Note

Different Schedule-dependent Effects of Epigenetic Modifiers on Cytotoxicity by Anticancer Drugs in Colorectal Cancer Cells

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SUMMARY

Limited information is currently available on how to apply epigenetic modifiers to current colorectal cancer (CRC) chemotherapy. The purpose of this study is to clarify the schedule-dependent effects of combined treatment with conventional anticancer drugs and epigenetic modifiers in human CRC cells. Cytotoxicity in 4 CRC cell lines (SW480, HT29, SW48, and HCT116) was measured using the WST-8 assay. As epigenetic modifiers, 3 DNA methyltransferase (DNMT) inhibitors such as decitabine (DAC), azacytidine (AC), and zebularine (Zeb), and 3 histone deacetylase (HDAC) inhibitors including trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), and valproic acid (VPA) were used. Combination effects were analyzed by the isobologram method. SW480 cells showed the lowest sensitivity to the anticancer drugs 5-fluorouracil, SN-38 (the active form of irinotecan), and oxaliplatin. In SW480 cells, epigenetic modifiers other than VPA showed the most significant synergistic effects when used before anticancer drugs, while VPA showed synergistic effects in co- or post-treatment. In the 3 other CRC cells, synergistic effects were less frequent and weaker. The dose of anticancer drugs may be reduced by combining epigenetic modifiers in SW480 cells, which are less sensitive to anticancer drugs, unlike the more sensitive HT29, SW48, and HCT116 cell lines. These results provide useful information for understanding how to incorporate epigenetic modifiers into current CRC chemotherapy.
Key words

DNA methyltransferase inhibitor; histone deacetylase inhibitor; schedule-dependent treatment; epigenetic therapy; colorectal cancer
INTRODUCTION

Epigenetic alterations such as DNA methylation and histone modifications have been identified as a crucial driving force in the initiation and progression of cancer.\textsuperscript{1)\textdegree} Since epigenetic alterations are reversible, epigenetic modifiers may be beneficial in chemotherapy.\textsuperscript{2)\textdegree} Several epigenetic modifiers including DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors have been approved by the Food and Drug Administration (FDA) for the treatment of cancer.

Combined chemotherapy, including 5-fluorouracil (5-FU) and either irinotecan (CPT-11) or oxaliplatin (L-OHP), has improved the survival period of metastatic colorectal cancer (CRC). However, resistance to these anticancer drugs, which limits the effectiveness of chemotherapy, has been reported.\textsuperscript{3,4)\textdegree} In order to overcome this issue, novel combination therapy using epigenetic modifiers is expected. Clinical trials on combination therapy have been performed using solid tumors including CRC.\textsuperscript{5,6)\textdegree} However, there is not enough integrated information on how to apply epigenetic modifiers to current CRC chemotherapy. We previously reported that a co-treatment with decitabine (DAC, 5-aza-2'-deoxycytidine), a DNMT inhibitor, and L-OHP exerted the most potent synergistic effects among the combinations tested using 5 epigenetic modifiers and 5-FU, CPT-11, or L-OHP in 4 different CRC cell lines.\textsuperscript{7)\textdegree}

The treatment schedule is important for effective cancer chemotherapy using combination...
therapy with several anticancer drugs. Regarding conventional cytotoxic anticancer drugs, information to show that the effects of anticancer drugs depend on the treatment schedule is increasing.\(^8\)\(^9\) In clinical settings, the FOLFIRI or FOLFOX regimen, which includes a bolus/infusion of 5-FU with leucovorin and CPT-11 or L-OHP, has been applied to the treatment of advanced CRC. In addition, the effects of DNMT or HDAC inhibitors combined with anticancer drugs were previously shown to be schedule-dependent.\(^10\)\(^11\) A pretreatment with DAC followed by idarubicin/cytarabine resulted in stronger anti-leukemic effects in patients with myeloid neoplasmas.\(^12\) However, integrated information on the schedule-dependent effects of combination therapy using epigenetic modifiers and anticancer drugs is limited. The present study aimed to elucidate which schedule is effective and what type of CRC cell is more sensitive to combination therapy using 6 kinds of epigenetic modifiers and 4 CRC cell lines examined in our previous study.\(^7\)
MATERIALS AND METHODS

Materials Three human colon adenocarcinoma cell lines (HT29, SW480, and SW48) and the human colon carcinoma cell line HCT116 were purchased from DS Pharmabiomedical (Osaka, Japan). McCoy’s 5A medium, Leibovitz L-15 medium (L-15), penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Life Technologies Corp. (Carlsbad, CA). L-OHP, DAC, 5-azacytidine (AC), zebularine (Zeb), trichostatin A (TSA), and suberoylanilide hydroxamic acid (SAHA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 5-FU was purchased from Nacalai Tesque Inc. (Kyoto, Japan). 7-Ethyl-10-hydroxycamptothecin (SN-38) and valproic acid (VPA) sodium salt were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). All other chemicals were of the highest grade commercially available.

Cells and cell cultures HT29 and HCT116 cells were cultured in McCoy’s 5A medium at 37°C in 5% CO₂-95% air. SW480 and SW48 cells were cultured in L-15 medium at 37°C in 100% air. Cell culture medium was supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Drug treatment Cells were seeded at a density of 7 x 10³ (SW480) or 4 x 10³ (HT29, HCT116, and SW48) on 96-well plates and cultured for 24 h before drug treatments. Drugs
were used as described in previous studies with some of the following modifications: (A) epigenetic modifiers followed by anticancer drugs, (B) anticancer drugs followed by epigenetic modifiers, and (C) a simultaneous treatment with epigenetic modifiers and anticancer drugs\textsuperscript{13}. In schedule (A), after cells had been treated with epigenetic modifiers for 24 h, they were washed twice with culture medium and anticancer drugs were then added. In schedule (B), the treatment order in treatment schedule (A) was changed to anticancer drugs followed by epigenetic modifiers. In schedule (C), 24 h after culture medium was added instead of drugs, cells were washed twice with culture medium and epigenetic modifiers and anticancer drugs were then added. In the three schedules, after the last drug treatment, cells were cultured for 48 h. In order to obtain the IC\textsubscript{50} values of anticancer drugs or epigenetic modifiers alone, culture medium was added instead of a counterpart of two combination drugs in the same treatment schedule described above. In the combination study, epigenetic modifiers were used at approximately their IC\textsubscript{20} and IC\textsubscript{50} values in SW480 cells or approximately their IC\textsubscript{20} values in HT29, HCT116, and SW48 cells; anticancer drugs were used at approximately their IC\textsubscript{20} and IC\textsubscript{50} values. In HT29 cells, a value less than IC\textsubscript{20} was used because the IC\textsubscript{50} value of DAC in HT29 cells was markedly larger than that in other cells.

**Cytotoxicity assay** A cytotoxicity assay was performed using the WST-8 assay with a
Cell Counting-8 kit (Dojindo Laboratories, Kumamoto, Japan) at day 4 after seeding.

Absorbance was measured at 450 nm/655 nm using the Model 550 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA) after an incubation with WST-8 solution at 37°C for 1-2 h. IC₅₀ values were obtained using the sigmoid inhibitory effect model with the non-linear least-squares fitting method (Solver, Microsoft Excel 2013).

Analysis of combination effects  
An isobologram analysis was performed using the method of Steel and Peckham because it has the ability to deal with any agents with unclear cytotoxic mechanisms and a number of dose-response curves of anticancer drugs (Supplementary Fig. 1). The isobologram was drawn as previously described. Briefly, concentrations producing 50% growth inhibition were shown as 1.0 on the ordinate and abscissa of isobolograms. Three isoeffect curves, mode I and mode II, were made based on the dose-response curves of each drug. The area surrounded by mode I and/or mode II lines was called the envelope of additivity. Combination index (CI) values were plotted in the isobologram. The CI value was calculated using CalcuSyn software (Biosoft, Cambridge, UK) and defined as follows: CI = Cₓ/Cₐₓ+Cᵧ/Cₐᵧ, where Cₓ and Cᵧ are the concentrations of drug x and drug y used in combination to achieve a% drug effect, while Cₐₓ and Cₐᵧ are the concentrations of a single agent to achieve a% when used alone. The data points of CI values on the left of the envelope of additivity, within it, or on its right were regarded as synergistic,
additive, or antagonistic, respectively.

CI values, which were regarded as synergistic or antagonistic in the isobologram, were further evaluated based on the method of Chou and Talay.\textsuperscript{15} CI values indicate the following: <0.1, very strong synergism; 0.1-0.3, strong synergism; 0.3-0.7, synergism; 0.7-0.85, moderate synergism; 0.85-0.9, slight synergism; 0.9-1.1, nearly additive; 1.1-1.2, slight antagonism; 1.2-1.45, moderate antagonism; 1.45-3.3, antagonism; 3.3-10, strong antagonism; >10, very strong antagonism. The dose reduction index (DRI), which represents the fold-reduction in each drug when two drugs are used in combination from the dose of each drug alone to achieve a given effect level, was also evaluated.\textsuperscript{15} The DRI value was defined as follows: CI = C_x/C_{a,x} + C_y/C_{a,y} = 1/DRI_x + 1/DRI_y. DRI more than 1 was regarded as a synergistic effect.

RESULTS

Cytotoxicity by Anticancer Drugs and Epigenetic Modifiers in 4 CRC Cell Lines

Among the 4 CRC cell lines tested (SW480, HT29, HCT116, and SW48), SW480 cells showed the largest IC\textsubscript{50} values for 3 anticancer drugs (5-FU, SN-38, and L-OHP) (Table 1). Therefore, SW480 cells were used in experiments on schedule-dependent combination effects with 6 epigenetic modifiers. The IC\textsubscript{50} value of DAC was the largest in HT29 cells (Table 1).
The IC₅₀ values of SAHA and VPA were the largest in SW480 cells (Table 1). The IC₅₀ values of AC, Zeb and TSA in SW480 cells were shown in Supplementary Table 1.

Schedule-dependent Effects of a Combined Treatment with Anticancer Drugs and Epigenetic Modifiers in 4 CRC Cell Lines

Combined data points in the isobologram were evaluated to establish whether they were synergistic, additive, or antagonistic (Supplementary Figs. 2, 3, 4, 5 and 6). The CI and DRI values of combined data points, which were regarded as synergistic or antagonistic in the isobologram analysis, were then evaluated. The schedules of all the combinations were carried out in 4 cell lines, but those of the most significant combination effects were shown in Table 2 to Table 5 for each cell except for HCT116 cells because synergistic effect was observed in only one combination schedule (DAC+L-OHP) in HCT116 cells. Tables 2 and 3 were the results of SW480 cells, while Table 4 was those of HT29 cells, and Table 5 was those of SW48 cells. The results of the other schedules were shown in Supplementary Tables 2-6. First, in SW480 cells, schedule-dependent combination effects using 3 anticancer drugs and 6 epigenetic modifiers were examined (Tables 2 and 3, Supplementary Tables 2 and 3). The intensity of the most significant combination effects by CI values was in the order of TSA>DAC≈AC>Zeb>SAHA>VPA (in the order of low CI values) (Tables 2 and 3). In synergistic combinations, the DRI values of anticancer drugs were larger than those of
epigenetic modifiers, except for DAC+L-OHP, AC→SN-38, and TSA→L-OHP. The effects of DAC and AC were similar. The effects of VPA were different from other epigenetic modifiers. SAHA is a more advanced anticancer drug than TSA. Therefore, schedule-dependent combined effects with DAC, SAHA or VPA and anticancer drugs were examined in HT29, HCT116, and SW48 cells (Tables 4 and 5, Supplementary Tables 4, 5 and 6). The isobolograms of the most significant synergistic combination in HT29 and SW48 cells were shown in Supplementary Figs. 5 and 6, respectively.

In pre-treatment with epigenetic modifiers, DAC, AC, Zeb, SAHA or TSA showed the most synergistic effects compared to 2 other schedules in SW480 cells (Tables 2 and 3). In particular, the combination with 5-FU showed the most synergistic effects although synergistic effects were also observed with 2 anticancer drugs (SN-38 and L-OHP). Among 3 CRC cells (HT29, HCT116 and SW48 cells), the pre-treatment with SAHA or VPA showed the most synergistic effects only in SW48 cells (Tables 4 and 5, Supplementary Tables 5 and 6). In co-treatment with epigenetic modifiers, in SW480 cells, VPA exerted the most synergistic effects with SN-38 although synergistic effects were observed with 3 anticancer drugs (Table 3). HT29 cells also showed synergistic effects in co-treatment with VPA and SN-38 (Table 4). Co-treatment with DAC showed the most synergistic effects in HT29, SW48 and HCT116 cells (Tables 4 and 5, Supplementary Tables 4). The DRI values of DAC were larger than those of anticancer drugs. In contrast, combination effects by post-treatment
with epigenetic modifiers were weaker than those by pre- or co-treatment in all CRC cells examined (Tables 2, 3, 4 and 5, Supplementary Tables 2, 3, 4, 5 and 6).

The most significant combination schedule of DAC in SW480 cells was different from that in 3 other CRC cells (Tables 2, 4 and 5, Supplementary Tables 2 and 4). The most significant combination schedule of SAHA in SW480 cells was similar to that in SW48 cells with SN38 or L-OHP (Tables 3 and 5). The most significant combination schedule of VPA in SW480 cells was similar to that in H29 cells only with SN-38 but different from that in SW48 cells with 3 anticancer drugs (Tables 3, 4 and 5). Taken together, synergistic effects were less frequent and weaker in HT29, HCT116, and SW48 cells than in SW480 cells.
DISCUSSION

Synergistic effects of anticancer drugs and epigenetic modifiers were the strongest and most frequent in SW480 cells, which are less sensitive to anticancer drugs, than in the other 3 CRC cells examined. The results obtained also revealed that the dose of anticancer drugs may be reduced by combining epigenetic modifiers in SW480 cells, which are less sensitive to anticancer drugs, in contrast to more sensitive CRC cells. Seventy CRC cell lines were profiled for mutation with microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) status.\(^1\) SW480 cells, which were the most sensitive in combination with epigenetic modifiers in this study, were non-hypermutated, low in both MSI and CIMP status. In contrast to SW480 cells, HCT116 and SW48 cells were hypermutated and high in both MSI and CIMP status, and HT29 cells were non-hypermutated, MSI-low and CIMP-high. These differences in types of CRC cells may be one of factors for different synergistic combination effects. However, differences in the combined effects of epigenetic modifiers and conventional anticancer drugs between resistant or sensitive cancer cells have yet to be clarified. It is necessary to find out other CRC cells that show synergistic effects and examine the characteristics of these cells. In the combination experiments of DAC using SW620 cells, derived from lymph node metastasis of primary CRC, SW480 cells, pre-treatment with DAC did not show synergistic effects (data not shown). Thus, the different characteristics between SW620 and SW480 cells may contribute to synergistic effects of DAC although SW620 and
SW480 cells are derived from same patients.\textsuperscript{18)}

Demethylating agents (DNMT inhibitors) and HDAC inhibitors act through the inhibition of enzymatic activities such as DNMTs and HDACs, respectively. The well-known DNMT inhibitors are DAC and AC, while limited data are available for Zeb.\textsuperscript{5)} Several clinical studies using DAC or AC in combination with other anticancer drugs were performed.\textsuperscript{19)} In colorectal cancer, DAC was used in combination with L-OHP and capecitabine (prodrug of 5-FU) and most of the response results from treated patients were to be classified as stable disease. SAHA is a first HDAC inhibitor approved by FDA. On the other hand, TSA is indicated to be limited therapeutic use due to poor bioavailability \textit{in vivo} and has toxic side effects at high dose.\textsuperscript{16)} SAHA has been used with conventional anticancer drugs in many clinical trials, but several trials were reported to be a lack of positive results.\textsuperscript{5)} VPA, which has been used as an anti-epileptic drug, has been also recognized as a HDAC inhibitor. VPA has been examined in combination with anticancer drugs although the number of clinical trials of VPA was lower than that of SAHA.\textsuperscript{5)} However, the role of VPA in chemotherapy appears to be moderate due to toxicity and limited efficacy in patients.

We found that the most significant combination schedules with anticancer drugs differed between VPA (co-treatment) and the other epigenetic modifiers examined (pre-treatment) in SW480 cells. As suggested mechanisms of synergistic effects, loosening-up the chromatin structure by histone acetylation and the activation of genes related to the effects of anticancer
drugs by DNA demethylation and histone acetylation may increase the efficacy of anticancer
drugs.\textsuperscript{11,20} In this context, pre-treatment with epigenetic modifiers before anticancer drugs
could be better although co-treatment could also enhance the cytotoxicity of anticancer drugs.
Regarding anticancer drugs targeting DNA including L-OHP, loosening-up the chromatin
structure may contribute to synergistic effects by pre- or co-treatment with HDAC inhibitors,
TSA, SAHA or VPA. HDAC inhibitors including TSA, SAHA and VPA were reported to show synergistic effects by pre- or co-treatment with anticancer drugs.\textsuperscript{11,20-24} The difference
in the most significant combination between VPA and TSA/SAHA is not clear, but the
differences in the proteome and acetylome profiling between SAHA and VPA might contribute.\textsuperscript{25} TSA, SAHA and VPA are all HDAC inhibitors. However, these drugs target
partially different HDAC classes; TSA, I, II; SAHA, I, II, IV; VPA, I, IIa.\textsuperscript{16} The two major
types of interactions between these inhibitors and HDACs were revealed: fast binding such as
SAHA and slow dissociation (causing large residence time in HDAC) such as TSA.\textsuperscript{26} These
different characteristics may contribute to the differences in the intensity of synergistic
combination among HDAC inhibitors. The results in co-treatment with HDAC inhibitors in
SW480 cells were synergistic in this study but not in our pervious study\textsuperscript{7}. This reason is not
clear but may be due to difference in treatment schedule such as co-treatment period for 2
(this study) or 3 (previous study) days. SAHA is known to induce cell-cycle arrest with
long-term treatment. Long-treatment with SAHA may not be effective in combination with
In previous reports on DNMT inhibitors, DAC showed synergistic effects by pre- or co-treatment with anticancer drugs,\textsuperscript{11,27-29} while AC and Zeb showed synergistic effects by co-treatment.\textsuperscript{28,30} The information on the pre-treatment with AC or Zeb is limited. Generally, DNMT inhibitors possess the ability to bind covalently with DNMT and obstruct DNA synthesis. Thus, co-treatment with DAC was reported to enhance the anticancer activity by DNA damage and induction of apoptosis in CRC cells.\textsuperscript{28,29} Molecular mechanistically, pre-treatment with DAC or co-treatment with AC synergistically enhanced anticancer activity via demethylation of candidate tumor-suppressor genes or genes related to the effects of anticancer drugs.\textsuperscript{11,27,30} In contrast, Qin \textit{et al.} reported that epigenetic synergy between DAC and carboplatin was independent of DNA demethylation.\textsuperscript{31} Zeb, a DNMT inhibitor, showed no hypomethylation activity and affected gene transcription less than DAC and AC.\textsuperscript{32} These may cause the less synergistic effects of Zeb than DAC and AC in this study. Zeb was also reported to show moderate synergistic or antagonistic interaction with 5-FU or L-OHP.\textsuperscript{28,29} In the present study, DAC and AC exerted synergistic effects to a similar extent in SW480 cells. DAC and AC are mechanistically similar DNA-hypomethylating agents. However, functional disparities have been demonstrated between DAC and AC due to their differential metabolism and incorporation into DNA and RNA, respectively, in cancer cells.\textsuperscript{33} DAC did not have direct connections to other compounds in the database to examine.
chemical-chemical connectivities, indicating that DAC has unknown effects that are unpredictable from existing drug information. DAC exerted synergistic effects with anticancer drugs in some combinations, even in HT29 cells, which showed the highest IC$_{50}$ value for DAC. The dose of DAC may be reduced by combining anticancer drugs in DAC-resistant HT29 cells.

The mechanisms by which synergistic effects are achieved in CRC need to be elucidated in more detail prior to clinical applications for the development of clinical regimens. In order to clarify schedule-dependent effects of epigenetic modifiers, it is necessary to examine molecular changes when cells are treated with epigenetic modifiers by microarray analysis using not only SW480 cells but also other CRC cells. Furthermore, the present results need to be evaluated using tumor xenograft models. Nevertheless, this in vitro study is important for considering the type of cancer cell and combination drug depending on treatment schedules for in vivo study. Despite some challenges, these results provide insights into the development of effective combinations for CRC cells, which show resistance to anticancer drugs. The results of the present study will contribute to our understanding of how to incorporate epigenetic modifiers into current CRC chemotherapy.
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Conflict of Interest  The authors declare no conflict of interest.

Supplementary Materials  The online version of this article contains supplementary materials.
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Table 1  IC_{50} Values of Anticancer Drugs and DAC, SAHA and VPA in 4 CRC Cell Lines.

<table>
<thead>
<tr>
<th>Anticancer drugs</th>
<th>DNMT inhibitor</th>
<th>HDAC inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μM)</td>
<td>(μM)</td>
</tr>
<tr>
<td>5-FU</td>
<td>SN-38</td>
<td>L-OHP</td>
</tr>
<tr>
<td>74±19</td>
<td>136±20</td>
<td>78±7.2</td>
</tr>
<tr>
<td>60±15</td>
<td>17±3.9</td>
<td>0.94±0.06</td>
</tr>
<tr>
<td>10±0.7</td>
<td>3.2±0.4</td>
<td>0.92±0.19</td>
</tr>
<tr>
<td>9.6±1.4</td>
<td>4.0±0.08</td>
<td>1.6±0.4</td>
</tr>
<tr>
<td>SW480</td>
<td></td>
<td></td>
</tr>
<tr>
<td>256±38</td>
<td>189±18</td>
<td>114±6.6</td>
</tr>
<tr>
<td>240±77</td>
<td>80±12</td>
<td>2.2±0.2</td>
</tr>
<tr>
<td>HCT116</td>
<td>38±7.5</td>
<td>49±8.7</td>
</tr>
<tr>
<td>SW48</td>
<td>15±5.4</td>
<td>7.9±0.3</td>
</tr>
</tbody>
</table>

Cell viability was measured using the WST-8 assay. Each value represents the mean ± SE of three independent experiments. The IC_{50} value was calculated as described in the Materials and Methods.
Table 2  The Most Significant Combination Schedule Effects with DNMT Inhibitors in SW480 Cells.

<table>
<thead>
<tr>
<th>Combination schedule</th>
<th>DAC (125, 1000 μM) → AntiC</th>
<th>AC (6.0, 30 μM) → AntiC</th>
<th>Zeb (500, 1000 μM) → AntiC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CI Symbols</td>
<td>DRI</td>
<td>CI Symbols</td>
</tr>
<tr>
<td>5-FU (20, 300 μM)</td>
<td>0.053, 0.19</td>
<td>5.6, 42</td>
<td>6.0, 96</td>
</tr>
<tr>
<td>SN-38 (30, 190 nM)</td>
<td>±, ++++</td>
<td>8.1</td>
<td>12</td>
</tr>
<tr>
<td>L-OHP (10, 110 μM)</td>
<td>0.16, 0.22</td>
<td>6.8, 10</td>
<td>10, 95</td>
</tr>
</tbody>
</table>

CI and DRI represent the minimum–maximum values and symbols represent the graded symbols defined below. Synergistic or antagonistic data in the isobologram analysis are shown as CI and DRI values. Additive combination data in the isobologram analysis are shown as ±. Graded symbols: +++++, very strong synergism; ++++, strong synergism; +++, synergism; +, moderate synergism; ±, slight synergism; -, nearly additive; -, slight antagonism; --, moderate antagonism; ---, antagonism; ----, strong antagonism; ------, very strong antagonism. AntiC, anticancer drug.
Table 3  The Most Significant Combination Schedule Effects with HDAC Inhibitors in SW480 Cells.

<table>
<thead>
<tr>
<th>Combination schedule</th>
<th>TSA (60, 100 nM) → AntiC</th>
<th>SAHA (5, 7 μM) → AntiC</th>
<th>VPA (5, 7 mM) + AntiC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CI Symbols</td>
<td>DRI TSA</td>
<td>AntiC</td>
</tr>
<tr>
<td>5-FU (20, 300 μM)</td>
<td>0.017, 0.14</td>
<td>++++, +++++</td>
<td>0.29, 0.39</td>
</tr>
<tr>
<td>SN-38 (30, 190 nM)</td>
<td>0.034, 0.34</td>
<td>4.4, 37</td>
<td>2.6, 3.5</td>
</tr>
<tr>
<td>L-OHP (10, 110 μM)</td>
<td>0.045, 0.34</td>
<td>3.7, 32</td>
<td>5.1, 222</td>
</tr>
</tbody>
</table>

CI and DRI represent the minimum–maximum values and symbols represent the graded symbols defined in Table 2. Synergistic or antagonistic data in the isobologram analysis are shown as CI and DRI values. Additive combination data in the isobologram analysis are shown as ±. AntiC, anticancer drug.
Table 4  The Most Significant Combination Schedule Effects with DAC and VPA in HT29 Cells.

<table>
<thead>
<tr>
<th>Combination schedule</th>
<th>DAC (20, 700 μM)+AntiC</th>
<th>VPA (3.0 mM)+AntiC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CI Symbols</td>
<td>DRI</td>
</tr>
<tr>
<td>5-FU (5.0, 200 μM)</td>
<td>0.32, 14</td>
<td>1.5, 65</td>
</tr>
<tr>
<td>SN-38 (15, 100 nM)</td>
<td>0.30, 8.4</td>
<td>0.87, 438</td>
</tr>
<tr>
<td>L-OHP (0.10, 2.5 μM)</td>
<td>0.79, 8.2</td>
<td>0.35, 160</td>
</tr>
</tbody>
</table>

CI and DRI represent the minimum–maximum values and symbols represent the graded symbols defined in Table 2. Synergistic or antagonistic data in the isobologram analysis are shown as CI and DRI values. Additive combination data in the isobologram analysis are shown as ±. NA, not available; AntiC, anticancer drug.
Table 5  The Most Significant Combination Schedule Effects with DAC, SAHA, and VPA in SW48 Cells.

<table>
<thead>
<tr>
<th>Combination schedule</th>
<th>DAC (0.5 μM)+AntiC</th>
<th>SAHA (0.5 μM)→AntiC</th>
<th>VPA (1.5 mM)→AntiC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CI Symbols</td>
<td>DRI</td>
<td>CI Symbols</td>
</tr>
<tr>
<td>5-FU (1.5, 12 μM)</td>
<td>NA</td>
<td>NA NA NA</td>
<td>NA</td>
</tr>
<tr>
<td>SN-38 (1.0, 9.0 nM)</td>
<td>0.30, 1.8</td>
<td>0.92, 822 1.3, 3.4</td>
<td>0.40, 1.7</td>
</tr>
<tr>
<td>L-OHP (0.40, 4.0 μM)</td>
<td>0.26</td>
<td>1371 3.8</td>
<td>0.54, 3.1</td>
</tr>
</tbody>
</table>

CI and DRI represent the minimum–maximum values and symbols represent the graded symbols defined in Table 2. Synergistic or antagonistic data in the isobologram analysis are shown as CI and DRI values. Additive combination data in the isobologram analysis are shown as ±. NA, not available; AntiC, anticancer drug.