the doublet combination of FK228 and oxaliplatin (Fig. 5A). Pre-treatment with 5-Aza-CdR exhibited additive effect in SNU-638 cells, whereas antagonistic interaction in SNU-719 cells at all concentration tested (Fig. 5B, 5C). Prolongation of pre-treatment duration up to 48 h showed similar results in both cell lines, suggesting that there would be little benefit to further pursuit these three-drug combinations in both cell lines (data not shown).

## DISCUSSION

We investigated the effects of combined treatments involving 5-Aza-CdR, FK228, and oxaliplatin in both EBV-negative SNU-638 and EBV-positive SNU-719 cells. In drug combination studies, drugs are generally combined at the concentrations producing equal to or less than 50% of activity of each drug given alone, which may avoid potential toxic effects when translated into patient treatment. We used drug concentrations lower than $IC_{50}$ of each drug, i.e., for doublet combination, 0.1-0.25 μM of 5-Aza-CdR was combined with 1-3 nM of FK228 compared to the $IC_{50,72\,h}$ of 0.89-5.76 μM and 2.68-3.06 nM for 5-Aza-CdR and FK228, respectively (Table 1, Fig. 1, 2, 3). When FK228 was combined with oxaliplatin, even lower concentration as low as 0.3-1.5 nM of FK228 and 0.05-0.25 μM of oxaliplatin was used compared to the oxaliplatin $IC_{50,72\,h}$ of 0.68-1.24 μM (Fig. 4, 5). In this work, we found that doublet combination of 5-Aza-CdR and FK228 exhibited synergistic effects in gastric cancer cell lines (Fig. 1). Combination of FK228 with other drugs at low concentration was reported to enhance the tumor response. Low concentration of 5-Aza-CdR is also known to sustain anti-tumor effects even after stopping a treatment due to the activation of immune response (Kanzaki *et al*., 2007; Wang *et al*., 2013).

Combinatorial treatment using 5-Aza-CdR/FK228, 5-Aza-CdR/cisplatin, and FK228/cisplatin exhibited synergistic effects when used to treat several different cancers (Karam *et al*., 2007; Shang *et al*., 2008; Wilson *et al*., 2012). The synergism between 5-Aza-CdR and FK228 has been mea-

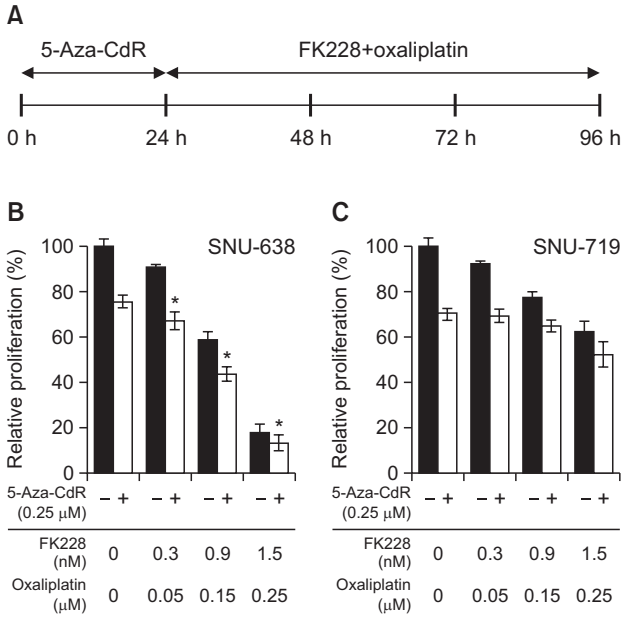


**Fig. 5.** Combination effects of 5-Aza-CdR pre-treatment combined with FK228/oxaliplatin treatment. Cells were treated with 0.25 μM of 5-Aza-CdR for 24 h, and then incubated for 72 h with FK228/oxaliplatin (A). SNU-638 (B) and SNU-719 (C) cells were exposed to drugs at the concentrations indicated. Relative rate of proliferation was determined using the MTS assay. Data are presented as the mean ± SD based on at least three replicate experiments. * indicates additive effects.

sured in clear cell renal carcinoma cells and triple-negative breast cancer cells, for which induction of secreted frizzled-related protein 1 (sFRP1), a negative regulator of the Wnt signaling pathway, was detected (Cooper *et al*., 2012). On the other hand, combination therapy with gene silencing reversal agents was reported to cause antagonistic effects. For example, combined treatment with 5-Aza-CdR and hydroxycarbamide has been shown to cause adverse effects on DNA methylation (Choi *et al*., 2007). Combined treatment of dimethoxycurcumin and 5-Aza-CdR exhibited antagonistic effects in primary leukemia cells (Hassan *et al*., 2016). Combination of FK228 with methotrexate and vincristine exhibited antagonistic effects when used on human leukemia and lymphoma cell lines (Kano *et al*., 2007). In our study, synergistic effects of 5-Aza-CdR and FK228 were shown and confirmed by Zta induction in SNU-719 cells (an EBV-positive cell line, Fig. 2). Zta expression in SNU-719 cells increased dramatically after combined treatment with 5-Aza-CdR and FK228 (Fig. 2). Since the activation of EBV lytic cycle is known to induce cell cycle arrest and apoptosis (Cayrol and Flemington, 1996; Hui *et al*., 2012; Wildeman *et al*., 2012), these findings suggest that combined treatment with 5-Aza-CdR and FK228 could provide a novel therapeutic strategy for treating EBV-positive gastric cancer. Additionally, the synergistic interaction of 5-Aza-CdR and FK228 in SNU-638 (EBV-negative cell line) can be due to the enhanced induction of sFRP1 (Cooper *et al*., 2012) and caspase-3 activation (Kalac *et al*., 2011).

In addition to 5-Aza-CdR/FK228 doublet combination, FK228 and oxaliplatin also demonstrated synergistic effects (Fig. 1, 4), and we next sought to assess the effects of triple combinations of 5-Aza-CdR, FK228 and oxaliplatin in gastric cancer cells. The triple combination of 5-Aza-CdR, FK228 and oxaliplatin not only fails to exhibit synergistic effects, but actually resulted in antagonistic effects; when 5-Aza-CdR/FK228 was given before oxaliplatin (Fig. 3) and when 5-Aza-CdR pre-treatment was combined with FK228 and oxaliplatin (Fig. 5). We speculated that the antagonistic effects measured for the triple combination treatment in our experiments may be a result of demethylating genes that are involved in tumor development (Ateeq *et al*., 2008), combined with the induction of P-glycoprotein by FK228 (Glaser, 2006; Ni *et al*., 2015). In addition, unexpected antagonism may be associated with cell cycle dependent interaction (Johnson *et al*., 1997; Sui *et al*., 2004; Xiong *et al*., 2007). Since 5-Aza-CdR and FK228 has been known to induce $G_0/G_1$ arrest in many types of cells (Lavelle *et al*., 2003; Son *et al*., 2010), 5-Aza-CdR/FK228 pre-treatment may interfere with antiproliferative effect of oxaliplatin, which is known to cause $G_2$/M arrest (Xu *et al*., 2015).

Collectively, our findings suggest that the strategies for optimizing the dose and schedule are critical for developing effective drug combination therapies. Oxaliplatin can be used as part of combinatorial therapy when combined with gene silencing reversal agents; however, it is critical to carefully optimize the dose and time schedule. Although several recent studies have reported that triple combinations of anticancer drugs, including platin-based drugs, can be effective for treating several cancers, we found that the triple combination of 5-Aza-CdR, FK228 and oxaliplatin presented antagonistic interactions, which implies that the development of clinical trial protocols for triplet combinations using gene-silencing reversal agents should be carefully evaluated in light of their potential antagonistic effects.

## CONFLICT OF INTEREST

The authors have no competing financial interests to declare.

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