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Original Article

Atractyloside mimics *BORIS* knockdown to induce DNA damage in colorectal cancer cells

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Abstract: The Brother of Regulator of Imprinted Sites (BORIS) is expressed abnormally in colorectal cancer and is predicted to be a potential diagnostic and prognostic target. However, little is known about BORIS-related signaling pathways and no bioactive drugs have been found to target BORIS. We screened the gene regulation panels of *BORIS*-silenced colorectal cancer cells by microarray assay and applied the regulated gene list in a connectivity map (CMap) database to screen for bioactive drugs which regulate gene panels similar to *BORIS* knockdown. Gene set enrichment analysis (GSEA) suggests a correlation between *BORIS* knockdown and apoptosis. Screening revealed atractyloside treatment as a drug similar to *BORIS* siRNA in regulating genes in colorectal cancer cells. Atractyloside treatment or *BORIS* knockdown induced the expression of *XRCC4*, which suggested DNA damage was induced by knockdown of the *BORIS* signaling pathway. H2A.X immunofluorescence stain indicated *BORIS* knockdown indeed created DNA damage. As atractyloside synergized with 5-Fluoruracil (5-FU) to suppress colorectal cancer cell proliferation, we concluded that the inhibition of BORIS downstream by atractyloside amplifies the effect of 5-FU by promoting DNA damage.

Keywords: Fluorouracil, atractyloside, colorectal neoplasms, microarray analysis

Introduction

Colorectal cancer is the third most prevalent common cancer in the world [1]. Though the first line of therapy strategies, including 5-Fluoruracil (5-FU), oxaliplatin, FOLFOX, and FOLFIRI chemotherapy regiments applied for the treatment of colorectal patients, drug resistance causes further progression in colorectal cancer patients [2]. Personal therapeutic strategy may improve the outcome of the colorectal patients. New targets and strategies for therapy are required. The Brother of Regulator of Imprinted Sites (BORIS) which is also known as CTCFL is expressed abnormally in colorectal cancer and is predicted to be a potential target of diagnosis and prognosis [3].

BORIS is reported to be required for certain cancers [4, 5]. *BORIS* knockdown suppressed the growth and induced apoptosis of breast cancer [5]. *BORIS* expression knockdown may have promise for colorectal cancer therapy. However, no siRNA product has been launched onto the market, because of the challenge of the effective delivery to the tumor. Bioactive small molecules targeting BORIS may be able to inhibit colorectal cancer cell growth in the same manner as *BORIS* siRNA.

The connectivity map (CMap) database collects more than 7000 transcriptional expression profiles from cultured human cells treated with 1309 compounds [6]. The aim is to enable the discovery of functional connections between drug, genes, and diseases by pattern-matching. It can find drugs that regulate similar gene groups such as *BORIS* siRNA.

Here, we found that *BORIS* knockdown induced apoptosis of colorectal cancer cells. To expand our view of the signaling pathway related with BORIS, altered gene expression by *BORIS* knockdown was assessed by microarray assay. Gene set enrichment analysis (GSEA) suggested the suppression of colorectal cancer cell proliferation by *BORIS* silencing was caused by apoptosis. To retrieve drugs targeting on *BORIS*,

Table 1. Primers and siRNA used in this study

Gene/siRNA	Forward (5' to 3')	Reverse (5' to 3')	Application
SEMA3A	GGTGCCTTATCAAGGAAGAGTCC	TACATGGCTGGATGACTTCTTGC	Realtime PCR
XRCC4	ATGGCTCCTCAGGAGAATCAGC	GAGGTCTTCTGGGCTGCTGTTT	Realtime PCR
BORIS	CAGGCCCTACAAGTGTAACGACTGCAA	GCATTCGTAAGGCTTCTCACCTGAGTG	Realtime PCR
GAPDH	CCCACTCCTCCACCTTTGAC	TGTTGCTGTAGCCAAATTCGT	Realtime PCR
Actin	AAAATCTGGCACCACACCTTC	TAGCACAGCCTGGATAGCAA	Realtime PCR
Negative siRNA	UUCUCCGAACGUGUCACGUdTdT	ACGUGACACGUUCGGAGAAdTdT	Knockdown
BORIS siRNA	GGAAAUACCACGAUGCAAATT	UUUGCAUCGUGGUAUUUCCtt	Knockdown[a]

[a]reference [5].

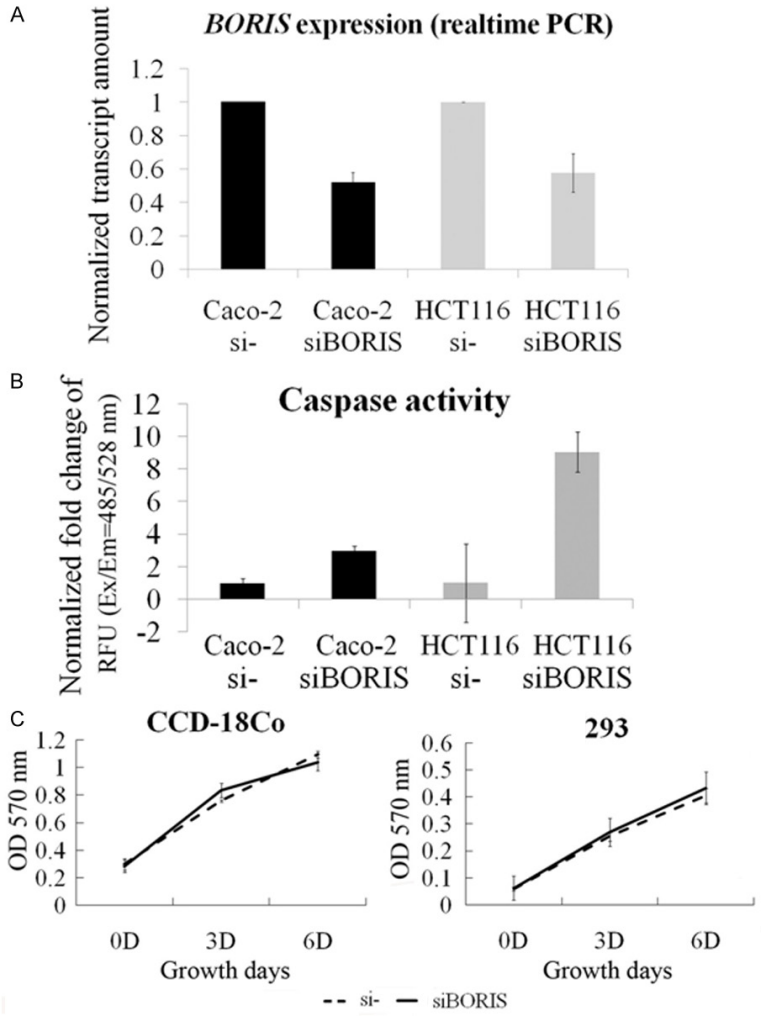

Figure 1. BORIS knockdown induced apoptosis of Caco-2 and HCT116 cells. A: BORIS knockdown efficiency was tested by real-time PCR. B: BORIS knockdown induced caspase 3/7 activity of Caco-2 and HCT116 cells. C: MTT was applied to test the cell viability of CCD-18Co or 293 cells after knockdown by BORIS siRNA. Negative siRNA was used as control. BORIS siRNA was used to silence the expression of BORIS.

drugs in CMap database were scanned. Atractyloside treatment was found to regulate a similar gene set as *BORIS* silencing and synergized with 5-FU for suppressing colorectal cancer cell growth. *BORIS* knockdown upregulated the expression of XRCC4 and induce DNA damage. Atractyloside treatment may regulate BORIS downstream signaling to amplify DNA damage.

Materials and methods

Cell culture

HCT116 and Caco-2 colorectal cancer cells, and non-cancer cell line CCD-18Co and 293 cells were cultured in DMEM high glucose medium supplied with 10% FBS and 5% CO_2. CCD-18Co is a normal colorectal cell line, which expresses partial length of BORIS [15], and was a negative control in our study. Though the 293 cell line does not originate from colon or rectum, it does not express *BORIS*. We did not detect BORIS in 293 cells by both quantitative real-time PCR and western blot assay, so 293 cell line served as one of the negative controls. Cells were plated on 6-well or 96-well plates for subsequent treatment. The Brother of Regulator of Imprinted Sites (BORIS) siRNA and Lipofectamine®RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) was applied to silence the expression of BORIS. Cell proliferation viability was assessed by Thiazolyl

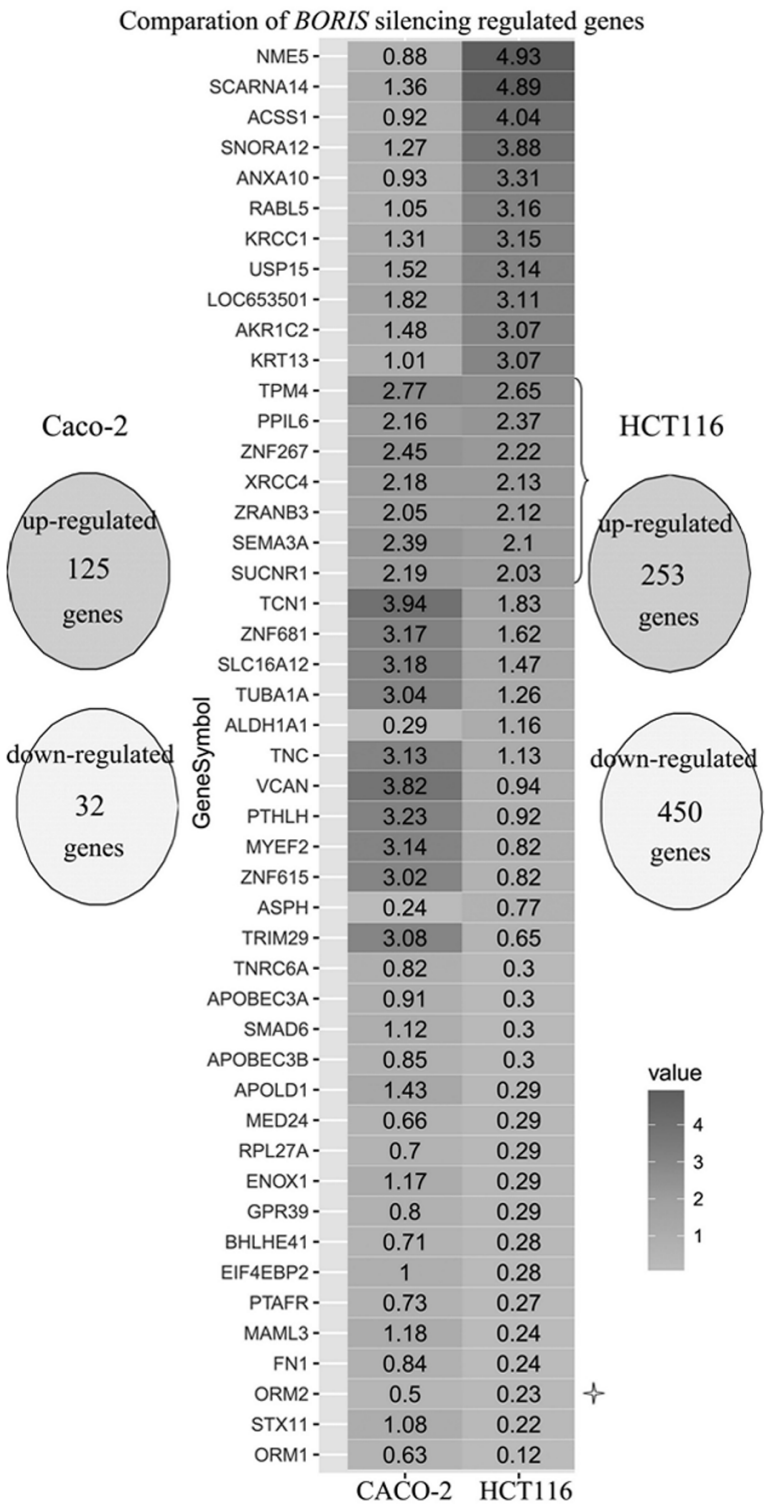

Figure 2. BORIS knockdown regulated genes in HCT116 and Caco-2. Comparison of common genes that had two-fold or more than three-fold change in HCT116 and Caco-2. The fold changes were calculated by normalizing the gene expression level in BORIS siRNA treated samples to negative siRNA controls. Genes showing a two-fold change in both HCT116 and Caco-2 are indicated by brace and asterisk.

Blue Tetrazolium Bromide (MTT) assay. siRNA used in this study are listed in **Table 1**. Atractyloside (potassium salt) was bought from Cayman Chemical Company (Ann Arbor, Michigan 48108 USA). 5-Fluoruracil (5-FU) was bought from Sigma-Aldrich Corporation (St. Louis, MO 63-103, United States). Medium were supplied with either or both Atractyloside (0 to 5 μM) or/and 5-FU (0 to 750 ng/ml) for treatment. DMSO was used as control. Drug treatments were performed for 3 to 8 days and stopped by MTT to test the inhibition effect.

Caspase 3/7 activity assay

Cells were seeded on white 96-well plates. Caspase-Glo® 3/7 kit was used to detect caspase 3/7 activity three days after knockdown of *BORIS*.

Microarray analysis

HCT116 and Caco-2 cells were silenced by siRNA of *BORIS* for 3 days and extracted for the RNA. Silenced samples and the controls were compared to screen for gene expression difference by Affymetrix PrimeView human gene expression array. Two-fold changed common genes or three-fold changed genes in either HCT116 or Caco-2 cells were used to establish a heatmap. Raw data have been deposited into GEO with accession number GSE86172.

GSEA analysis

The microarray results were analyzed subsequently by GSEA analysis. The enriched

Table 2. Common *BORIS* pathways regulated in both Caco-2 and HCT116

	Caco-2	HCT116
Common GS follow link to MSigDB	HAMAI_APOPTOSIS_VIA_TRAIL_UP	HAMAI_APOPTOSIS_VIA_TRAIL_UP
SIZE	162	75
ES	0.330	0.376
NES	2.367	2.520
FDR q-val	0.023	0.017
FWER *p*-val	0.034	0.013
LEADING EDGE	tags=75%, list=44%, signal=130%	tags=91%, list=54%, signal=188%

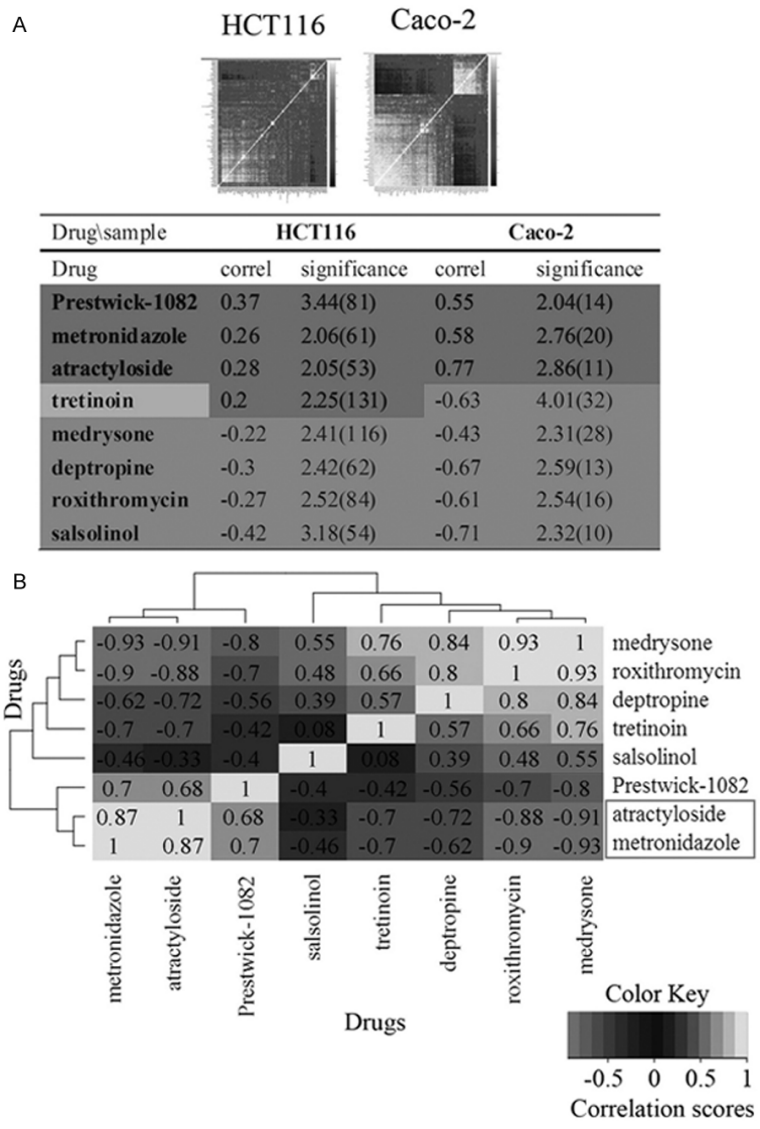

Figure 3. Selection of drugs acting similarly to BORIS knockdown. A: The listed drugs as act similar to BORIS knockdown to regulate genes in HCT116 and Caco-2 cells. B: Cluster analysis by correlation between the drugs was applied to classify the drugs. The correlation scores between the drugs were downloaded from SPIEDw. The intensity of background indicates a similarity correlation index between the drugs. High similarity was indicated by a light background.

common pathways in both two cell lines were selected for further examination.

SPIEDw assay

SPIEDw (http://www.spied.org.uk/cgi-bin/wSPIED.cgi) is a platform-independent implementation of connectivity map (CMap) database. Up and down-regulation of genes in the microarray were analyzed by appropriate SPIEDw input format. The entire outcome was scored according to the relation.

Quantitative real-time PCR

RNA was extracted by TRIzol® (Thermo Fisher Scientific) and ethanol precipitation. After reverse transcribed to cDNA equally between control and the treatment, candidate gene expression was assessed by quantitative real-time PCR. *GAPDH* and actin were used as house keeping genes. Primers used in real-time PCR are listed in **Table 1**.

Immunofluorescence stain

Caco-2 cells were plated on coverslips in 24-well plates. Three days after siRNA transfection, cells were fixed and provided for immunofluorescence stain of H2A.X. The antibody was anti-phospho-His-

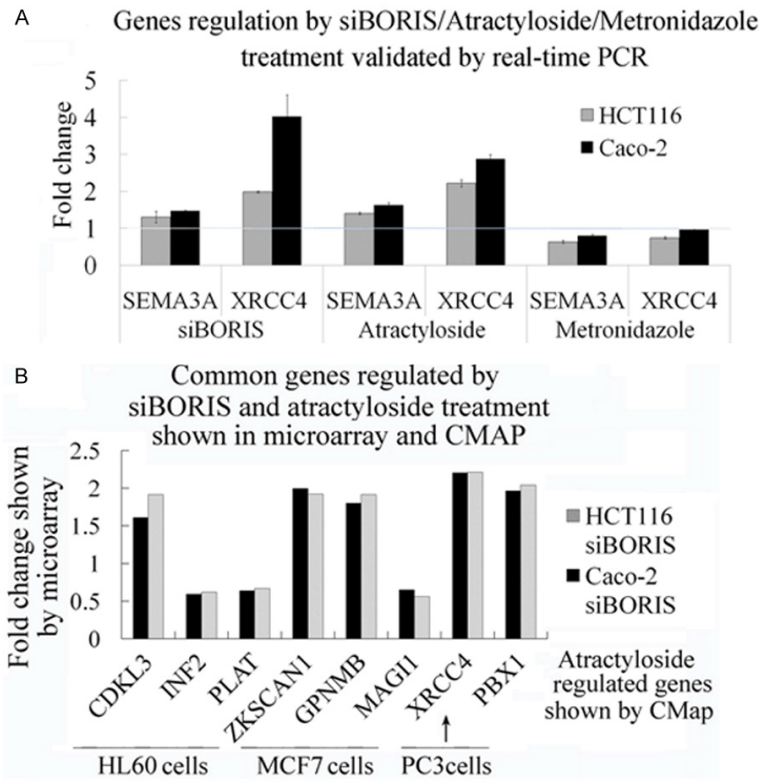

Figure 4. Genes regulated by BORIS knockdown or atractyloside treatment in both HCT116 and Caco-2 cells. A: SEMA3A and XRCC4 regulation by BORIS knockdown/atractyloside/metronidazole treatment validated by real-time PCR. B: Common genes regulated by siBORIS in our microarray result and by atractyloside treatment shown in the CMap database.

tone H2A.X (Ser139) antibody, clone JBW301, bought from EMD Millipore. Spots were counted and the percentage of spot per cell was calculated to indicate DNA damage status in silenced cells.

Statistical analysis

All assays were performed in triplicate and presented as means and standard deviations. Values were calculated by unpaired Student's t-test and considered significantly different if $P<0.05$.

Results

BORIS knockdown induced apoptosis

The Brother of Regulator of Imprinted Sites (BORIS) is reported to be required for some types of the cancers including colorectal cancer [5, 7, 8]. We verified the significance of *BORIS* in colorectal cancer cells by silencing *BORIS* (**Figure 1A**). *BORIS* siRNA induced caspase 3/7 activity in Caco-2 and HCT116 cells (**Figure 1B**). However, *BORIS* knockdown did not affect the viability of CCD-18Co or 293 cells (**Figure 1C**), which is a non-cancer colon cell line without *BORIS* expression. The requirement of *BORIS* in colorectal cancer cells suggested the potential for *BORIS* siRNA in colorectal cancer therapy. However, the mechanism by which *BORIS* silencing suppresses colorectal cancer proliferation remains unrevealed.

Bioactive small molecules selection by gene pattern match as BORIS siRNA

To study gene regulation by BORIS knockdown, microarray assay was applied to screen the gene expression regulated by *BORIS* siRNA in colorectal cancer cells. We found that 157 and 703 genes were regulated by *BORIS* siRNA in Caco-2 and HCT116 cells respectively (**Figure 2**). Eight common genes were regulated by *BORIS* siRNA in HCT116 and Caco-2 cells (**Figure 2**). Seven of them were up-regulated and one was down-regulated with more than a two-fold change. Then pathways commonly regulated by *BORIS* knockdown were analyzed by GSEA analysis, which showed that *BORIS* knockdown regulated the apoptosis pathway in both HCT116 and Caco-2 cells (**Table 2**).

Bioactive small molecules which mimic the effect of *BORIS* knockdown may be a substitute for *BORIS* siRNA. The connectivity map (CMap) database was screened for drugs that regulate similar gene panels as *BORIS* silencing. Online software of SPIEDw was applied to perform the screen. Genes that were regulated two-fold in HCT116 and Caco2 by *BORIS* knockdown were interrogated for drugs acting similar to *BORIS* siRNA. Prestwick-1082, metronidazole, and atractyloside were predicted to be positively correlated with *BORIS* siRNA in either HCT116 or Caco-2 cells (**Figure 3A**). As the rank and correlation significance between the drug treat-

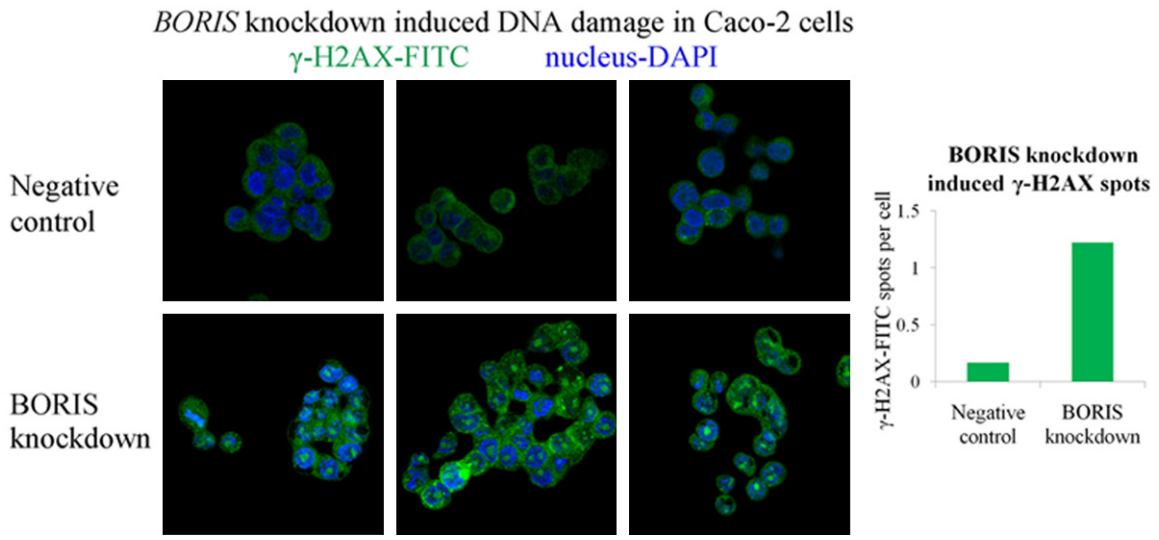

Figure 5. BORIS knockdown induced DNA damage in Caco-2 cells. Caco-2 cells were treated with BORIS siRNA or negative siRNA control. H2A.X was stained by H2A.X primary antibody and FITC conjugated secondary antibody. Nuclei were stained with DAPI. H2A.X spots in nucleus were counted to indicate DNA damage.

ment and *BORIS* knockdown were inconsistent in the two cell lines, cluster analysis by correlation between the drugs was applied to classify the drugs to search for the best candidates (**Figure 3B**). Metronidazole and atractyloside were found to exert similar gene regulation and may be the candidates to mimick the effect of *BORIS* siRNA to regulate the gene expression in colorectal cancer cells (**Figure 3B**).

DNA damage induced by BORIS knockdown

SEMA3A and *XRCC4* are two common genes regulated by *BORIS* silencing in both HCT-116 and Caco-2 cells. Regulation of these two genes was similar to *BORIS* silencing with atractyloside but not with metronidazole treatment (**Figure 4A**). *BORIS* knockdown or atractyloside treatment induced the expression of *XRCC4* significantly (**Figure 4A**). When comparing the expression panels between our microarray results of siBORIS treatment and atractyloside treatment reported in CMap, eight common genes were found to be regulated in the same direction. *XRCC4* was also noted to be up-regulated by atractyloside treatment in PC3 cells (**Figure 4B**).

XRCC4 is a DNA repair protein, which is crucial for repairing spontaneous and ionizing-radiation-induced double-strand DNA breaks (DSBs) and for V(D)J recombination to ensure a diverse repertoire of T and B cells [9]. Induction of *XRCC4* suggested that *BORIS* knockdown might induce DNA damage. The status of DNA damage was investigated on *BORIS* silenced cells by H2A.X immunofluorescence stain. The results indicated that H2A.X positive nuclear spots were induced by *BORIS* knockdown (**Figure 5**).

Atractyloside synergized with 5-FU in colorectal cancer cell suppression

The effect of atractyloside and metronidazole on cell growth was evaluated Atractyloside (0.5 μM) facilitated 5-Fluoruracil (5-FU, 0.6 μM) inhibition of colorectal cancer proliferation in both HCT116 and Caco-2 cells (**Figure 6**). Metronidazole had no effect (data not shown). This agrees with our result that atractyloside up-regulated the expression of *SEMA3A* and *XRCC4* similar to *BORIS* knockdown, while metronidazole did not (**Figure 4A**).

Discussion

The combination of atractyloside and 5-FU had a synergistic effect to inhibit colorectal cancer cells (**Figure 6**). Atractyloside was determined by screening to be a drug that regulated a similar geneset as *BORIS* knockdown (**Figure 3**), while atractyloside alone did not inhibit the growth viability of colorectal cancer cells. Atractyloside inhibits the transfer of ADP to ATP and then blocks the energy production in cells

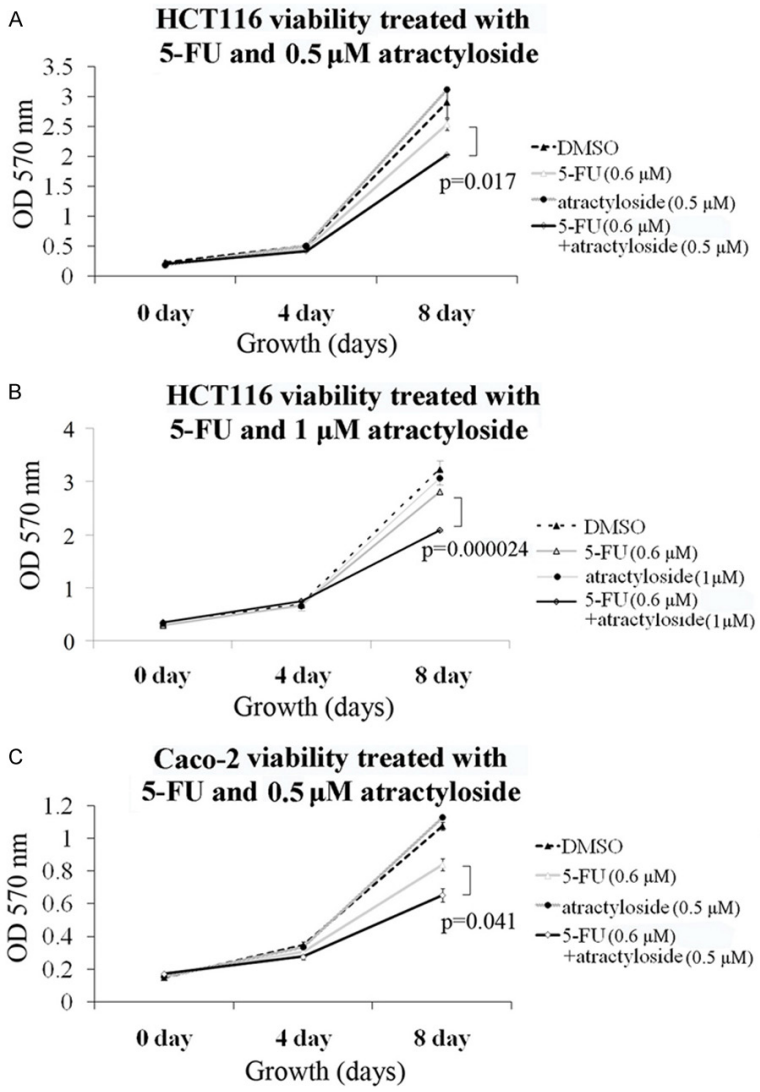

Figure 6. Atractyloside synergized with 5-Fluoruracil (5-FU) to suppress colorectal cancer cell proliferation. A: Atractyloside (0.5 μM) synergized with (0.6 μM) to suppress HCT116 cells. B: Atractyloside (1 μM) synergized with (0.6 μM) to suppress HCT116 cells. C: Atractyloside (0.5 μM) synergized with (0.6 μM) to suppress Caco-2 cells.

[10]. We also found that *XRCC4* was up-regulated by atractyloside treatment (**Figure 4A**). XRCC4 is a DNA repair protein [9]. The up-regulation of *XRCC4* reflected that DNA damage was created. Since 5-FU treatment induces DNA damage [11], the damage created by 5-FU may be amplified by atractyloside treatment.

The enhancement of H2A.X indicated that *BORIS* knockdown promoted DNA damage (**Figure 5**). *BORIS* knockdown may function similar to the combined treatment of atractyloside and 5-FU to block energy generation and DNA damage repair. How BORIS suppresses apoptosis and inhibits DNA damage needs further study. It is reported that BORIS regulates gene expression by demethylation [12-14]. Knockdown of *BORIS* might suppress the expression of DNA repair genes and enhanced DNA damage. As the expression panels between *BORIS* siRNA and atractyloside treatment were predicted to be similar (**Figure 3**), BORIS might act on mitochondria to regulate the energy supply. We indeed visualized the cytoplasmic localization of BORIS in colorectal cancer cells in a previous study [15]. We deduce that *BORIS* knockdown may synergize with present chemotherapy regimens to facilitate cure of colorectal cancer patients. Considering the aberrant expression of *BORIS* in cancer but not in normal colorectal tissue, BORIS may serve as a predictive and therapeutic target for colorectal cancer.

Conclusions

In conclusion, we provide evidence that BORIS is important for colorectal cancer cell survival. The analysis of microarray data by GSEA analysis suggests a correlation between *BORIS* knockdown and apoptosis. The further selection of drugs that regulate a similar gene panel as *BORIS* knockdown, reveals that atractyloside regulates *BORIS* signaling. Atractyloside synergizes 5-FU to suppress colorectal cancer cell growth, suggesting that the block of *BORIS* signaling augments the effect of 5-FU. Our study indicates that the treatment, targeting *BORIS* or its downstream effectors, may be applicable for tumor suppression.

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Disclosure of conflict of interest

None.

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