Combination of the histone deacetylase inhibitor depsipeptide and 5-fluorouracil upregulates major histocompatibility complex class II and p21 genes and activates caspase-3/7 in human colon cancer HCT-116 cells

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Abstract. Epigenetic anticancer drugs such as histone deacetylase (HDAC) inhibitors have been combined with existing anticancer drugs for synergistic or additive effects. In the present study, we found that a very low concentration of depsipeptide, an HDAC inhibitor, potentiated the antitumor activity of 5-fluorouracil (5-FU) in a human colon cancer cell model using HCT-116, HT29, and SW48 cells via the inhibition of colony formation ability or cellular viability. Exposure to a combination of 5-FU (1.75 μM) and 1 nM depsipeptide for 24 and 48 h resulted in a 3- to 4-fold increase in activated caspase-3/7, while 5-FU alone failed to activate caspase-3/7. Microarray and subsequent gene ontology analyses revealed that compared to 5-FU or depsipeptide alone, the combination treatment of 5-FU and depsipeptide upregulated genes related to cell death and the apoptotic process consistent with the inhibition of colony formation and caspase-3/7 activation.

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These analyses indicated marked upregulation of antigen processing and presentation of peptide or polysaccharide antigen via major histocompatibility complex (MHC) class (GO:0002504) and MHC protein complex (GO:0042611). Compared with vehicle controls, the cells treated with the combination of 5-FU and depsipeptide showed marked induction (3- to 8.5-fold) of expression of MHC class II genes, but not of MHC class I genes. Furthermore, our global analysis of gene expression, which was focused on genes involved in the molecular regulation of MHC class II genes, showed enhancement of pro-apoptotic PCAF and CIITA after the combination of 5-FU and depsipeptide. These results may indicate a closer relationship between elevation of MHC class II expression and cellular apoptosis induced by the combination of depsipeptide and 5-FU. To the best of our knowledge, this is the first study to report that the combination of 5-FU and depsipeptide induces human colon cancer cell apoptosis in a concerted manner with the induction of MHC class II gene expression.

Introduction

The International Agency for Research on Cancer (IARC) reported that colorectal cancer (CRC) is the third most commonly diagnosed cancer and the fourth leading cause of cancer-related death worldwide. Drug combinations and administration protocols, and the development of molecular targeted drugs have greatly improved the chemotherapeutic efficiency for CRC.

Fluoropyrimidines, especially 5-fluorouracil (5-FU), remain the most important chemotherapeutic agents in the treatment of CRC. 5-FU is reported to induce cellular toxicity by inhibiting its target enzyme, thymidylate synthase (TS); it also induces cell cycle arrest and apoptosis via the incorporation of fluoronucleotides into RNA and DNA leading to thymidylate depletion (1). 5-FU is the central component in the FOLFIRI regimen with the addition of irinotecan, and the FOLFOX regimen with the addition of oxaliplatin. These combination chemotherapies have resulted in improved response rates and significant improvements in progression-free

and overall survival (2,3). However, despite recent advances in CRC chemotherapy, the occurrence of drug resistance hinders effective control of the disease. Moreover, most advanced CRC patients cannot be cured with these refined chemotherapies.

Histone acetylation and deacetylation are biological processes that regulate the modification of chromatin structures and are important for gene regulation. Recently, a number of anticancer drugs such as inhibitors of DNA methyltransferase (DNMT) and histone deacetylase (HDAC) have been developed. DNMT and HDAC inhibitors cause DNA demethylation and repressive complex disintegration. This leads to the reactivation of critical genes and the reversal of genome-wide epigenetic alterations in cancer through resetting multiple cellular processes including, lineage commitment, immunomodulation, major cell signaling pathways, cell proliferation, and cell death (4,5). Overexpression or underexpression of pro-apoptotic and anti-apoptotic molecules, such as p53 and Bcl-2, can be modulated by various HDAC inhibitors (6). Inhibitors of HDACs are known to exert effects including apoptosis, retardation of cell cycle progression, and cancer cell differentiation, which are specific depending on cancer cell type (7,8). Therefore, a number of HDAC inhibitors have been investigated clinically. Vorinostat, entinostat, panobinostat, valproic acid, depsipeptide, and belinostat have been approved and clinically used. HDAC inhibitors are also used in combination therapy with DNMT inhibitors and other HDAC inhibitors (9). A basic study on combinations of valproic acid and gemcitabine has also been conducted (10).

DNMT inhibitors, such as 5-azacytidine, 5-aza-2'-deoxycytidine, and zebularine have been studied for various cancers in clinical trials (11). The therapeutic benefits of HDAC inhibitors have been verified as a monotherapy for cutaneous T-cell lymphoma (CTCL), and in other malignancies (12). Epigenetic agents are also known to have the ability to reverse the resistance of anticancer drugs (13,14). Furthermore, synergistic or additive effects have been reported when combining epigenetic agents with existing anticancer drugs (15-17).

Depsipeptide (Dep, also known as FR901228, FK228 and romidepsin), isolated from *Chromobacterium violaceum* strain WB968, is a unique bicyclic peptide with a non-cysteine disulfide bridge. It is a stable prodrug, which is activated by reduction in cancer cells, to inhibit HDAC class I enzymes, and has been shown to convert H-ras-transformed NIH 3T3 cells to normal morphology. Thus, Dep is a promising anticancer agent, and indeed, has been approved by the FDA for the treatment for CTCL and peripheral T-cell lymphoma (18,19). The glutathione-mediated activation also implicates its clinical usefulness for counteracting glutathione-mediated drug resistance in chemotherapy (20).

In addition to their use as monotherapy, there are several studies on the effects and mechanisms of the combination of 5-FU and HDAC inhibitors for CRC treatment. Lee *et al* reported that trichostatin A enhanced 5-FU cytotoxicity by downregulating the expression of both TYMS mRNA and TS protein in colon cancer cells (21). Tumber *et al* reported that belinostat, an HDAC inhibitor, synergized with 5-FU to inhibit colon cancer cell growth *in vitro* and *in vivo*, and this combination therapy increased DNA fragmentation and poly(ADP-ribose) polymerase cleavage and downregulated TYMS in HCT116 cells (22). Di Gennaro *et al* found that

modulation of TYMS and p53 expression by vorinostat resulted in synergistic antitumor effects in combination with 5-FU or raltitrexed (23). Fazzone *et al* reported that panobinostat suppressed TYMS gene expression and synergized with fluoropyrimidines in colon cancer cells (24). Little is known concerning the combinatorial effects of 5-FU and Dep for CRC, which prompted us to investigate the combination of HDAC inhibitors, namely, Dep, apicidin, and oxamflatin, with 5-FU. We previously found that among these HDAC inhibitors, Dep potentiated the cytotoxicity of 5-FU against human colon cancer cells, HCT-116.

Thus, in the present study, we aimed to evaluate the mode of the combined effect of 5-FU and Dep in HCT-116 cells (i.e., additive or synergistic), and to elucidate the genetic mechanism of the drug-drug interaction in a cell-based model using microarray analysis.

Materials and methods

Cell culture and reagents. Human colon carcinoma HCT-116 (no. CCL-247) , HT29 (no. HTB-38) and SW48 cell lines (no. CCL-231) [all from American Type Culture Collection (ATCC), Manassas, VA, USA] were cultured in Dulbecco's modified Eagle's medium (DMEM; 4.5 g/l D-glucose; Gibco, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (HyClone, South Logan, VT, USA) and antibiotics (Gibco) at 37°C in a CO_2 incubator. Dep was purchased from Selleck Chemicals LLC (Houston, TX, USA). 5-FU was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of the highest grade available.

Drug exposure. HCT-116 cells were exposed for 7 days to either vehicle alone, 5-FU alone, Dep alone, or a combination of 5-FU and Dep for assessment of inhibition of colony formation and gene expression analysis. The concentrations used for colony formation analysis were 0.875, 1.25, 1.75 and 2.5 μM for 5-FU; 0.5, 1, 1.5 and 2 nM for Dep; and 0.875 μM 5-FU plus Dep (0.1 or 1 nM), 1.25 μM 5-FU plus Dep (0.1 or 1 nM), and 1.75 μM 5-FU plus Dep (0.1 or 1 nM). Concentrations used for gene expression analysis and caspase-3 activation assay were 1.75 μM for 5-FU; 1 nM for Dep; and 1.75 μM 5-FU plus Dep 1 nM.

HT29 cells were exposed for 10 days to either vehicle alone, 5-FU alone, Dep alone, or a combination of 5-FU and Dep for assessment of inhibition of colony formation. The concentrations used for colony formation analysis were 0.313, 0.438, 0.625 and 0.875 μM for 5-FU; 0.125, 0.25, 0.375 and 0.5 nM for Dep; and 0.313 μM 5-FU plus Dep (0.375 nM), 0.438 μM 5-FU plus Dep (0.375 nM), and 0.625 μM 5-FU plus Dep (0.375 nM).

SW48 cells were exposed for 7 days to either vehicle-only, 5-FU alone, Dep alone, or a combination of 5-FU and Dep for crystal violet assay. The concentrations used for the crystal violet assay were, 0.438, 0.625, 0.875 and 1.75 μM for 5-FU; 0.125, 0.25, 0.375 and 0.5 nM for Dep; and 0.438 μM 5-FU plus Dep (0.2 nM), 0.625 μM 5-FU plus Dep (0.2 nM), and 0.875 μM 5-FU plus Dep (0.2 nM).

Colony formation assay. HCT-116 cells were trypsinized, counted, and seeded for the colony formation assay in 60-mm

Table I. Nucleotide sequences of the oligonucleotide primers used for determining mRNA levels in quantitative real-time PCR.

Accession no.	Gene symbol	Forward primer (5'-3')	Reverse primer (3'-5')
NM_001101	ACTB	GGCACCACACCTTCTACAATGAG	GGATAGCACAGCCTGGATAGCA
NM_001188	BAK1	TGAAAAATGGCTTCGGGGCAAGGC	TCATGATTTGAAGAATCTTCGTACCA
NM_138763	BAX	GGCCCACCAGCTCTGAGCAGA	GCCACGTGGGCGTCCCAAAGT
NM_000633	BCL2	CGACTTCGCCGAGATGTCCAG	ACTTGTGGCCCAGATAGGCACCCAG
NM_000389	CDKN1A (p21)	CAAGCTCTACCTTCCCACGG	GGTAGAAATCTGTCATGCTGGTC
NM_000077	CDKN2A (p16)	ATGGAGCCTTCGGCTGACT	ATCATCATGACCTGGATCGGC
NM_004964	HDAC1	GCTCCATCCGTCCAGATAAC	TGCCACAGAACCACCAGTAG
NM_002116	HLA-A	CAGCTTGTAAAGTGTGAGACAGC	GGAAAGATGATTGGGGAGGGAG
NM_005514	HLA-B	GTAGGAGGAAGAGTTCAGGTGG	ACATTATGCTAACAGGGACGCA
NM_002117	HLA-C	CTGGTTGTCCTAGCTGTCCTTG	AGCTGTCTCAGGCTTTACAAGTG
NM_002121	HLA-DPB1	TCCACCAACCTGATCCGTAATG	AGAATCAGACTGTGCCTTCCAC
NM_002123	HLA-DQB1	CAAAGGAGTCAGAAAGGGCTTC	GGGGATGAAAGGAGATGACCTG
NM_019111	HLA-DRA	CCGATCACCAATGTACCTCCAG	CAGGAAGGGGAGATAGTGGAAC
NM_002124	HLA-DRB1	CTCACAGTGGAATGGAGAGCAC	GAATAACTGCCAAGCAGGAAAGC
NM_000546	TP53	GCTCAGATAGCGATGGTCTGG	GATGGTGGTACAGTCAGAGCC
NM_001071	TYMS	GTTGCTGTGGTTTATCAAGGGAT	TTGGTCAACTCCCTGTCCTG

dishes at 20,000 cells/dish. HT29 cells were trypsinized, counted, and seeded for the colony formation assay in 60-mm dishes at 10,000 cells/dish. After incubation for 7 to 10 days, the colonies were stained with crystal violet and the numbers of positive colonies were counted. Colonies containing more than 50 cells were regarded as positive and were scored, and each assay was conducted in triplicate and three separate assays were performed on three different days.

Crystal violet assay. We measured cell viability in SW48 cells by direct staining of the cells using crystal violet assay (25) as SW48 cells have poor colony-forming ability. SW48 cells were trypsinized, counted, and seeded for the crystal violet assay in 6-well plate at 50,000 cells/well. After incubation for 7 days, the medium was removed and any adherent cells were fixed to the plate with 10% formaldehyde in PBS. The cells were then stained with an aqueous solution of 0.5% crystal violet followed by elution of the dye with 33% aqueous acetic acid. Absorbance at 550 nm was determined with a microplate reader, Infinite F500 (Tecan Group Ltd., Männedorf, Switzerland). Each assay was conducted in triplicate and three separate assays were performed on three different days.

Caspase-3/7 activation assay. Luminescent caspase-3/7 activation assay was conducted according to the manufacturer's instructions (Caspase-Glo™ 3/7 assay; Promega, Madison, WI, USA). HCT-116 cells were inoculated into each well of a white-walled 96-well plate (4,000 cells/well), treatment reagents were added and incubated was carried out for 0, 24 and 48 h. After incubation for 1 h with Caspase-Glo™ reagent, the enzymatic activity of caspase-3/7 was measured using a microplate reader, Infinite F500 (Tecan Group Ltd.). The background luminescence associated with the cell culture and assay reagent (blank reaction) was subtracted from the experimental values. Mean values of four replicates were used to represent caspase-3/7 activity. To enable normalization of data to total cellular protein content, the Lowry assay (26) was conducted for all samples according to the manufacturer's instructions (Protein Assay Lowry kit; Nacalai Tesque, Kyoto, Japan). Data are expressed as relative light units (RLU)/μg of protein.

RNA extraction and quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Total RNA was extracted from three independent HCT-116 cell cultures using the RNeasy Mini kit following the manufacturer's instructions (Qiagen, Dusseldorf, Germany). Total RNAs (1 μg) were used for cDNA synthesis using a cDNA synthesis kit (Roche, Basel, Switzerland). Using first-strand cDNA, real-time PCR was performed using a 7500 Real-Time PCR system (Applied Biosystems, Tokyo, Japan). The sequences of amplification primers are shown in Table I.

Microarray analysis. For DNA microarray analysis, total RNA (50 ng) was labeled with cyanine-3 using a Low Input Quick Amp Labeling kit (Agilent Technologies, Inc., Santa Clara, CA, USA). The labeled targets were hybridized to SurePrint G3 Human 8x60K oligo DNA microarrays and hybridized microarrays were scanned using an Agilent Microarray Scanner (both from Agilent Technologies, Inc.). Image analysis and raw array data generation were processed using Feature Extraction software (Agilent Technologies, Inc.). Normalization of the raw array data was performed using GeneSpring software.

Gene ontology analysis. Following quantile normalization of the raw data, data sets were further reduced by filtering noise of the signal, including gIsSaturated, gIsFeatNonUnifOL, gIsFeatPopnOLg, and gIsWellAboveBG.

Finally, we identified gene lists that showed at least 1.5-fold upregulation or downregulation.

The PANTHER classification system (www.pantherdb.org/tools/genexAnalysis.jsp) was used for functional classification of genes.

Mi *et al* discussed the procedure for classification of proteins using PANTHER, a web tool for analyzing protein family trees and functions (27). The overall process of PANTHER Protein Library data generation consisted of three major steps: family clustering, phylogenetic tree building, and annotation of tree nodes. The requirements for being family clusters in PANTHER are as follows:

i) The family must contain at least five members among which at least one gene must be listed as a Gene Ontology (GO) reference genome.

ii) In order to support phylogenetic inference, the family should be aligned with high quality sequence data.

iii) A certain length of aligned sequences stored in at least 30 sites should be aligned across 75% or more of the family members for creation of proper family clusters.

A statistical enrichment test was performed for each molecular function, biological process, or cellular component. The genes associated with a particular ontology term were evaluated according to the likelihood of the numerical values of genes that were drawn randomly from the overall distribution of values. The Mann-Whitney U test was used to determine the P-value. A number of genes, which were induced in large extent by our microarray analysis, were classified according to a number of statistical tests that were performed by the PANTHER classification system. As a result, PANTHER classification concluded that induction of a considerable number of major histocompatibility complex (MHC) class II genes was a characteristic change in global gene expression caused by the present drug combination of 5-FU and Dep (discussed in Results). The details of using this tool are described in the PANTHER user manual for PANTHER 9.0.

Statistical analysis. Data are represented as the mean ± standard error of mean (SEM) and analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test as a post hoc test. A P-value of <0.05 was considered to indicate a statistically significant result.

Results

Cellular sensitivity of the HCT-116 cells to 5-FU. The approximate IC_{50} value of the HCT-116 cells to 5-FU was 1.90 μM. However, Dep was almost 2,000-fold active in terms of molar concentration to inhibit colony formation ability of HCT-116 compared with 5-FU. Fig. 1A and B shows the dose-response curves of 5-FU and Dep, respectively. Fig. 1C shows the reduction of survival fraction for the combination of 5-FU and Dep, 5-FU alone, and Dep alone. The combination of 1.75 μM 5-FU and 1 nM Dep suppressed colony formation to 30% compared with vehicle-only. This combination suppressed colony formation ability with statistical significance compared with 5-FU only (P<0.01). HCT-116 cells became less sensitive to 5-FU in combination with 0.1 nM Dep compared with 5-FU only, which was not marked.

Cellular sensitivity of the HT29 cells to 5-FU. The approximate IC_{50} value of HT29 to 5-FU was 0.68 μM. However, Dep was almost 2,000-fold active in terms of molar concentration to inhibit colony formation ability of HT29 cells compared with 5-FU. Fig. 1D and E shows the dose-response curves of 5-FU and Dep, respectively. Fig. 1F illustrates the reduction in survival fraction for the combination of 5-FU and Dep, 5-FU alone, and Dep alone. The combination of 0.625 μM 5-FU and 0.375 nM Dep suppressed colony formation to 35% compared with vehicle-only. This combination suppressed colony formation ability with statistical significance compared with 5-FU-only (P<0.01).

Cellular sensitivity of the SW48 cells to 5-FU. The approximate IC_{50} value of SW48 to 5-FU was 1.15 μM. However, Dep was almost 5,000-fold active in terms of molar concentration to inhibit cellular viability of the SW48 cells compared with 5-FU. Fig. 1G and H shows the dose-response curves of 5-FU and Dep, respectively. Fig. 1I illustrates the reduction in survival fraction for the combination of 5-FU and Dep, 5-FU alone, and Dep alone. The combination of 0.875 μM 5-FU and 0.2 nM Dep suppressed cellular viability to 25% compared with vehicle alone. This combination suppressed cellular viability with statistical significance compared with 5-FU alone (P<0.01).

Caspase-3/7 activation in HCT-116 cells following treatment with 5-FU (1.75 μM) alone, Dep (1 nM) alone, and a combination of 5-FU and Dep. As shown in Fig. 2, the time course of caspase-3/7 activation was examined in the HCT-116 cells following treatment with 5-FU (1.75 μM) alone, Dep (1 nM) alone, and the combination of 5-FU and Dep.

At 24 h, the combination of 5-FU and Dep markedly enhanced caspase-3/7 activation compared to the vehicle control (3.8-fold-change, P<0.01). Dep alone also showed a 2.0-fold increase (P<0.01) compared to the vehicle control.

At 48 h, the combination of 5-FU and Dep increased caspase-3/7 activation compared to the vehicle control (2.8-fold-change, P<0.01). 5-FU alone and Dep alone slightly increased caspase-3/7 activation compared to the vehicle control (1.4-fold, P<0.05; and 1.5-fold, P<0.01, respectively).

Comparison of the gene expression profile and gene ontology of the cells treated with the control and 5-FU (1.75 μM) + Dep (1 nM) combination. To examine the gene expression profile after drug exposure, we conducted a microarray analysis of the cells treated for 7 days with the vehicle control, 5-FU alone, Dep alone, and the combination of 5-FU with Dep as described in Materials and methods. Results of the gene expression profile were analyzed by gene ontology in order to clarify what mRNAs were upregulated (Table II) or downregulated (Table III) after treatment with each drug alone or with combination of 5-FU and Dep. To indicate the biological significance of cellular processes affected by the combination treatment, we listed the fold enrichment in descending order in Tables II and III.

Analysis of PANTHER GO-Slim Biological Process and Cellular Component revealed a marked increase in mRNA levels of MHC class-related gene ontologies (GO:000254 and GO:0042611, respectively).

The mechanism of action, cell cycle- and apoptosis-related gene expression in HCT-116 cells following treatment with 5-FU (1.75 μM) alone, Dep (1 nM) alone, and combination
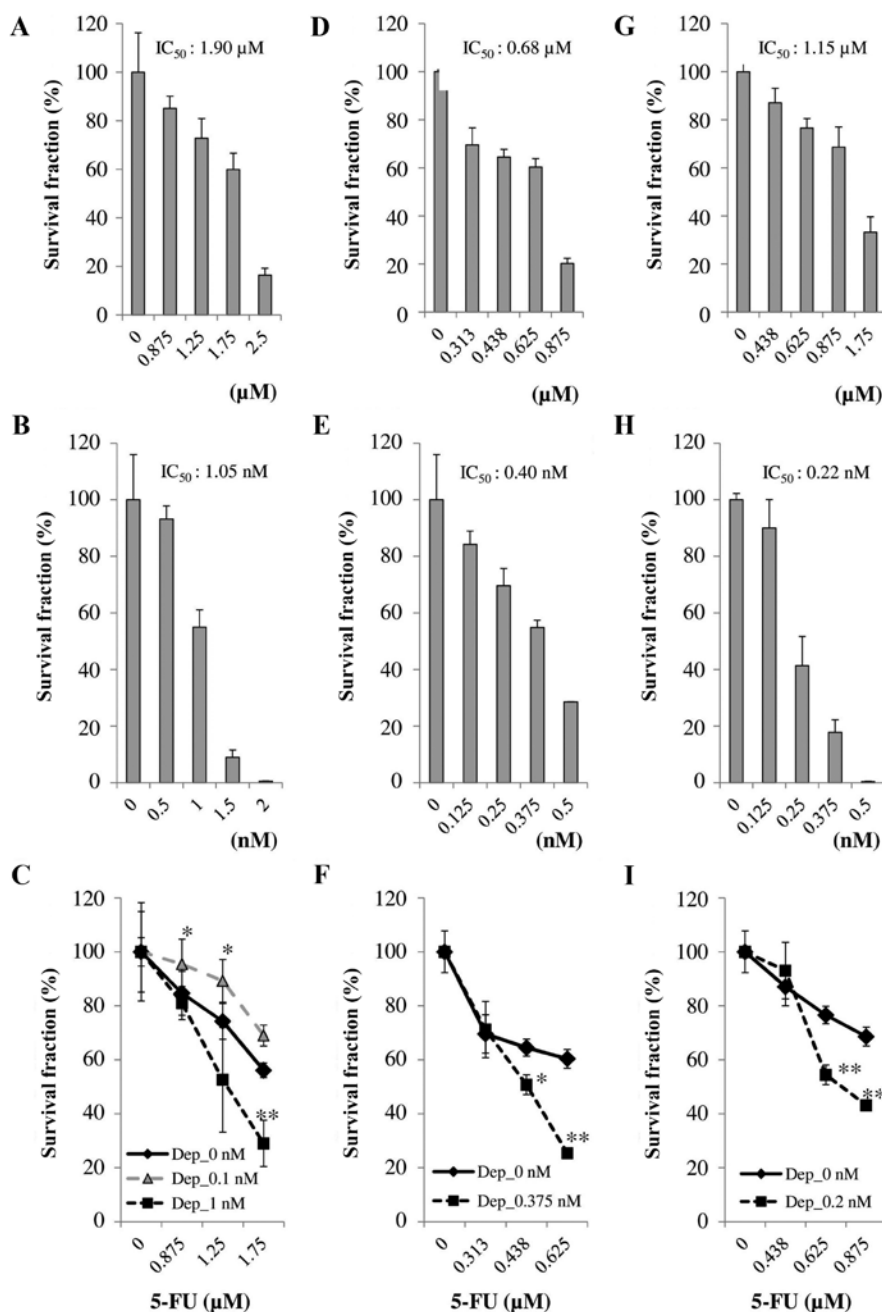
Figure 1. Effect of (A) 5-fluorouracil (5-FU), (B) depsipeptide (Dep) and the combination of (C) 5-FU and Dep on the inhibition of colony formation ability of human colon cancer HCT-116 cells. Effect of (D) 5-FU, (E) Dep and the combination of (F) 5-FU and Dep on the inhibition of colony formation ability of human colon cancer HT29 cells. Effect of (G) 5-FU, (H) Dep and the combination of (I) 5-FU and Dep on inhibition of cellular viability of the human colon cancer SW48 cells. The x-axis represents drug concentrations, and y-axis represents survival fraction (i.e., colony-forming ability relative to 0 μM 5-FU). *P<0.05 vs. 0 nM Dep, **P<0.01 vs. 0 nM Dep by one-way ANOVA.
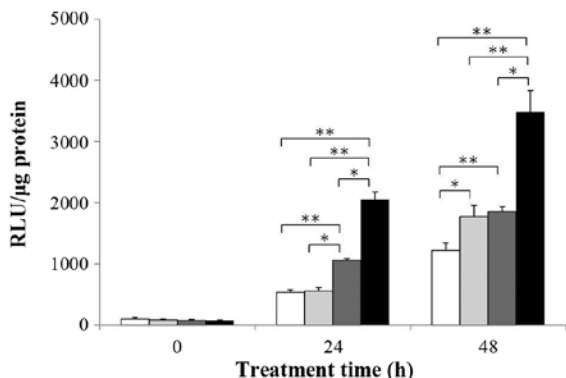
Figure 2. Time-course of caspase-3/7 activation in HCT-116 cells following treatment with the vehicle control (white bar), 5-fluorouracil (5-FU) (1.75 μM) alone (light gray bar), depsipeptide (Dep) (1 nM) alone (dark gray bar) and the combination of 5-FU (1.75 μM) + Dep (1 nM) (black bar). Data are expressed as relative light units (RLU)/μg of protein. The x-axis represents the length of the treatment period (h), and the y-axis represents RLU/μg of protein. *P<0.05 or **P<0.01 indicates a significant difference.

Table II. GO analysis for comparison between control and 5-FU (1.75 μM) + Dep (1 nM) combination. Top 1,000 genes with upregulation of over 1.5-fold-change.

Ontology terms (class ID)	F1.75D1		F1.75D0		F0D1	
	Fold enrichment	P-value	Fold enrichment	P-value	Fold enrichment	P-value
PANTHER GO-Slim Biological Process						
Antigen processing and presentation of peptide or polysaccharide antigen via MHC class (GO:0002504)	4.98	2.42E-02	0.56	1.00E+00	>5.00	3.78E-03
Phospholipid metabolic process (GO:0006644)	2.78	2.58E-03	1.77	1.00E+00	2.11	6.54E-01
Cellular component morphogenesis (GO:0032989)	1.98	4.08E-03	1.66	5.20E-01	1.21	1.00E+00
Anatomical structure morphogenesis (GO:0009653)	1.91	1.78E-03	1.66	1.75E-01	1.29	1.00E+00
Death (GO:0016265)	1.86	7.23E-03	1.21	1.00E+00	1.32	1.00E+00
Cell death (GO:0008219)	1.83	1.19E-02	1.18	1.00E+00	1.33	1.00E+00
Apoptotic process (GO:0006915)	1.77	4.59E-02	1.21	1.00E+00	1.32	1.00E+00
Cellular component organization (GO:0016043)	1.61	1.71E-03	1.44	1.71E-01	1.49	5.37E-02
Phosphate-containing compound metabolic process (GO:0006796)	1.57	4.78E-02	1.32	1.00E+00	1.88	2.19E-05
Cellular component organization or biogenesis (GO:0071840)	1.52	9.82E-03	1.52	1.25E-02	1.43	1.37E-01
Developmental process (GO:0032502)	1.41	1.22E-03	1.25	7.35E-01	1.25	6.68E-01
Cell communication (GO:0007154)	1.39	1.64E-04	1.13	1.00E+00	1.13	1.00E+00
Cellular process (GO:0009987)	1.22	2.39E-04	1.19	5.49E-03	1.23	1.72E-04
PANTHER GO-Slim Cellular Component						
MHC protein complex (GO:0042611)	>5.00	2.23E-05	1.13	1.00E+00	>5.00	1.98E-05
Intermediate filament cytoskeleton (GO:0045111)	3.70	3.58E-03	4.06	7.06E-04	1.15	1.00E+00
Cytoskeleton (GO:0005856)	2.18	4.74E-08	1.68	8.97E-03	1.79	9.17E-04
Actin cytoskeleton (GO:0015629)	2.14	1.70E-03	1.62	6.18E-01	1.36	1.00E+00
Organelle (GO:0043226)	1.40	4.23E-03	1.44	1.18E-03	1.41	3.42E-03
Intracellular (GO:0005622)	1.28	3.67E-02	1.32	7.91E-03	1.22	2.77E-01
Cell part (GO:0044464)	1.25	4.49E-02	1.35	2.80E-04	1.17	8.30E-01
PANTHER GO-Slim Molecular Function						
Hydrogen ion transmembrane transporter activity (GO:0015078)	4.62	3.24E-02	2.09	1.00E+00	1.56	1.00E+00
Actin binding (GO:0003779)	2.63	9.73E-03	2.07	7.69E-01	1.70	1.00E+00
Cytoskeletal protein binding (GO:0008092)	2.60	4.21E-04	1.88	7.31E-01	1.79	1.00E+00
Structural constituent of cytoskeleton (GO:0005200)	2.06	1.11E-05	1.59	2.26E-01	1.42	1.00E+00
Protein binding (GO:0005515)	1.37	1.30E-03	1.22	8.41E-01	1.14	1.00E+00
Hydrolase activity (GO:0016787)	1.37	1.10E-02	1.29	2.30E-01	1.27	5.10E-01

GO, Gene Ontology. F1.75D1, 5-FU (1.75 μM) + Dep (1 nM) combination; F1.75D0, 5-FU (1.75 μM) alone, F0D1, Dep (1 nM) alone.

of 5-FU and Dep. We examined levels of mRNAs encoded by various genes in the cells treated with 5-FU or Dep alone, and the combination of 5-FU and Dep by qRT-PCR method (Fig. 3).

The levels of TS mRNA encoded by the *TYMS* gene were decreased by 42% in the cells treated with the combination of 5-FU and Dep with statistical significance (P<0.01).

Table III. GO analysis for comparison between control and 5FU (1.75 μM) + Dep (1 nM) combination. Top 1,000 genes with downregulation over 1.5-fold-change.

Ontology terms (class ID)	F1.75D1		F1.75D0		F0D1	
	Fold enrichment	P-value	Fold enrichment	P-value	Fold enrichment	P-value
PANTHER GO-Slim Biological Process						
Chromatin organization (GO:0006325)	3.17	4.23E-07	1.02	1.00E+00	1.52	1.00E+00
Organelle organization (GO:0006996)	1.99	6.67E-04	1.11	1.00E+00	1.19	1.00E+00
Cell cycle (GO:0007049)	1.65	1.56E-03	1.03	1.00E+00	1.11	1.00E+00
Cellular component organization (GO:0016043)	1.55	1.13E-02	1.16	1.00E+00	1.23	1.00E+00
Cellular component organization or biogenesis (GO:0071840)	1.48	3.27E-02	1.16	1.00E+00	1.14	1.00E+00
Transcription from RNA polymerase II promoter (GO:0006366)	1.43	1.80E-02	1.20	1.00E+00	1.61	1.41E-05
Nucleobase-containing compound metabolic process (GO:0006139)	1.41	6.86E-06	1.07	1.00E+00	1.27	3.06E-02
RNA metabolic process (GO:0016070)	1.38	6.85E-03	1.09	1.00E+00	1.44	3.19E-04
Transcription, DNA-dependent (GO:0006351)	1.38	3.81E-02	1.20	1.00E+00	1.64	4.53E-07
Biological regulation (GO:0065007)	1.27	1.13E-02	1.24	4.79E-02	1.34	9.88E-05
Primary metabolic process (GO:0044238)	1.25	1.70E-05	1.11	1.00E+00	1.10	1.00E+00
Metabolic process (GO:0008152)	1.22	3.98E-06	1.13	1.80E-01	1.11	8.58E-01
Cellular process (GO:0009987)	1.19	1.06E-02	1.19	9.44E-03	1.06	1.00E+00
PANTHER GO-Slim Molecular Function						
Chromatin binding (GO:0003682)	2.87	4.26E-03	1.48	1.00E+00	1.75	1.00E+00
Nucleic acid binding (GO:0003676)	1.41	8.32E-05	0.97	1.00E+00	1.33	6.16E-03
DNA binding (GO:0003677)	1.40	1.67E-02	1.09	1.00E+00	1.54	5.30E-05
Binding (GO:0005488)	1.34	2.77E-08	1.10	1.00E+00	1.26	6.70E-05
Catalytic activity (GO:0003824)	1.22	1.17E-02	1.19	8.98E-02	1.03	1.00E+00
PANTHER GO-Slim Molecular Function Not extracted						

GO, Gene Ontology. F1.75D1, 5-FU (1.75 μM) + Dep (1 nM) combination; F1.75D0, 5-FU (1.75 μM) alone, F0D1, Dep (1 nM) alone.

Slight, but statistically significant downregulation was observed for HDAC1 mRNA in the cells treated with the 5-FU and Dep combination.

Dep alone and the combination of 5-FU and Dep decreased cellular p16 mRNA levels by 60 and 64%, respectively.

Regardless of the presence of Dep, 5-FU induced p21 levels; marked changes in p21 levels by 396% were observed without Dep, and by 764% with combination of 5-FU and Dep with statistical significance (P<0.01).

Dep reduced TP53 levels by 75%. 5-FU alone also slightly decreased TP53 levels by 42%.

5-FU considerably decreased the BCL2 mRNA level to 59%, which was apparently restored in the presence of Dep (to 103% in the combination with 5-FU).

An unremarkable change was observed in BAK1 mRNA in the cells following treatment with either 5-FU alone or Dep alone (138 and 86%, respectively). The combination of these drugs slightly increased the BAK1 mRNA level to 160%, which was statistically significant compared with the vehicle control (P<0.05), and with Dep only (P<0.01). Regarding BAX, 5-FU increased the BAX mRNA level to 163%. The combination of Dep with 5-FU decreased BAX mRNA to 49% of the vehicle control, which was statistically significant compared with 5-FU only (P<0.01), and with the vehicle control (P<0.05).

MHC class II and I mRNA levels in the HCT-116 cells following treatment with 5-FU (1.75 μM) alone, Dep (1 nM) alone, and a combination of 5-FU and Dep. We examined levels of MHC class II and I mRNA by qRT-PCR in the cells treated with 5-FU or Dep alone, and a combination of 5-FU and Dep (Fig 4).

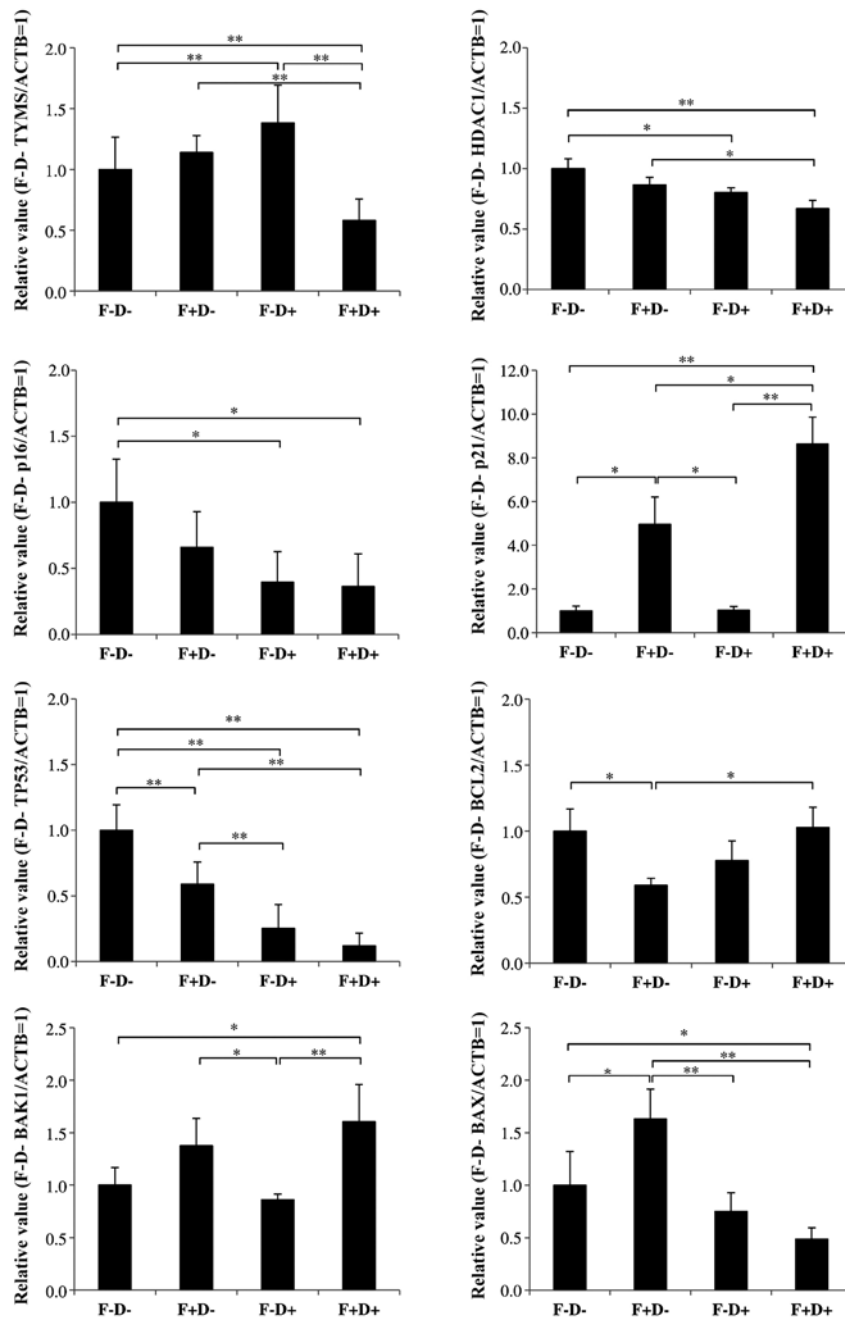MHC class II genes, including *HLA-DPB1*, *HLA-DQB1*, *HLA-DRA*, and *HLA-DRB1* were markedly upregulated

Figure 3. The mechanism of action, cell cycle- and apoptosis-related gene-expression in HCT-116 cells following treatment with the vehicle control (F⁻D⁻), 5-fluorouracil (5-FU) (1.75 μM) alone (F⁺D⁻), depsipeptide (Dep) (1 nM) alone (F⁻D⁺) and the combination of 5-FU (1.75 μM) + Dep (1 nM) (F⁺D⁺). mRNA levels were assessed by qRT-PCR and were normalized by the ACTB mRNA level at each point. The y-axis represents mRNA levels of the genes of interest relative to ACTB mRNA. Data are expressed as mean ± SEM. *P<0.05 or **P<0.01 indicates a significant difference by one-way ANOVA.

in combination with 5-FU and Dep (P<0.01) to 432, 298, 667 and 847%, respectively, compared to the vehicle controls.

Dep alone slightly increased levels of MHC class II genes, including *HLA-DPB1*, *HLA-DQB1*, *HLA-DRA*, and *HLA-DRB1* by 52, 38, 157 and 168%, respectively. Compared with Dep alone, the combination with 5-FU greatly elevated mRNA levels of these MHC class II genes, namely, *HLA-DPB1*, *HLA-DQB1*, *HLA-DRA*, and *HLA-DRB1* with statistical significance (P<0.01). In the HT29 and SW48 cells, increased expression level of HLA-DRA (MHC class II gene) was observed with statistical significance in combination of 5-FU and Dep by real-time PCR (data not shown).

No change in the levels of MHC class I genes including *HLA-A*, *HLA-B-HLA-C* were observed following treatment with 5-FU, Dep, or 5-FU plus Dep.

Summary of changes in expression of molecular regulation of MHC class II genes by microarray analysis in the HCT-116 cells. We further analyzed the results of our global analysis of gene expression, which were focused on genes involved in the molecular regulation of MHC class II genes. As described in Table IV, we found the highest degree of increased expression levels of PCAF, CREB3, CREB5, and CIITA after the combination of 5-FU and Dep. PCAF, a histone acetyltrans-
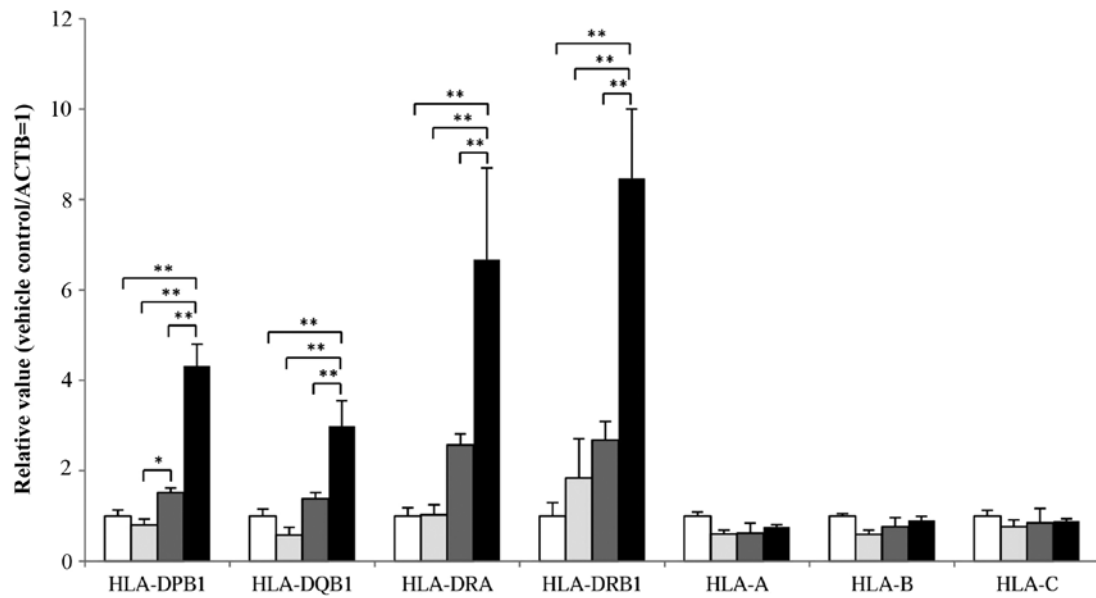
Figure 4. Expression of major histocompatibility complex (MHC) class II and I genes in the HCT-116 cells following treatment with the vehicle control (white bar), 5-fluorouracil (5-FU) (1.75 μM) alone (light gray bar), depsipeptide (Dep) (1 nM) alone (dark gray bar), and the combination of 5-FU (1.75 μM) + Dep (1 nM) (black bar). The mRNA levels were normalized by the ACTB mRNA level at each point. The x-axis indicates the MHC class II and MHC class I genes. The y-axis represents mRNA levels of the genes of interest relative to the ACTB mRNA. Data are expressed as mean ± SEM. [*]P<0.05 or [**]P<0.01 indicates a significant difference by one-way ANOVA.

Table IV. Summary of changes in gene expression for molecular regulation of MHC class II genes by microarray analysis in the HCT-116 cells.

Gene name (synonym)	Accession no.	Description	Fold-change		
			F1.75D1	F1.75D0	F0D1
CIITA	NM_000246	Class II, major histocompatibility complex, transactivator	2.80	0.80	0.91
CREB1	NM_13444	cAMP responsive element binding protein 1	0.91	0.91	1.17
CREB2 (ATF4)	NM_001675	Activating transcription factor 4	0.22	0.66	0.55
CREB3	NM_006368	cAMP responsive element binding protein 3	2.13	1.05	1.09
CREB5	NM_182898	cAMP responsive element binding protein 5	6.51	0.75	0.64
EP300 (KAT3B)	NM_001429	E1A binding protein p300	0.59	0.69	0.80
KAT2B (PCAF)	NM_003884	K(lysine) acetyltransferase 2B	3.15	1.37	1.47
NFYA	NM_002505	Nuclear transcription factor Y, α	1.27	1.02	1.15
NFYB	NM_006166	Nuclear transcription factor Y, β	0.88	1.14	0.97
NFYC	NM_014223	Nuclear transcription factor Y, γ	0.56	1.08	0.87
PCBP4 (CBP)	NM_033010	poly(rC) binding protein 4	1.43	1.05	1.10
RFXANK	NM_003721	Regulatory factor X-associated ankyrin-containing protein	0.37	0.63	0.68
RFXAP	NM_000538	Regulatory factor X-associated protein	1.29	0.82	0.79
RX5	NM_000449	Regulatory factor X, 5 (influences HLA class II expression)	0.95	1.51	0.97

MHC, major histocompatibility complex. F1.75D1, 5-FU (1.75 μM) + Dep (1 nM) combination; F1.75D0, 5-FU (1.75 μM) alone, F0D1, Dep (1 nM) alone.

ferase, is related to apoptotic cell processes, and CREB3, CREB5 and CIITA are related to MHC class II gene expression. On the contrary, ATF4, a member of the CREB family, was slightly downregulated following treatment with 5-FU or Dep alone, and markedly downregulated by the combination of 5-FU and Dep.

Discussion

In the present study, we found the HDAC inhibitor, Dep, potentiated 5-FU cytotoxicity via inhibition of colony formation ability in human colon cancer HCT-116 cells (Fig. 1). The primary findings of our study were that Dep augments

the effects of 5-FU in HCT-116 cells, and that activation of caspase-3/7 is the underlying mechanism. Moreover, the importance of MHC class II and p21 (*CDKN1A*) upregulation (Figs. 4 and 3, respectively) in the effect of the combination of 5-FU and Dep was revealed. Dep was previously reported to induce caspase-dependent apoptosis in small cell lung cancer cells via the mitochondrial pathway by suppressing the expression of BCL2 family members (28). In contrast, the present study did not show a marked reduction in HCT-116 cells. These results indicated that the sensitization of HCT-116 cells to the combination of 5-FU and Dep was not likely to be mediated by changes in expression of BCL2 family members (28).

Exposure of cancer cells to 5-FU elevated the expression of p21 (*CDKN1A*), and the combination of Dep with 5-FU further enhanced this mRNA expression of p21 (*CDKN1A*) (Fig. 3). Induction of p21 using a tetracycline-inducible vector system showed that p21 plays an important role in the induction of growth arrest in tumor cells (29). A critical role of p21 has been described as a critical mediator of cytotoxicity of 5-FU (30). These results are consistent with our present results that the 5-FU-induced and Dep-enhanced p21 (*CDKN1A*) expression are both associated with cell cycle arrest.

Our present microarray and the successive gene ontology analyses revealed that combination of 5-FU and Dep markedly increased the mRNA levels of MHC class II genes, namely, *HLA-DPB1*, *HLA-DQB1*, *HLA-DRA*, and *HLA-DRB1* (Fig. 4). This marked activation of MHC class II gene expression, however, was not noted following treatment with 1.75 μM 5-FU alone, or 1 nM depsipeptide alone. The HCT-116 cells treated with the combination underwent caspase 3/7-associated apoptosis. Therefore, activation of MHC class II expression may have some relationship with the cell apoptotic process. Ting and Trowsdale described positively regulated control of MHC class II expression, which was mediated by DNA binding factors including NF-Y, CREB, and RFX, and the coactivator, CIITA, general transcription factors, and histone acetyltransferases including P300/CBP-associated factor (PCAF) (31). Among them, PCAF was reported to accelerate cancer cell apoptosis by counteracting the anti-apoptotic cellular process (32). In addition, an HDAC inhibitor was reported to enhance MHC class II expression in both CIITA-dependent and -independent manners (33). Our present results of augmentation of MHC class II gene expression after the treatment with 5-FU combined with an HDAC inhibitor, depsipeptide, may be collateral antitumor events for the induction of cellular apoptosis by 5-FU. Our global analysis of gene expression indicated marked induction of MHC class II members together with PCAF (also called KAT2B), CREB3, CREB5, and CIITA (Table IV), which may be correlated with a number of apoptotic cell processes, by the combination of 5-FU and depsipeptide. In fact, higher 5-FU concentrations revealed dose-dependent MHC class II induction (unpublished data). Collectively, our present results may indicate a closer relationship between the elevation of MHC class II expression and cellular apoptosis induced by antitumor agent, 5-FU. Furthermore, we are investigating whether induction of MHC class II proteins after 5-FU-based chemotherapy is a prognostic marker.

On the other hand, previous reports have documented the importance of histone acetylation as a positive regulator of MHC class II transcription and that HDAC inhibitors augment the expression of tumor cell MHC class II (34-36). Dep may affect the differentiation of colon cancer cells because of the close correlation already shown between the expression of *HLA-DR* antigen and the differentiation of tumor cells using gastric carcinoma models (37,38).

West *et al* recently proposed that HDAC inhibitors require the immune system to exert their anticancer effects (39). Downregulation of immune-system-related genes including MHC class II genes was shown to associate cooperatively with the metastatic potential of early stage colorectal carcinoma (40).

Our present results are in agreement with concepts raised very recently that immune systems where MHC class II genes are involved have a considerable relationship with successful cancer chemotherapy and/or less cancer cell malignancies.

Therefore, our present results imply that 5-FU combined with Dep synergistically enhances the expression of tumor cell surface immunological proteins (i.e., marked inductions of MHC class II genes). This is consistent with what is known concerning the concerted mechanisms of therapeutic drugs and immune systems in cancer chemotherapy.

In conclusion, the combination of 5-FU and Dep sensitized human colon cancer HCT-116, HT29, and SW48 cells. The sensitization in the HCT-116 cells likely occurred through apoptosis induction shown by caspase-3/7 activation, induction of cell cycle arrest shown by p21 (*CDKN1A*) upregulation, and induction of MHC class II gene expression. MHC class II gene induction may render cancer chemotherapy more effective through a possible cooperation of the immune system with cancer chemotherapy. Epigenetic modulation of existing anticancer drugs may be considered for development of novel cancer chemotherapy.

Acknowledgements

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