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**Original article**

# Antagonizing BCLXL functional dependence overcomes 5-fluorouracil resistance in colon cancer cells

**Running title:** Functional BCLXL determines 5-FU resistance

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**Abstract**

**Background** 5-Fluorouracil (5-FU) is a cytotoxic anticancer drug commonly used for patients with advanced colon cancer. This drug effectively reduces the size of the tumor to some degree, but cancer cells can gradually acquire resistance, resulting in disease progression.

**Methods** We established a 5-FU-resistant colon cancer cell line, which was resistant to different concentrations of this agent and sought to identify the mechanism of 5-FU resistance.

**Results** Even though BCL2 protein expression was considerably elevated in 5-FU-resistant cells, well-designed functional apoptosis assays and BH3 profiling demonstrated BCLXL dependence without increased expression. Functional BCLXL inhibition using an antagonist and siRNA in 5-FU-resistant cells not only sensitized the cells to apoptosis but also overcame 5-FU resistance. The apoptotic BIM protein was preferentially sequestered, thereby resulting in dependence on BCLXL for survival.

**Conclusions** BH3 profiling clarified the association between BCLXL dependence and 5-FU resistance even under the complex conditions of altered expression of several apoptosis-related proteins; thus, this assay could help in

the understanding of the functional role of anti-apoptotic proteins. The results also have clinical implications for the targeting of BCLXL in colon cancer.

**Keywords:** BH3 profiling, BCLXL, colon cancer, 5-Fluorouracil, drug resistance

## INTRODUCTION

Colon cancer is the third most common cancer in the world (Arnold *et al*, 2017) and the second most common cancer in Japan (Hori *et al*, 2015). The prognosis of advanced colon cancer with metastasis remains poor, with the overall 5-year survival rate being only 18.8% (Watanabe *et al*, 2017). Standard treatment of unresectable advanced or recurrent colon cancer is systemic chemotherapy. The recent development of several chemotherapy regimens such as FOLFIRI and FOLFOX, which are combined with bevacizumab, cetuximab, or panitumumab, has clearly prolonged patient survival (Watanabe *et al*, 2017). In Japan, fourth or fifth line continuous chemotherapy regimens are administered for unresectable advanced colon cancer such that all usable anticancer drugs are consumed. Among several anticancer drugs, 5-fluorouracil (5-FU) is a core cytotoxic drug that is included in all first line regimens. 5-FU is an analogue of uracil, which is one of the four bases found in RNA; it is also utilized more in tumors than in normal tissue (Rutman *et al*, 1954). After entering the cell, 5-FU is converted to several metabolites including fluorouridine triphosphate (FUTP), fluorodeoxyuridine triphosphate (FdUTP), and fluorodeoxyuridine monophosphate (FdUMP) (Longley *et al*, 2003). Each metabolite leads to

misincorporation into RNA and DNA, thereby inhibiting thymidine synthase (TS), which is consequently followed by apoptosis. In general, cytotoxic anti-cancer drugs show good efficacy with respect to killing cancer cells at the beginning of treatment. However, tumors gradually fail to respond to these drugs after developing drug resistance. Therefore, understanding the mechanism of resistance to anticancer drugs is indispensable for uncovering more efficacious treatments for refractory cancer. In the present study, we established a colon cancer cell line with acquired resistance to different concentrations of 5-FU, and analyzed the mechanisms of dose-dependent 5-FU resistance.

## MATERIALS AND METHODS

### *Cell lines*

The colorectal adenocarcinoma cell line HT-29 (ATCC® HTB-38™) was obtained from the American Type Culture Collection, and cultured at 37 ºC in a humidified 5% $CO_2$ incubator in DMEM (Sigma, St Louis, MO, USA) medium supplemented with 10% FBS (Sigma), 10 mM L-glutamine (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma).

### *Cell viability assay*

Five thousand HT-29 cells were treated for 72 hours with 5-FU (Sigma) at the indicated concentrations in 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, cell proliferation was determined using the Premix WST-1 Cell Proliferation Assay (Takara Bio Inc., Kusatsu, Shiga, Japan) and an Infinite M1000 PRO microplate reader (TecanJapan, Kawasaki, Kanagawa, Japan). The half-maximal inhibitory concentration ($IC_{50}$) was defined as the drug concentration resulting in 50% cell survival relative to that of untreated cells. Triplicate wells were treated with various drug concentrations, and average $IC_{50}$ values were determined. Antagonists of BCL2 and BCL-XL, ABT-199 (Selleck Chemicals, Houston, TX, USA) and WEHI-539 hydrochloride (MedChem Express, Monmouth Junction, NJ, USA), respectively, were used and $IC_{50}$ values were obtained.

### *Generation of 5-FU-resistant cell line*

The 5-FU-resistant HT-29 cell line was developed as previously reported (Liu *et al*, 2014). Briefly, parental HT-29 cells were treated with gradually increasing concentrations of 5-FU. The initial 5-FU concentration added to cells

was 10% of the $IC_{50}$, and this concentration was gradually increased. Cell lines resistant to both 4 and 16 μM of continuous 5-FU were established, and the $IC_{50}$ for both cell lines was determined.

***Apoptosis assays***

Five hundred thousand parental and 5-FU-resistant-HT-29 cells were allowed to adhere to 6-well plates for 24 hours, and cells were treated with either 5-FU or WEHI-539 hydrochloride as indicated. Cells were then stained with a phycoerythrin-conjugated Annexin V antibody (BD Pharmingen, San Diego, CA, USA). Apoptotic cells were analyzed using a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) with FACSDiva software (BD Biosciences). Apoptosis was also assessed using the Caspase-Glo 3/7 Assay (Promega, Madison, AL, USA). Five thousand cells were plated in white-walled 96-well round plates (Thermo Fisher Scientific) and treated with drugs as indicated. After incubation, 100 μl of Caspase-Glo reagent was added to each well, and the contents of the well were gently mixed with a plate shaker at 300 rpm for 30 seconds; this was followed by incubation at room temperature for 1 hour. The luminescence of each sample was measured using an Infinite M1000

PRO microplate reader. The caspase inhibitor Q-VD-OPH (Bay bioscience, Kobe, Hyogo, Japan) was also used.

***Flow cytometry***

Parental and 5-FU-resistant HT-29 cells were incubated with both anti-CD338 (clone 5D3; BioLegend, San Diego, CA, USA) and anti-CD243 (clone UIC2; BioLegend), both anti-CD133 (clone 293C3; Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-CD147 (clone HIM6; BioLegend), or isotype-matched IgG antibodies (Pacifica Blue; clone MPC-11, BioLegend, APC; clone MOPC-173, BioLegend, Alexa Fluor 488; clone MOPC-21, BioLegend, respectively) for 30 minutes at 4 ºC. Stained cells were analyzed using a BD FACSCanto II with FACSDiva software. For cell cycle assays, cells were fixed with cold 70% ethanol for 2 hours, followed by staining with FxCycle PI/RNase Staining Solution (Thermo Fisher Scientific), according to the manufacturer's protocol. For examining DNA damage, cells were stained with anti-phospho (Ser139) histone H2A.X-conjugated Alexa Fluor 488 antibody (clone 2F3; BioLegend) and analyzed by flow cytometry.

***Real time PCR***

Total RNA from parental and 5-FU-resistant HT-29 cells were isolated using TRIzol reagent (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific). The primer sequences for real time PCR were designed based on Primer bank (http://pga.mgh.harvard.edu/primerbank) and previous studies (Supplementary Table 1) (Smith *et al*, 2014; Xu *et al*, 2013; Lu *et al*, 2011; Wang *et al*, 2014). Real time PCR was conducted in quadruplicate using Power SYBR Green PCR Mix (Thermo Fisher Scientific). Changes in relative gene expression between cDNA samples were determined using the ΔΔCt method, as previously described (Kamihara *et al*, 2016).

***Western blotting***

Western blotting was performed as previously described (Kawano *et al*, 2003). Briefly, separated proteins were transferred to nylon membranes and blotted with specific antibodies to detect BCL2 (Thermo Fisher Scientific, #13−8800), BCLW (Cell Signaling Technology, Denver, MA, USA, #2724),

BCLXL (Cell Signaling Technology, #2764), MCL1 (Cell Signaling Technology, #5453), BID (Cell Signaling Technology, #2002), BIM (Cell Signaling Technology, #2933), BAD (Cell Signaling Technology, #9292), HRK (R & D Systems, Minneapolis, MN, USA, #AF851), NOXA (Cell Signaling Technology, #14766), PUMA (Cell Signaling Technology, #12450), BMF (Abcam, Cambridge, UK, #EPR10930(2)), BAK (Cell Signaling Technology, #12105), BAX (Cell Signaling Technology, #5023), and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA, #sc1615). After incubation with either horseradish peroxidase-linked anti-rabbit IgG (Cell Signaling Technology, #7074S) or anti-mouse IgG (Cell Signaling Technology, #7076S), the membrane was stained with ECL Select Western Blotting Detection Reagent (GE Healthcare UK Ltd, Little Chalfont, UK). Finally, bands were imaged by either exposing membranes to BIOMAX XAR film (Sigma) and developing using a Kodak X-OMAT 1000 Processor (Amersham, Piscataway, NJ, USA) or by using a LAS-4000UV mini (GE Healthcare UK Ltd) and MultiGauge software (Fujifilm, Tokyo, Japan).

***BCL2-homology domain 3 (BH3) profiling***

We conducted plate-based BH3 profiling as previously described (Ryan & Letai,

2013). Ten BH3 peptides including one control peptide derived from the PUMA peptide, in which two amino acids are substituted, were obtained as HPLC-purified products from Sigma (Supplementary Table 2). All peptides were dissolved in dimethyl sulfoxide (DMSO, Sigma) as 1 mM stock solutions and stored at −80 ºC. Each peptide was prepared in 15 µl DTEB buffer (135 mM trehalose, 10 mM HEPES-KOH [pH 7.5], 50 mM KCl, 20 µM EDTA, 20 µM EGTA, 0.1% BSA, and 5 mM succinate; all from Sigma) containing 0.005% digitonin (Sigma), 20 µg/ml oligomycin (Sigma), 10 mM 2-mercaptethanol and 2 µM JC-1 (Invitrogen, Carlsbad, CA, USA), and aliquoted into 384-well plates (Corning Incorporated, Corning, NY, USA). Two thousand parental and 5-FU-resistant HT-29 cells were then suspended in 15 µl DTEB buffer and added to 384-well plates. Fluorescence intensities at 590 nm were analyzed using an Infinite M1000 PRO microplate reader, and the reader was programmed to take measurements every 5 minutes for 120 minutes. The percentage of relative mitochondrial depolarization was calculated based on the area under the curve (AUC) using the following equation: % mitochondrial depolarization = 100 × [DMSO (AUC) − X (AUC)] / [DMSO (AUC) − p-trifluoromethoxy carbonyl cyanide phenyl hydrazine (FCCP) (AUC)], where DMSO (AUC) is the AUC with the

negative control, X(AUC) indicates the AUC with the tested BH3 peptide, and FCCP(AUC) indicates the AUC with the positive control.

***Inhibition of BCL-XL expression through small-Interfering RNA (siRNA) transfection***

One million parental and 5-FU-resistant HT-29 cells, in 6-well plates, were transfected with either siRNAs targeting human BCL-XL (Dharmacon, Lafayette, CO, USA; D-003458-03, D-003458-30) or a non-targeting siRNA (Dharmacon; D-001210-03-05) using RNAi Max reagent (Invitrogen), according to manufacturer's instructions.

***Immunoprecipitation***

BCL-XL immunoprecipitation experiments were conducted using the ImmunoCruz IP/WB Optima F System (Santa Cruz Biotechnology, #SC-45043). Separated total proteins (1000 μg) were incubated with a complex of BCL-XL antibody and IP matrix overnight at 4 ºC. After incubation and centrifugation, the eluted supernatant was subjected to western blotting for BID, BIM, and BCLXL.

### *Statistical analysis*

All data represent the mean ± SD. For multiple comparisons, post-hoc analysis was conducted using the Tukey test. For comparisons between two groups, two-tailed unpaired Student's t-tests were performed. $IC_{50}$ values for each tested drug and dose-response curves were analyzed using Graph-Pad PRISM 5 (GraphPad Software, Inc., La Jolla, CA, USA). All statistical analyses were performed using SPSS software, version 21 (IBM, Armonk, NY, USA).

## RESULTS

### *Survival is enhanced in 5-FU-resistant HT-29 cells in the presence of 5-FU*

Initially, we established HT-29 cell lines that were resistant to two different concentrations of 5-FU, specifically 4 and 16 μM, in the presence of continuous exposure. Doubling times of 5-FU-resistant HT-29 cells were increased compared to those of parental cells (4 μM 5-FU-resistant, 32.2 hours; 16 μM 5-FU-resistant, 33.6 hours; parental, 22.3 hours). The 5-FU $IC_{50}$ values of resistant HT-29 cells were markedly higher than those of parental cells (4 μM 5-FU-resistant, 145.9 μM; 16 μM 5-FU-resistant, > 1000 μM; parental, 7.19 μM; Figure 1A).

***CD338 and CD243 are not differentially regulated in 5-FU-resistant HT-29 cells***

CD338, which is also called both ATP-binding cassette sub-family G member 2 and breast cancer resistance protein, functions as a xenobiotic transporter that plays a major role in multi-drug resistance (Yuan *et al*, 2008). CD243, which is also called multidrug resistance protein 1, is a membrane-associated protein that is responsible for decreasing drug accumulation. As these cell surface proteins are known to contribute to 5-FU resistance in some cancer cells (Theile *et al*, 2009), we first determined the expression of these proteins in 5-FU-resistant HT-29 cells. No increase in expression was observed in 5-FU-resistant HT-29 cells compared to that in parental cells, suggesting that 5-FU efflux is unchanged during the acquisition of resistance (Figure 1B).

***Expression of CD133 and CD147 is similar between parental and 5-FU-resistant HT-29 cells***

Cancer stem cells (CSCs) are thought to account for the drug resistance

of cancer cells. Given the fact that some surface proteins such as CD133 (Atashpour *et al*, 2015) and CD147 are markers of CSCs (Xu *et al*, 2014; Chen *et al*, 2015), we further examined whether these proteins are upregulated in 5-FU-resistant HT-29 cells. Whereas CD133 expression remained virtually unchanged compared to that in parental HT-29 cells, decreased CD147 expression was observed in 5-FU-resistant HT-29 cells (Figure 1C), suggesting that mechanisms other than CD147 expression in CSCs contribute to 5-FU resistance.

***Expression of mRNA associated with 5-FU metabolic enzymes in 5-FU-resistant HT-29 cells***

The mRNA expression of four genes encoding enzymes associated with 5-FU metabolism, including thymidine phosphorylase (TP), TS, dihydropyrimidine dehydrogenase (DPD), and oronate phosphoribosyl transferase (OPRT), was examined by real time PCR. As shown in Figure 2A, increased TS and TP mRNA expression was observed in 5-FU-resistant HT-29 cells. A previous study (Longley *et al*, 2003) showed that upregulation of TS in response to 5-FU treatment allows cells to escape from the incorporation of

5-FU into RNA and DNA. Given the similar induction of TS mRNA in 4 μM and 16 μM 5-FU-resistant HT-29 cells (11.31-fold and 11.62-fold compared to that in parental HT-29 cells, respectively) in our study, we reasoned that there might be another mechanism associated with the dose dependency of 5-FU resistance. mRNA expression of DPD and OPRT, which encoded key enzymes for de novo pyrimidine biosynthesis during 5-FU resistance (Tsutani *et al*, 2008), were essentially unchanged compared to expression in parental HT-29 cells.

***S-phase cell cycle arrest does not occur in 5-FU-resistant HT-29 cells***

5-FU is known to induce S-phase cell cycle arrest (Yamane *et al*, 1999), following DNA double strand breaks, with subsequent apoptosis. With 4 μM 5-FU treatment, the proportion of cells in S-phase in the two 5-FU-resistant HT-29 cell lines was not increased by much when compared to that in parental cells (Figure 2B). Upon treatment with 16 μM 5-FU, only 16 μM 5-FU-resistant HT-29 cells could completely escape from S-phase cell cycle arrest (% S-phase: parental, 61.7%, 4 μM 5-FU-resistant, 42.3%; 16 μM 5-FU-resistant, 16.5%). To confirm the occurrence of DNA double strand breaks with 5-FU treatment, cells were stained with an anti-phosphorylated (Ser139) histone H2A.X antibody. As

expected, phosphorylation of histone H2A.X decreased in a concentration-dependent manner in 4 μM and 16 μM 5-FU-resistant HT-29 cells (Figure 2C and 2D), suggesting that 5-FU has a diminished effect on the cell cycle in resistant HT-29 cells.

***Apoptosis-related mRNA and protein expression***

To clarify the effect of 5-FU resistance on the expression of apoptosis-related genes, mRNA and protein levels of both apoptotic and anti-apoptotic markers were examined (Figure 3A, 3B, and Supplemental Figure 1). mRNA expression of some apoptosis-related genes such as *BID*, *BIM*, *NOXA*, *PUMA*, *BMF*, *BAK*, and *BAX* were similar or slightly higher in 5-FU-resistant HT-29 cells compared to that in parental cells. Among these, mRNA expression of the anti-apoptotic BCL-2 was much higher in 5-FU-resistant HT-29 cells than in parental cells, and there was a dose-dependent induction based on the concentration of 5-FU to which cells were resistant (4 μM 5-FU-resistant, 28.7-fold; 16 μM 5-FU-resistant, 79.7-fold increase compared to that in parental cells). Regarding protein analysis, BCL2 protein expression in 5-FU-resistant HT-29 cells was also induced, whereas the expression of three anti-apoptotic

proteins, BCLW, BCLXL, and MCL1, remained similar compared to that in parental cells. The apoptotic related protein levels of BIM and NOXA were increased in 5-FU-resistant HT-29 cells compared to that in parental cells, whereas others were not changed.

***BH3 profiling reveals that 5-FU-resistant HT-29 cells are dependent on BCL-XL for survival***

From the aforementioned results, cell survival in 5-FU-resistant HT-29 cells appeared to be dependent on the induction of BCL-2 expression and induced BIM and NOXA expression. To clarify the relationship between 5-FU resistance and apoptosis, we conducted BH3 profiling of parental and 5-FU-resistant HT-29 cells (Figure 4A and 4B). When treated with BAD, PUMA, and BMF BH3 peptides, all of which correlate with dependency on BCL2, BCLXL, and BCLW, 5-FU-resistant HT-29 cells were more primed than parental cells. Furthermore, when treated with the HRK peptide, which specifically binds BCLXL, 5-FU-resistant HT-29 cells also became more primed than parental cells. To further verify BCLXL protein dependency, XXA1 peptide, which had previously been structurally identified (Dutta *et al*, 2015) as specific inhibitor of

BCLXL, was tested using BH3 profiling. Dose-dependent mitochondrial priming was observed in 5-FU-resistant HT-29 cells upon treatment with XXA1 at a concentration of 0.1–100 μM, whereas no mitochondrial priming was observed in parental HT-29 cells treated with XXA1 peptide at a concentration up to 10 μM (Figure 4A). Taken together, BH3 profiling results suggested strong dependency on BCLXL for cell survival, regardless of protein and mRNA expression profiles in 5-FU-resistant HT-29 cells.

***Inhibition of BCL-XL selectively induces apoptosis in 5-FU-resistant HT-29 cells***

To clarify whether induced expression of BCL2 in 5-FU-resistant HT-29 cells, as shown Figure 3, contributes to cell survival, we treated cells with the BCL2 specific inhibitor ABT-199 (Figure 5A). No remarkable differences in $IC_{50}$ values were observed between parental and 5-FU-resistant HT-29 cells ($IC_{50}$: parental, 16.5 μM; 4 μM 5-FU-resistant, 15.0 μM; 16 μM 5-FU-resistant, 13.5 μM). We then treated these cells with the BCLXL-specific inhibitor WEHI-539. The $IC_{50}$ of WEHI-539 in 5-FU-resistant HT-29 cells was markedly decreased compared to that in parental cells (4 μM 5-FU-resistant, 41.29 μM; 16 μM

5-FU-resistant, 0.66 μM; parental, > 100 μM; Figure 5B), and the $IC_{50}$ of WEHI-539 tended to be lower in 16 μM 5-FU-resistant HT-29 cells than in 4 μM 5-FU-resistant cells. We next examined the effect of WEHI-539 on apoptosis in 5-FU-resistant HT-29 cells. An increased number of Annexin V-positive cells was observed in both 4 μM and 16 μM 5-FU-resistant HT-29 cells, whereas no additional Annexin V-positive cells were noted in parental cells (Figure 5C). Furthermore, the activities of caspase-3 and caspase-7, both of which are key effectors of apoptosis, were upregulated in 5-FU-resistant HT-29 cells (Figure 5D). This induction of caspase-3 and caspase-7 activity upon treatment with a BCLXL inhibitor was completely inhibited through co-treatment with the caspase inhibitor Q-VD-OPH. These results indicated that BCLXL, but not BCL2, predominantly exerts anti-apoptotic functions in resistant HT-29 cells in a manner that is dependent on the concentration of 5-FU, to which cells are resistant.

***Restoration of 5-FU sensitivity by inhibiting BCL-XL in 5-FU-resistant HT-29 cells***

We then inhibited BCL-XL protein expression in 16 μM 5-FU-resistant

HT-29 cells through siRNA transfection (Figure 5E), which was followed by treatment with 5-FU. The $IC_{50}$ of 5-FU in resistant HT-29 cells markedly decreased with BCL-XL inhibition, as compared to that in both cells transfected with non-targeting siRNA (siNT) and untransfected HT-29 cells (siBCLXL#1 transfected, 174.9 μM; siBCLXL#2 transfected, 236.5 μM, siNT transfected, > 1000 μM, untransfected, > 1000 μM; Figure 5F). Furthermore, with 5-FU treatment, an increase in Annexin V-positive cells was observed with BCLXL inhibition (Figure 5G), suggesting that BCLXL downregulation could restore 5-FU sensitivity in resistant HT-29 cells by enhancing vulnerability to apoptosis.

***Sequestration of BIM by BCL-XL mediates sensitization to apoptosis in 5-FU-resistant HT-29 cells***

To confirm that an interaction between BCLXL and an apoptotic protein would account for functional BCLXL dependency, we performed immunoprecipitation on cell extracts of 5-FU-resistant cells using a BCLXL antibody, and examined the binding of apoptosis-related proteins including BID and BIM. As shown in Figure 6, BIM preferentially bound BCLXL in 5-FU-resistant HT-29 cells, compared to binding in parental cells. In summary,

BCL-XL dependence in 5-FU-resistant HT-29 cells was mediated through the sequestration of induced BIM by BCL-XL.

## DISCUSSION

Here, we present a new strategy to overcome 5-FU resistance, namely BH3 profiling. Results of this approach identified strong dependence on BCLXL, regardless of induced expression of BCL2, in 5-FU-resistant colon cancer cells. Similar to a previous study (Ryan *et al*, 2010), our results suggested that examination of only apoptosis-related proteins cannot explain cancer cell survival mechanisms.

In clinical settings, anti-cancer drugs are continued until they elicit no response by the cancer cells or cause severe adverse effects in the treated patient. At this time, other drugs with different mechanisms of cytotoxicity are used. During prolonged drug treatment, some cancer cell clones can acquire drug resistance; these cells gradually become predominant in the population, whereas other cancer cells that are sensitive to drug die. There are several mechanisms through which cancer cells acquire drug resistance, such as drug efflux, the existence of cancer stem cells, drug metabolism, and anti-apoptotic

functions; these functions result in complex situations for clinical treatment. To elucidate such mechanisms, we adopted a method for establishing two cell lines with 5-FU dose-dependent resistance. We achieved this by continuously treating the cells with different concentrations . Overexpression of multi-drug resistant proteins (CD338, CD243) or stem cell markers (CD133, CD147) was not observed in either 5-FU-resistant colon cancer cell line (Figure 1B, 1C), suggesting that population selection did not occur during 5-FU treatment.

Induction of TS was observed in 5-FU-resistant colon cancer cells. As TS is the target of 5-FU, this induction could help cells escape the cytotoxicity of this drug to some extent. A previous clinical study showed that higher levels of TS mRNA are correlated to 5-FU resistance (Leichman *et al*, 1997), which also supports this notion. However, based on the results of our vitro study, a similar degree of TS expression was observed between lower dose (4 μM) and higher dose (16 μM) 5-FU-resistant colon cancer cells; thus, the expression of this marker could not explain the dose-dependent resistance to 5-FU, suggesting the existence of other dominant mechanisms.

Expression analysis of apoptosis-related proteins and mRNA indicated the marked induction of anti-apoptotic BCL2 expression in 5-FU-resistant colon

cancer cells. A previous study showed that BCL2 is expressed at low levels in wild type HT-29 colon cancer cells (Nita *et al*, 1998). A clinical immunohistochemical study showed that approximately half of all colorectal carcinoma specimens were BCL2-negative (Zhao *et al*, 2005). Another study also reported that BCL2 stabilization in HT-29 cells contributes to 5-FU resistance (Wu *et al*, 2015). Consistent with these studies, our results confirmed the low expression of BCL2 in parental HT-29 cells and also the induction of BCL2 in 5-FU-resistant cells. However, results showed that ABT-199 had no inhibitory effect, even with the induction of BCL2, in 5-FU-resistant HT-29 cells.

In general, a complex network comprising many apoptosis-related proteins makes it difficult to predict apoptotic responses by quantifying only protein expression. Recently, a new functional assay, namely BH3 profiling, was previously reported (Ryan *et al*, 2010), and this technique was shown to predict sensitivity to a single drug in patients with acute myelogenous leukemia (Vo *et al*, 2012), multiple myeloma (Ni Chonghaile *et al*, 2011), and gastric cancer (Kubo *et al*, 2016). Based on our BH3 profiling results, 5-FU-resistant HT-29 colon cancer cells have specific BCLXL dependence, irrespective of the induction and expression of several apoptosis-related proteins. Disparate results between the

quantification of protein expression and BH3 profiling provides great insight in clinical settings, as expression analysis including immunohistochemistry and real time PCR has been previously conducted for assessing cancer cells. Therefore, to accurately elucidate protein dependence for cancer cell survival, BH3 profiling might become an auxiliary tool for targeted therapy.

BIM was induced in 5-FU-resistant colon cancer cells and this enhanced sequestration by BCLXL. BIM, which is usually bound to microtubules under physiological conditions and which is recruited to mitochondria after treatment with cytotoxic drugs (Klotz *et al*, 2012), functions as a pro-apoptotic activator of BAK and BAX (Ryan *et al*, 2010) . In our study, sequestration of induced BIM by BCLXL in 5-FU-resistant HT-29 cells resulted in the evasion of apoptosis caused by 5-FU; consequently, this BIM sequestration resulted in addiction to BCLXL.

There are some limitations to our study. We established other 5-FU-resistant colon cancer cell lines, DLD1 and HCT-15, and conducted BH3 profiling. As these parental cell lines were already primed by treatment with 10 μM XXA1 peptide, no additional mitochondrial priming was found in 5-FU-resistant cells (data not shown). This suggested that the pre-existence of BCLXL dependence would not enhance addiction to this protein. Therefore, in

clinical settings, conducting BH3 profiling on colon cancer samples before and after chemotherapy will be needed. Although BCLXL inhibition in 5-FU-resistant HT-29 cells could reverse sensitivity (Figure 5F and 5G), the detailed mechanism through which BCLXL dependence affects cell survival in only 5-FU-resistant colon cancer cells remains unknown. A previous study reported that BCLXL can prolong the G0 phase during the induction of cell cycle entry after release from contact inhibition (Zinkel *et al*, 2006). In our study, the proportions of cells in each cell cycle phase were similar between parental and 5-FU-resistant HT-29 cells without 5-FU treatment, suggesting other unknown BCLXL-mediated functions during treatment with 5-FU.

In conclusion, our work utilizing BH3 profiling demonstrated a function for BCLXL in the acquisition of 5-FU drug resistance in HT-29 colon cancer cells. In addition, we showed that the sequestration of the apoptosis-related BIM protein by BCLXL results in dependence on the latter protein. Clinical studies targeting BCLXL in laryngeal (NCT01633541) and small cell lung cancer (NCT03080311) are ongoing. Assessing BCLXL dependence in colon cancer cells through BH3 profiling will enable a more-detailed stratification of individual sensitivities to this class of BCLXL selective inhibitors, thereby increasing the

efficacy of precision medicine.

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**Conflict of Interest**

The authors declare that they have no conflict of interest.

**Titles and legends to figures**

**Figure 1. 5-Fluorouracil (5-FU)-resistant HT-29 cells show similar expression of surface antigens associated with multidrug resistance and stem cell markers**. **(A)** Parental and two 5-FU-resistant HT-29 cell lines were treated with different concentrations of 5-FU for 72 hours, which was followed by measurements of cell viability. Each point indicates the mean of three independent experiments. All data represent the mean ± SD (n = 3). **(B, C)** Flow cytometric, two-color analyses of CD243 and CD338 **(B)**, and CD133 and CD147 **(C)** in parental and 5-FU-resistant HT-29 cells. APC, allophycocyanin.

**Figure 2. High thymidylate synthase (TS) gene expression and lack of S-phase cell cycle arrest in 5-Fluorouracil (5-FU)-resistant HT-29 cells. (A)** Real time PCR analysis of genes associated with 5-FU metabolism in parental and 5-FU-resistant HT-29s. All data represent the mean ± SD (n = 3). **(B)** Cell cycle analysis of parental and 5-FU-resistant HT-29 cells. **(C, D)** Parental and 5-FU-resistant HT-29 cells were stained with anti-phospho (Ser139) histone H2A.X-conjugated Alexa Fluor 488 antibody, and analyzed by flow cytometry. Representative data are shown. Light gray histogram represents isotype control.

Dark gray, red, and blue histograms depict fluorescence intensity when cells were treated with either medium only, 4 μM 5-FU, or 16 μM 5-FU, respectively. **(D)** Phospho (Ser139) H2A.X fluorescence intensity relative to isotype control-treated fluorescence intensity. P values (*$P$ < 0.01) represent the significance when compared to parental HT-29 cells based on analysis of variance of variance with Tukey's multiple-comparison test.

**Figure 3. High BCL2 gene and protein expression in 5-Fluorouracil (5-FU)-resistant HT-29 cells. (A)** Real time PCR analysis of anti-apoptosis-related genes in parental and 5-FU-resistant HT-29 cells. All data represent the mean ± SD (n = 3). **(B)** Apoptosis-related protein analysis of parental and 5-FU-resistant HT-29s. Actin was used as a loading control. P values represent the significance when compared to parental HT-29 cells (*$P$ < 0.01) and 4 μM 5-FU-resistant HT-29 cells (**$P$ < 0.01), respectively, based on analysis of variance with Tukey's multiple-comparison test.

**Figure 4. BH3 profiling determines that mitochondrial priming is dependent on BCLXL in 5-Fluorouracil (5-FU)-resistant HT-29 cells. (A)**

Heat map for mitochondrial responses in parental and 5-FU-resistant HT-29 cells. A series of different concentrations (0.01–100 μM) of each BH3 peptides including the control peptide PUMA2A was tested. **(B)** Bar graph representing the percentage of mitochondrial depolarization. All data represent the mean ± SD (n = 3). P values represent the significance when compared to parental HT-29 cells (*$P < 0.01$) and 4 μM 5-FU-resistant HT-29 (**$P < 0.01$), respectively, based on analysis of variance with Tukey's multiple-comparison test.

**Figure 5. A BCLXL, but not a BCL2, inhibitor can sensitize 5-Fluorouracil (5-FU)-resistant HT-29 cells to apoptosis. (A)** Parental and 5-FU-resistant HT-29 cells were treated with different concentrations of the BCL2 inhibitor ABT-199 for 72 hours, which was followed by measurement of cell viability. Each point indicates the mean of three independent experiments. **(B)** Parental and 5-FU-resistant HT-29 cells were treated with different concentrations of the BCLXL inhibitor WEHI-539 for 72 hours, which was followed by measurement of cell viability. **(C)** Parental and 5-FU-resistant HT-29 cells were treated with or without 10 μM WEHI-539 for 48 hours, which was followed by the analysis of Annexin V-positive cells using flow cytometry. P

values represent the significance when compared to parental HT-29 cells (*$P$ < 0.01) and 4 μM 5-FU-resistant HT-29 cells (**$P$ < 0.01), respectively, based on analysis of variance with Tukey's multiple-comparison test. P values (†$P$ < 0.01) represent the significance when compared to vehicle-treated cells, analyzed by a two-tailed unpaired Student's t-test. **(D)** Caspase3/7 activity in parental and 5-FU-resistant HT-29 cells when treated with 10 μM WEHI-539, with or without the caspase inhibitor (50 μM Q-VD-OPH) for 24 hours. Values are fold-changes in Caspase3/7 activity compared to that with medium treatment only. P values represent comparisons similar to that shown in (C). **(E)** Western blot analysis of BCLXL siRNA-transfected 16 μM 5-FU-resistant HT-29 cells. **(F)** BCLXL siRNA-transfected 16 μM 5-FU-resistant HT-29 cells were treated with different concentrations of 5-FU for 72 hours, which was followed by measurements of cell viability. (**G)** BCLXL siRNA-transfected 16 μM 5-FU-resistant HT-29 cells were treated with either 200 or 400 μM 5-FU for 72 hours, which was followed by analysis of Annexin V-positive cells using flow cytometry. P values represent significance when compared to non-targeting siRNA (siNT)-transfected cells (*$P$ < 0.01) or cells treated with medium only (†$P$ < 0.01).

**Figure 6. Induced (apoptotic) BIM preferentially binds (anti-apoptotic) BCLXL in 5-Fluorouracil (5-FU)-resistant HT-29 cells. (A, B)** BCLXL immunoprecipitation analysis of parental and two 5-FU-resistant HT-29 cell lines. Actin was used as a loading control for input.

## REFERENCES


Arnold M, Sierra MS, Laversanne M, Soerjomataram I, Jemal A, Bray F (2017) Global patterns and trends in colorectal cancer incidence and mortality. *Gut* **66**(4): 683–691, doi: 10.1136/gutjnl-2015-310912.

Atashpour S, Fouladdel S, Movahhed TK, Barzegar E, Ghahremani MH, Ostad SN, Azizi E (2015) Quercetin induces cell cycle arrest and apoptosis in CD133(+) cancer stem cells of human colorectal HT29 cancer cell line and enhances anticancer effects of doxorubicin. *Iran J Basic Med Sci* **18**(7): 635–643.

Chen J, Pan Y, He B, Ying H, Wang F, Sun H, Deng Q, Liu X, Lin K, Peng H, Cho WC, Wang S (2015) Inhibition of CD147 expression by RNA interference reduces proliferation, invasion and increases chemosensitivity in cancer stem cell-like HT-29 cells. *Int J Oncol* **47**(4): 1476–1484, doi: 10.3892/ijo.2015.3138.

Dutta S, Ryan J, Chen TS, Kougentakis C, Letai A, Keating AE (2015) Potent and specific peptide inhibitors of human pro-survival protein Bcl-xL. *J Mol Biol* **427**(6 Pt B): 1241–1253, doi: 10.1016/j.jmb.2014.09.030.

Hori M, Matsuda T, Shibata A, Katanoda K, Sobue T, Nishimoto H, Japan Cancer Surveillance Research Group (2015) Cancer incidence and incidence rates in Japan in 2009: a study of 32 population-based cancer registries for the Monitoring of Cancer Incidence in Japan (MCIJ) project. *Jpn J Clin Oncol* **45**(9): 884–891, doi: 10.1093/jjco/hyv088.

Kamihara Y, Takada K, Sato T, Kawano Y, Murase K, Arihara Y, Kikuchi S, Hayasaka N, Usami M, Iyama S, Miyanishi K, Sato Y, Kobune M, Kato J (2016) The iron chelator deferasirox induces apoptosis by targeting oncogenic Pyk2/beta-catenin signaling in human multiple myeloma. *Oncotarget* **7**(39): 64330–64341, doi: 10.18632/oncotarget.11830.

Kawano Y, Kobune M, Yamaguchi M, Nakamura K, Ito Y, Sasaki K, Takahashi S, Nakamura T, Chiba H, Sato T, Matsunaga T, Azuma H,

Ikebuchi K, Ikeda H, Kato J, Niitsu Y, Hamada H (2003) Ex vivo expansion of human umbilical cord hematopoietic progenitor cells using a coculture system with human telomerase catalytic subunit (hTERT)-transfected human stromal cells. *Blood* **101**(2): 532–540.

Klotz DM, Nelson SA, Kroboth K, Newton IP, Radulescu S, Ridgway RA, Sansom OJ, Appleton PL, Nathke IS (2012) The microtubule poison vinorelbine kills cells independently of mitotic arrest and targets cells lacking the APC tumour suppressor more effectively. *J Cell Sci* **125**(Pt 4): 887–895, doi: 10.1242/jcs.091843.

Kubo T, Kawano Y, Himuro N, Sugita S, Sato Y, Ishikawa K, Takada K, Murase K, Miyanishi K, Sato T, Takimoto R, Kobune M, Nobuoka T, Hirata K, Takayama T, Mori M, Hasegawa T, Kato J (2016) BAK is a predictive and prognostic biomarker for the therapeutic effect of docetaxel treatment in patients with advanced gastric cancer. *Gastric Cancer* **19**(3): 827–838, doi: 10.1007/s10120-015-0557-1.

Leichman CG, Lenz HJ, Leichman L, Danenberg K, Baranda J, Groshen S, Boswell W, Metzger R, Tan M, Danenberg PV (1997) Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal cancer response and resistance to protracted-infusion fluorouracil and weekly leucovorin. *J Clin Oncol* **15**(10): 3223–3229.

Liu W, Fang Y, Wang XT, Liu J, Dan X, Sun LL (2014) Overcoming 5-Fu resistance of colon cells through inhibition of Glut1 by the specific inhibitor WZB117. *Asian Pac J Cancer Prev* **15**(17): 7037–7041.

Longley DB, Harkin DP, Johnston PG (2003) 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* **3**(5): 330–338.

Lu M, Gao J, Wang XC, Shen L (2011) Expressions of thymidylate synthase, thymidine phosphorylase, class III beta-tubulin, and excision repair cross-complementing group 1 predict response in advanced gastric cancer patients receiving capecitabine plus paclitaxel or cisplatin. *Chin J Cancer Res* **23**(4): 288–294, doi: 10.1007/s11670-011-0288-8.

Ni Chonghaile T, Sarosiek KA, Vo TT, Ryan JA, Tammareddi A, Moore Vdel G, Deng J, Anderson KC, Richardson P, Tai YT, Mitsiades CS, Matulonis UA, Drapkin R, Stone R, Deangelo DJ, McConkey DJ, Sallan SE, Silverman L, Hirsch MS, Carrasco DR, Letai A (2011) Pretreatment mitochondrial priming correlates with clinical response to cytotoxic chemotherapy. *Science* **334**(6059): 1129–1133, doi: 10.1126/science.1206727.

Nita ME, Nagawa H, Tominaga O, Tsuno N, Fujii S, Sasaki S, Fu CG, Takenoue T, Tsuruo T, Muto T (1998) 5-Fluorouracil induces apoptosis in human colon cancer cell lines with modulation of Bcl-2 family proteins. *Br J Cancer* **78**(8): 986–992.

Rutman RJ, Cantarow A, Paschkis KE (1954) Studies in 2-acetylaminofluorene carcinogenesis. III. The utilization of uracil-2-C14 by preneoplastic rat liver and rat hepatoma. *Cancer Res* **14**(2): 119–123.

Ryan J, Letai A (2013) BH3 profiling in whole cells by fluorimeter or FACS. *Methods* **61**(2): 156–164, doi: 10.1016/j.ymeth.2013.04.006.

Ryan JA, Brunelle JK, Letai A (2010) Heightened mitochondrial priming is the basis for apoptotic hypersensitivity of CD4+ CD8+ thymocytes. *Proc Natl Acad Sci U S A* **107**(29): 12895–12900, doi: 10.1073/pnas.0914878107.

Smith SM, Lyu YL, Cai L (2014) NF-kappaB affects proliferation and invasiveness of breast cancer cells by regulating CD44 expression. *PloS One* **9**(9): e106966, doi: 10.1371/journal.pone.0106966.

Theile D, Grebhardt S, Haefeli WE, Weiss J (2009) Involvement of drug transporters in the synergistic action of FOLFOX combination chemotherapy. *Biochem Pharmacol* **78**(11): 1366–1373, doi: 10.1016/j.bcp.2009.07.006.

Tsutani Y, Yoshida K, Sanada Y, Wada Y, Konishi K, Fukushima M, Okada M (2008) Decreased orotate phosphoribosyltransferase activity produces 5-fluorouracil resistance in a human gastric cancer cell line. *Oncol Rep* **20**(6): 1545–1551.

Vo TT, Ryan J, Carrasco R, Neuberg D, Rossi DJ, Stone RM, Deangelo DJ, Frattini MG, Letai A (2012) Relative mitochondrial priming of myeloblasts and normal HSCs determines chemotherapeutic success in AML. *Cell* **151**(2): 344–355, doi: 10.1016/j.cell.2012.08.038.

Wang KZ, Zhu J, Dagda RK, Uechi G, Cherra SJ 3rd, Gusdon AM, Balasubramani M, Chu CT (2014) ERK-mediated phosphorylation of TFAM downregulates mitochondrial transcription: implications for Parkinson's disease. *Mitochondrion* **17**: 132–140, doi: 10.1016/j.mito.2014.04.008.

Watanabe T, Muro K, Ajioka Y, Hashiguchi Y, Ito Y, Saito Y, Hamaguchi T, Ishida H, Ishiguro M, Ishihara S, Kanemitsu Y, Kawano H, Kinugasa Y, Kokudo N, Murofushi K, Nakajima T, Oka S, Sakai Y, Tsuji A, Uehara K, Ueno H, Yamazaki K, Yoshida M, Yoshino T, Boku N, Fujimori T, Itabashi M, Koinuma N, Morita T, Nishimura G, Sakata Y, Shimada Y, Takahashi K, Tanaka S, Tsuruta O, Yamaguchi T, Yamaguchi N, Tanaka T, Kotake K, Sugihara K, Japanese Society for Cancer of the Colon and Rectum (2017) Japanese Society for Cancer of the Colon and Rectum (JSCCR) guidelines 2016 for the treatment of colorectal cancer. *Int J Clin Oncol*, doi: 10.1007/s10147-017-1101-6. [Epub ahead of print].

Wu DW, Huang CC, Chang SW, Chen TH, Lee H (2015) Bcl-2 stabilization by paxillin confers 5-fluorouracil resistance in colorectal cancer. *Cell Death Differ* **22**(5): 779–789, doi: 10.1038/cdd.2014.170.

Xu M, Chen X, Chen N, Nie L, Li X, Li Q, Zeng H, Zhou Q (2013) Synergistic silencing by promoter methylation and reduced AP-2alpha transactivation of the proapoptotic HRK gene confers apoptosis resistance and enhanced tumor growth. *Am J Pathol* **182**(1): 84–95, doi: 10.1016/j.ajpath.2012.09.018.

Xu T, Zhou M, Peng L, Kong S, Miao R, Shi Y, Sheng H, Li L (2014) Upregulation of CD147 promotes cell invasion, epithelial-to-mesenchymal transition and activates MAPK/ERK signaling pathway in colorectal cancer. *Int J Clin Exp Pathol* **7**(11): 7432–7441.

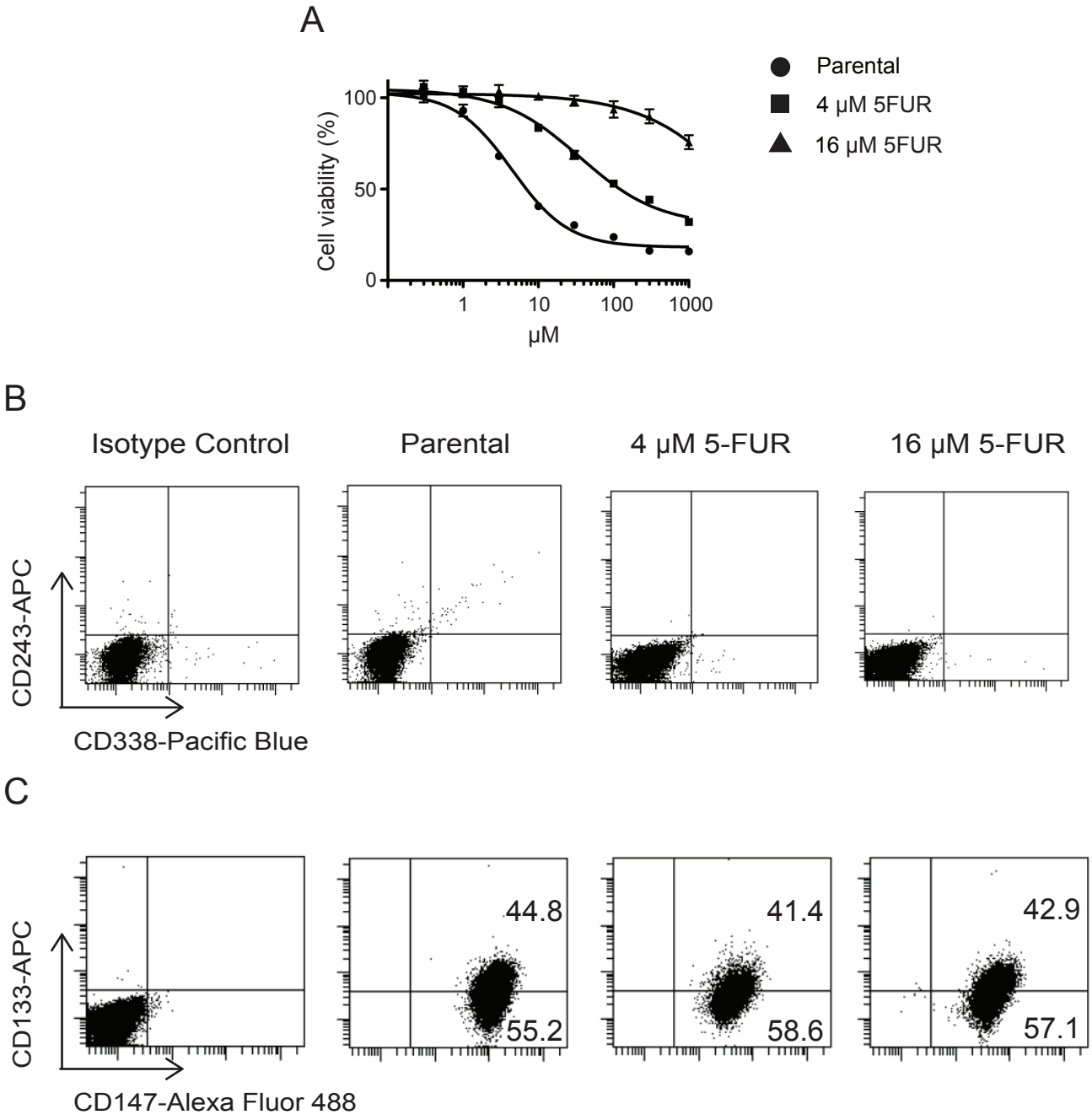
Yamane N, Makino M, Kaibara N (1999) S-phase accumulation precedes apoptosis induced by preoperative treatment with 5-fluorouracil in human colorectal carcinoma cells. *Cancer* **85**(2): 309–317.

Yuan JH, Cheng JQ, Jiang LY, Ji WD, Guo LF, Liu JJ, Xu XY, He JS, Wang XM, Zhuang ZX (2008) Breast cancer resistance protein expression and 5-fluorouracil resistance. *Biomed Environ Sci* **21**(4): 290–295, doi: 10.1016/S0895-3988(08)60044-6.

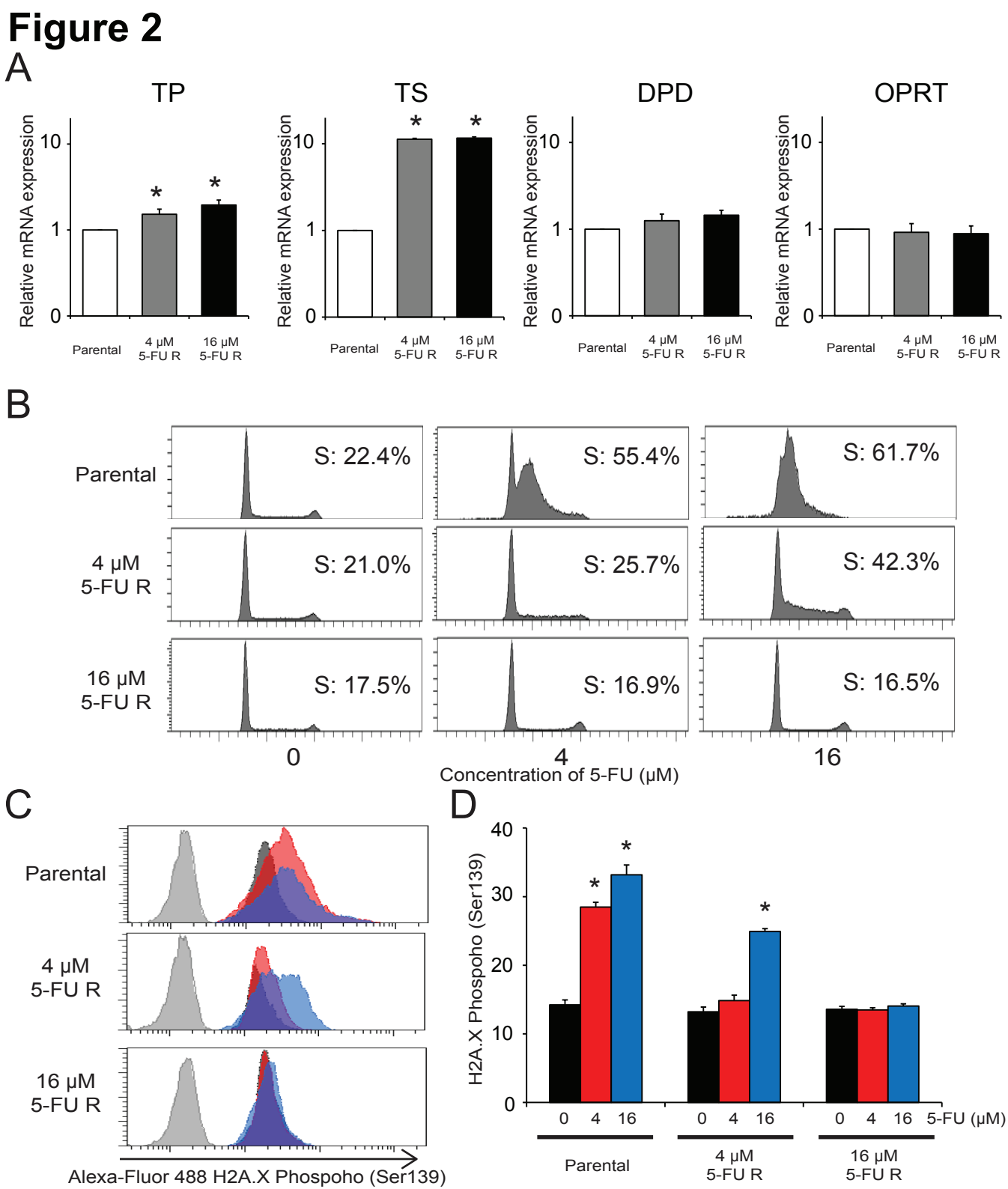
Zhao DP, Ding XW, Peng JP, Zheng YX, Zhang SZ (2005) Prognostic significance of bcl-2 and p53 expression in colorectal carcinoma. *J Zhejiang Univ Sci B* **6**(12): 1163–1169.

Zinkel S, Gross A, Yang E (2006) BCL2 family in DNA damage and cell cycle control. *Cell Death Differ* **13**(8): 1351–1359.

# Figure 1

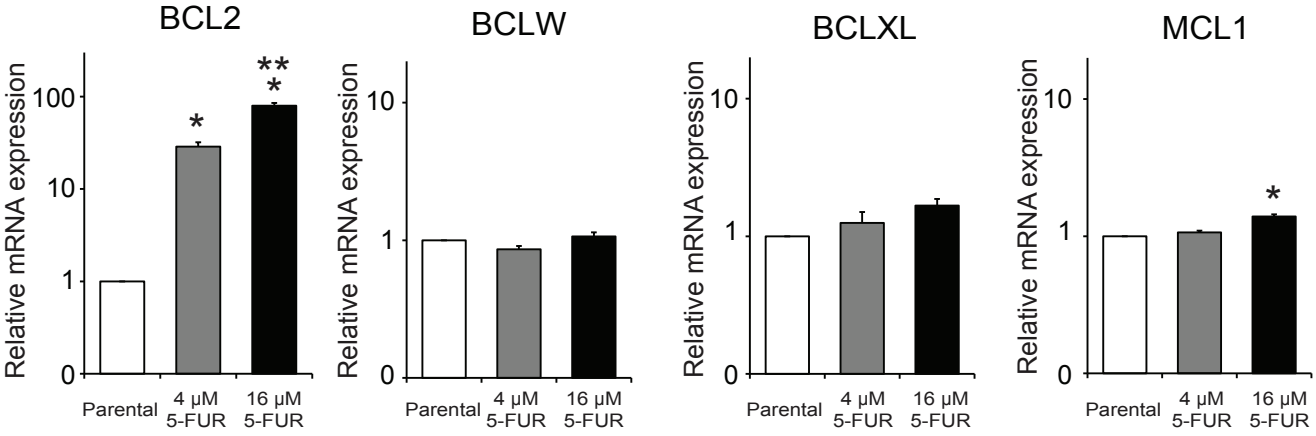

A
Parental
4 μM 5FUR
16 μM 5FUR
Cell viability (%)
100
50
0
1
10
100
1000
μM
B
Isotype Control
Parental
4 μM 5-FUR
16 μM 5-FUR
CD243-APC
CD338-Pacific Blue
C
CD133-APC
CD147-Alexa Fluor 488
44.8
55.2
41.4
58.6
42.9
57.1

# Figure 2


A
TP
TS
DPD
OPRT
Relative mRNA expression
10
1
0
*
Parental
4 μM 5-FU R
16 μM 5-FU R
B
Parental
4 μM 5-FU R
16 μM 5-FU R
S: 22.4%
S: 55.4%
S: 61.7%
S: 21.0%
S: 25.7%
S: 42.3%
S: 17.5%
S: 16.9%
S: 16.5%
0
4
16
Concentration of 5-FU (μM)
C
Parental
4 μM 5-FU R
16 μM 5-FU R
Alexa-Fluor 488 H2A.X Phospoho (Ser139)
D
H2A.X Phospoho (Ser139)
40
30
20
10
0
0 4 16 5-FU (μM)
Parental
4 μM 5-FU R
16 μM 5-FU R

# Figure 3

A


BCL2
BCLW
BCLXL
MCL1
Relative mRNA expression
100
10
1
0
*
**
Parental
4 μM 5-FUR
16 μM 5-FUR

B

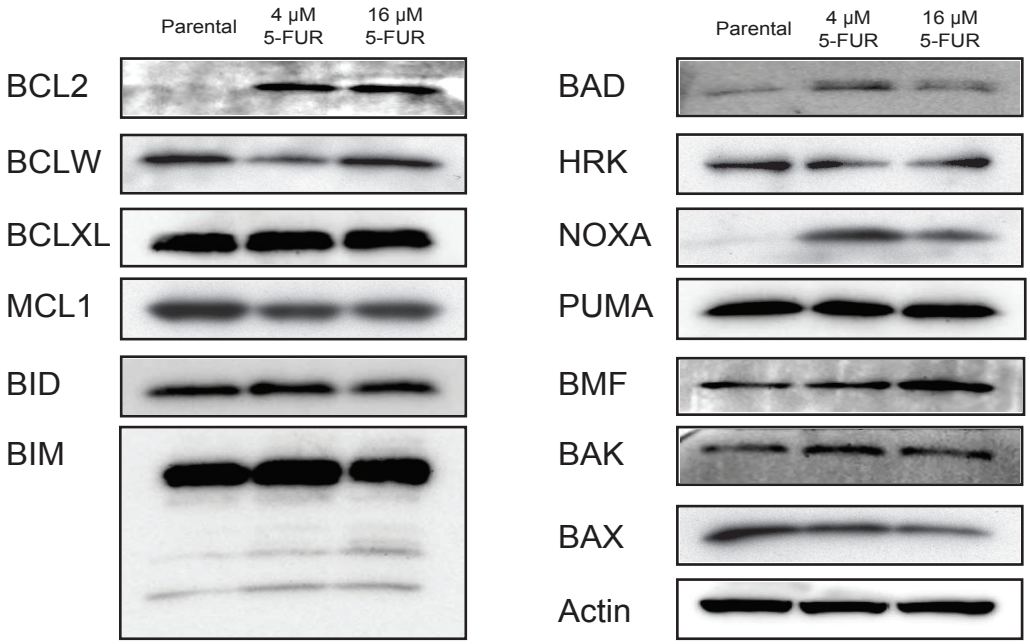
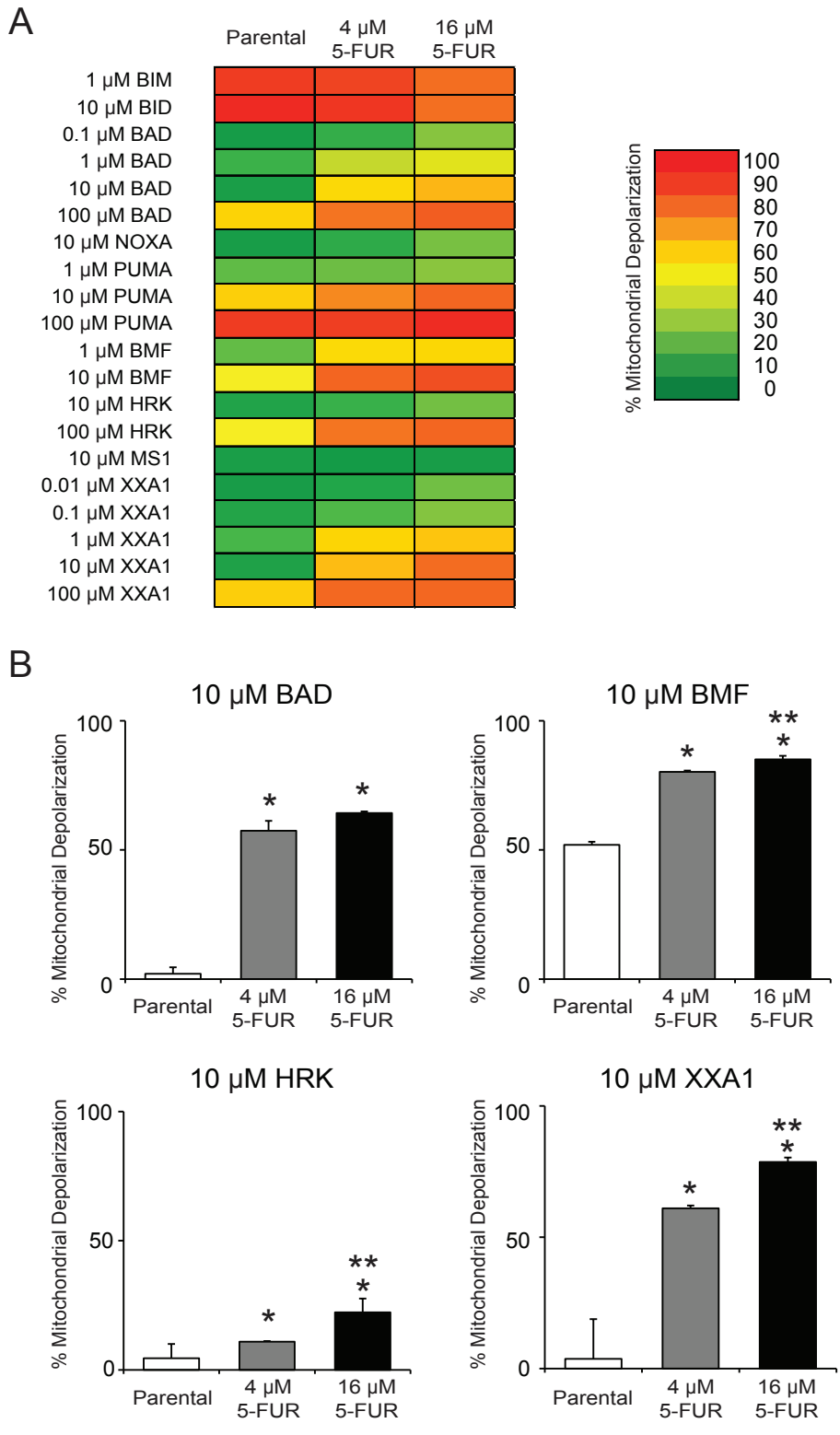
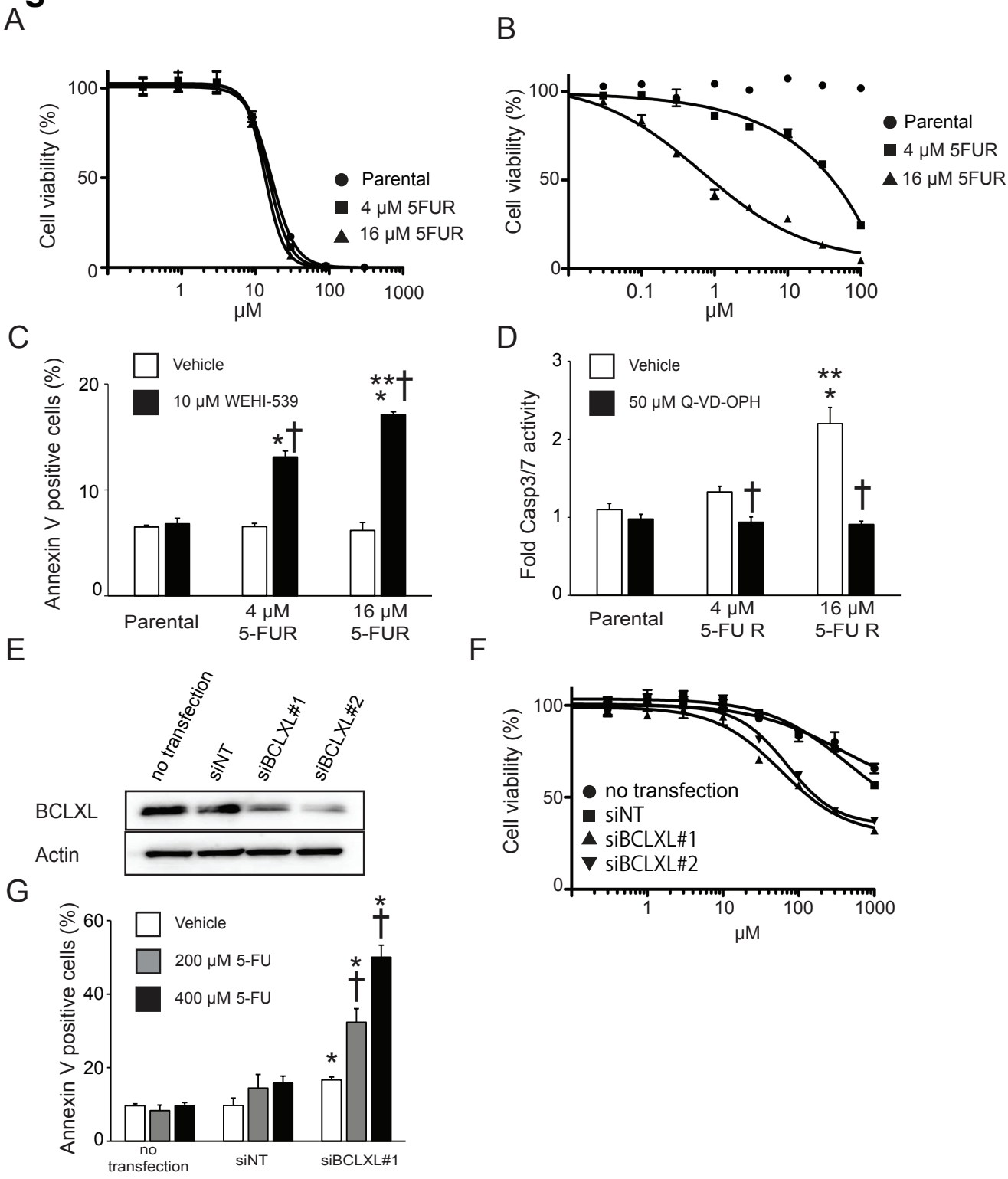

Parental
4 μM 5-FUR
16 μM 5-FUR
BCL2
BCLW
BCLXL
MCL1
BID
BIM
BAD
HRK
NOXA
PUMA
BMF
BAK
BAX
Actin

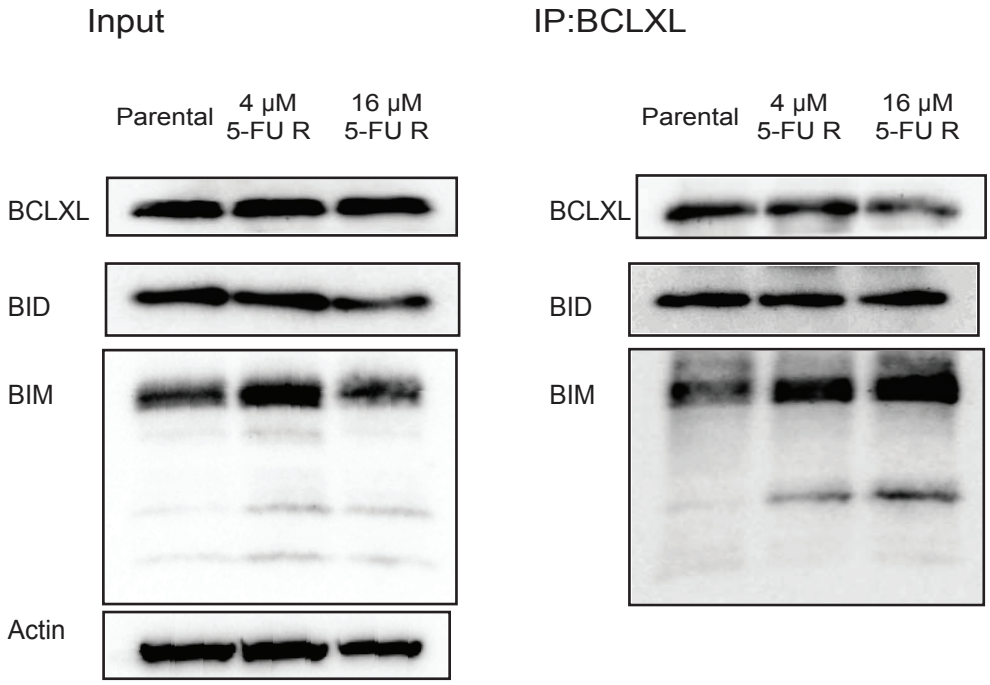
**Figure 4**


A
Parental
4 μM 5-FUR
16 μM 5-FUR
1 μM BIM
10 μM BID
0.1 μM BAD
1 μM BAD
10 μM BAD
100 μM BAD
10 μM NOXA
1 μM PUMA
10 μM PUMA
100 μM PUMA
1 μM BMF
10 μM BMF
10 μM HRK
100 μM HRK
10 μM MS1
0.01 μM XXA1
0.1 μM XXA1
1 μM XXA1
10 μM XXA1
100 μM XXA1
% Mitochondrial Depolarization
100
90
80
70
60
50
40
30
20
10
0
B
10 μM BAD
10 μM BMF
10 μM HRK
10 μM XXA1
% Mitochondrial Depolarization
100
50
0
Parental
4 μM 5-FUR
16 μM 5-FUR
*
**

# Figure 5


A
Cell viability (%)
100
50
0
1
10
100
1000
μM
Parental
4 μM 5FUR
16 μM 5FUR
B
Cell viability (%)
100
50
0
0.1
1
10
100
μM
Parental
4 μM 5FUR
16 μM 5FUR
C
Annexin V positive cells (%)
20
10
0
Vehicle
10 μM WEHI-539
*†
**†
Parental
4 μM 5-FUR
16 μM 5-FUR
D
Fold Casp3/7 activity
3
2
1
0
Vehicle
50 μM Q-VD-OPH
**
*
†
†
Parental
4 μM 5-FU R
16 μM 5-FU R
E
no transfection
siNT
siBCLXL#1
siBCLXL#2
BCLXL
Actin
F
Cell viability (%)
100
50
0
1
10
100
1000
μM
no transfection
siNT
siBCLXL#1
siBCLXL#2
G
Annexin V positive cells (%)
60
40
20
0
Vehicle
200 μM 5-FU
400 μM 5-FU
*
*†
*†
no transfection
siNT
siBCLXL#1

# Figure 6


Input
IP:BCLXL
Parental
4 μM 5-FU R
16 μM 5-FU R
Parental
4 μM 5-FU R
16 μM 5-FU R
BCLXL
BID
BIM
Actin
BCLXL
BID
BIM

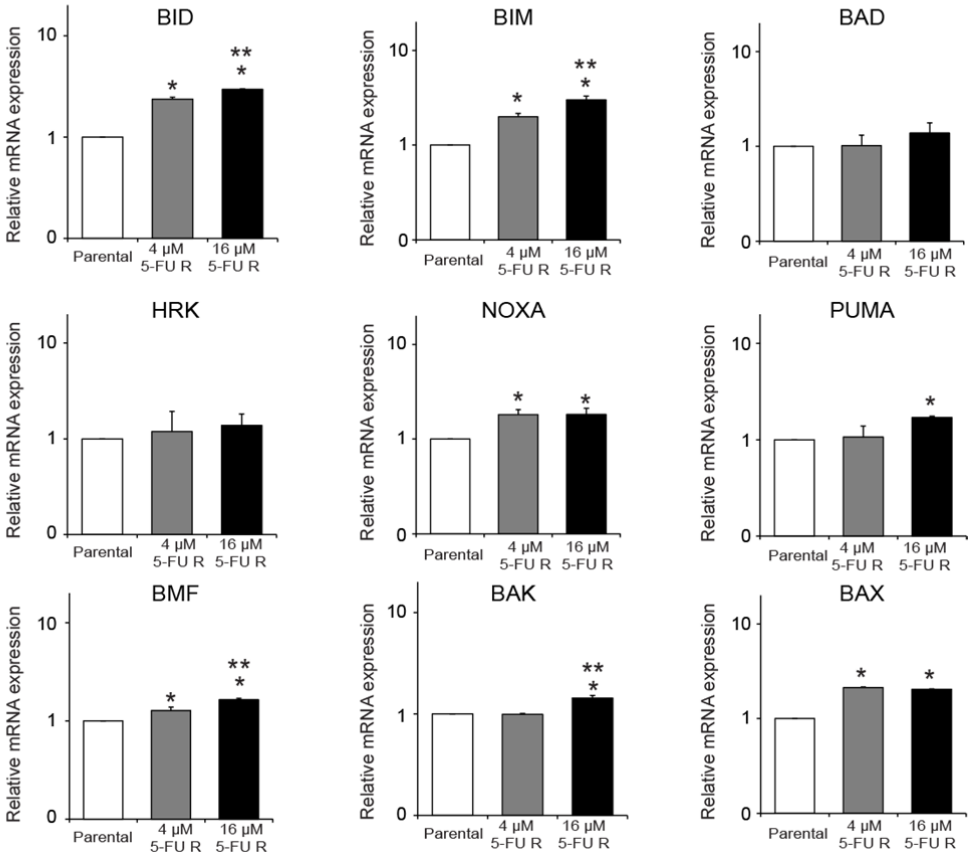
**Supplementary Appendix**

**Supplementary Figure. 1**

**Real time PCR analysis of apoptosis-related genes in parental and 5-Fluorouracil (5-FU)-resistant HT-29 cells.**

All data represent the mean ± SD (n = 3). P values represent the significance when compared to parental HT-29 cells (*$P$ < 0.01), and 4 μM 5-FU resistant HT-29 cells (**$P$ < 0.01), respectively, based on analysis of variance of variance with Tukey's multiple-comparison test.

## Supplement Figure 1



| Supplementary Table 1. Real Time PCR primer sets | | | |
|---|---|---|---|
| **Gene name** | **Primer sequence** | **Gene name** | **Primer sequence** |
| BID | F: ATGGACCGTAGCATCCCTCC | BCL2 | F: GGTGGGGTCATGTGTGTGG |
| | R: GTAGGTGCGTAGGTTCTGGT | | R: CGGTTCAGGTACTCAGTCATCC |
| BIM | F: TAAGTTCTGAGTGTGACCGAGA | BCLW | F: GCGGAGTTCACAGCTCTATAC |
| | R: GCTCTGTCTGTAGGGAGGTAGG | | R: AAAAGGCCCCTACAGTTACCA |
| BAD | F: CCCAGAGTTTGAGCCGAGTG | BCLXL | F: GATCCCCATGGCAGCAGTAAAGCAAG |
| | R: CCCATCCCTTCGTCGTCCT | | R: CCCCATCCCGGAAGAGTTCATTCACT |
| HRK | F: GGCAGGCGGAACTTGTAGGAAC | MCL1 | F: TGCTTCGGAAACTGGACATCA |
| | R: TCCAGGCGCTGTCTTTACTCTCC | | R: TAGCCACAAAGGCACCAAAAG |
| NOXA | F: ACCAAGCCGGATTTGCGATT | TP | F: TGGCTGCAAGGTGCCAATG |
| | R: ACTTGCACTTGTTCCTCGTGG | | R: AGCACTTGCATCTGCTCTGG |
| PUMA | F: GCCAGATTTGTGAGACAAGAGG | TS | F: GCCTCGGTGTGCCTTTCA |
| | R: CAGGCACCTAATTGGGCTC | | R: CCCGTGATGTGCGCAAT |
| BMF | F: GAGCCATCTCAGTGTGTGGAG | DPD | F: GGCGGACATCGAGAGTATCCT |
| | R: GCCAGCATTGCCATAAAAGAGTC | | R: TTCTTGGCCGAAGTGGAACAC |
| BAK | F: GTTTTCCGCAGCTACGTTTTT | OPRT | F: TTGGTGACGGGTCTGTACGA |
| | R: GCAGAGGTAAGGTGACCATCTC | | R: GAAGACGCGGTCGAGACAC |
| BAX | F: CCCGAGAGGTCTTTTTCCGAG | ACTB | F: GGCATCCTCACCCTGAAGTA |
| | R: CCAGCCCATGATGGTTCTGAT | | R: GAAGGTGTGGTGCCAGATTT |

| Supplementary Table 2. Sequences of BH3 peptides | |
|---|---|
| **Name** | **Sequence** |
| BIM | MRPEIWIAQELRRIGDEFNA |
| BID | EDIIRNIARHLAQVGDSMDRY |
| BAD | LWAAQRYGRELRRMSDEFEGSFKGL |
| NOXA | AELPPEFAAQLRKIGDKVYC |
| PUMA | EQWAREIGAQLRRMADDLNA |
| BMF | HQAEVQIARKLQLIADQFHRY |
| HRK | WSSAAQLTAARLKALGDELHQ |
| MS1 | RPEIWMTQGLRRLGDEINAYYAR |
| XXA1 | RPEIWYAQGLKRFGDEFNAYYAR |
| PUMA2A | EQWAREIGAQARRMAADLNABIM |

All BH3 peptides were 20–25 amino acid residues in length.

PUMA2A served as a control peptide for the 2-amino acid substitution in Puma.

The conserved LXXXXD motif was present in all peptides, except for PUMA2A.

All peptides were N-terminally acetylated and C-terminally amidated.