Tropical Journal of Pharmaceutical Research May 2018; 17 (5): 761-766

ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org

http://dx.doi.org/10.4314/tjpr.v17i5.2

Original Research Article

Blockage of bone marrow kinase in chromosome X enhances ABC294640-induced growth inhibition and apoptosis of colorectal cancer cells

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Sent for review: 5 January 2018 *Revised accepted: 25 April 2018*

Abstract

Purpose: *To investigate the role of bone marrow kinase in chromosome X (BMX) in colorectal cancer (CRC) cell resistance to ABC294640 treatment.*
Methods: *HCT-116R, LS174T and WiDr cells were transfected with either BMX-specific siRNA or scrambled siRNA, and then BMX mRNA and protein expressions were detected by quantitative polymerase chain reaction (qPCR) and western blotting, respectively. The cells were treated with ABC294640 and cell viability evaluated using cell counting and colony formation assays. Apoptosis was determined by detecting caspase 3/7 activity. To evaluate tumor growth of HCT-116R cells, a xenograft model was utilized to measure tumor size.*
Results: *Pharmacological inhibition of sphingosine kinase type 2 (SK2) with ABC294640 significantly decreased cell viability ($p < 0.001$) when compared with control group. SK2 inhibition also remarkably induced apoptosis in HCT-116 CRC cells in a dose-dependent manner ($p < 0.01$ and $p < 0.001$). However, no significant effects were observed in HCT-116R, LS174T, or WiDr cells following ABC294640 treatment. BMX mRNA and protein expression increased in ABC294640-resistant cell lines. In addition, silencing BMX expression with siRNA potentiated ABC294640-induced inhibition of tumor growth in CRC cells in vitro and in vivo.*
Conclusion: *ABC294640-induced BMX upregulation impedes the antitumor effect of ABC294640 in CRC cells. Therefore, these results may provide a novel therapeutic strategy for CRC using a combination of ABC294640 treatment and BMX blockade.*

Keywords: *ABC294640, Apoptosis, Bone marrow kinase in chromosome X, Cell viability, Colorectal cancer*

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies, ranking second in incidence and mortality among all types of malignant worldwide [1]. Without reliable biomarkers, diagnosis of CRC usually occurs in the advanced stage, leading to high mortality in these patients. Currently, surgery and conventional chemotherapy remain the primary treatments for CRC [2]. However, tumor relapse and/or metastasis severely limit their clinical

effectiveness, which demonstrates that finding effective cancer therapies remains an important medical challenge [3]. Therefore, more investigation of mechanisms of CRC pathogenesis is needed to develop effective treatments for CRC.

Sphingosine 1-phosphate (S1P) is a pleiotropic molecule and involved in various intracellular functions, including cell survival, cell motility, and angiogenesis [4]. S1P is formed via sphingosine phosphorylation by sphingosine kinase type 1 (SK1) and sphingosine kinase type 2 (SK2) [5]. The two kinases are highly conserved but have different subcellular localizations functions, and pharmacology [6]. Initially, overexpression of SK2 was reported to induce apoptosis, mediated by its BH3 domain [7]. Subsequently, it was found that SK2 downregulation had dramatic antitumor function in several tumor types, including colon cancer [8]. Loss of SK2 had stronger anticancer effects than suppression of SK1, providing a rationale for targeting SK2 in cancer therapy [9]. ABC294640 is an inhibitor of SK2 which showed very good bioavailability when administered orally as well as a favorable safety profile in various pre-clinical models [10, 11]. However, the drug resistance of cancer cell to ABC294640 is not clear.

Bone marrow kinase in chromosome X (BMX) is a member of Tec family [12]. It has two critical domains, a PH-like domain that mediates membrane binding and an SH2 domain important for the binding of tyrosine-phosphorylated proteins [13]. BMX is regulated by several oncogenes such as Src and phosphoinositide 3-kinase (PI3K), and is activated by inflammatory pathways [14]. It been implicated in tumor progression in prostate cancer, bladder cancer, and glioblastoma [15]. Additionally, BMX is reported to be involved in drug resistance. For example, overexpression of BMX abolished the miR-495-induced inhibition of drug resistance [16]. In addition, it suppressed the ephrin receptor A3 (EPHA3)-induced drug sensitivity of small cell lung cancer [17]. In the present study, the effect of BMX on the CRC response to ABC294640 treatment was explored.

EXPERIMENTAL

siRNA and transfection

HCT-116R, LS174T and WiDr cells (3×10^5 cells/well) were cultured in 6-well plates overnight. Then the cells were transfected with either BMX-specific siRNA or scrambled siRNA (30 nM; Ribo-Bio, China) using lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The protein and RNA were extracted after transfection. BMX siRNA sequences were as follows: GUACCAGUCUAGCGCAAUAUU (sense) and UAUUGCGCUAGACUGGUACUU (anti-sense).

Quantitative RT-PCR (qPCR)

In accordance with the specifications, total RNA was isolated using TRIzol reagent (Invitrogen). Reverse transcription of RNA was carried out with a first strand cDNA kit (Sigma, Munich, Germany) followed by PCR amplification with a SYBR Green PCR kit (Thermo Fisher Scientific) run on an ABI 7300 Thermocycler (Thermo Fisher Scientific). The primers were as following: β-actin Forward: 5'-AAGGCCAACCGCGAGAAG AT-3'; and Reverse:5'-TGATGACCTGGCCGTC AGG-3'; BMX Forward:5'-GAGCCGAAGTCA GTGGTTGA-3'; and Reverse: 5'-ACTTCCCGTC CACGAAGAAC-3'. Differences in relative mRNA expression were calculated with the $2^{-\Delta\Delta Ct}$ method [18].

Caspase 3/7 activity assay

For detection of apoptosis, caspase-Glo® 3/7 Assay kit from Promega was used. Cells (5×10^3 cells/well) were cultured in 96-well plates and treated with either ABC294640 (0.3 or 3 μM) or left untreated for 48 hr. The Caspase-Glo® 3/7 Reagent was added directly into the wells followed by the luminometer readings.

Protein isolation and western blot analysis

Cells were lysed with ice cell lysis buffer from cell Signaling Technology (Danvers, MA, USA). Protein was quantified using a bicinchoninic acid assay kit (EMD Millipore, Billerica, MA, USA). Total protein was subjected to 10 % SDS polyacrylamide gel electrophoresis and were transferred onto PVDF membranes. Membranes were probed with specific antibodies at 4 °C overnight (anti-GAPDH, 1:5000 dilution, #D16H11; anti-BMX, 1:1000 dilution, #24773, Cell Signaling Technology). The membranes were then incubated with secondary antibodies at room temperature for 2 h (1:5,000 dilution; #7074, Cell Signaling Technology). Bands were visualized using RapidStep™ ECL Reagent (EMD Millipore).

Tumor xenografts

Six-week old nude mice (Charles River Laboratories) received a single subcutaneous injection of 1×10^6 HCT-116R cells in the right flank. Tumor volumes were assessed with digital

calipers and calculated as previously described [19]. Tumors were measured 3 times weekly, including total mouse weights. At the end of the study, the tumors were excised, and weighed. The study was approved by Animal Ethic Committee of the First Affiliated Hospital of Wenzhou Medical University (approval ref no. 2017-0003), and were in accordance with "Principles of Laboratory Animal Care" (NIH publication no. 85 - 23, revised 1985) [19].

RESULTS

Effect of ABC294640 on CRC cells

Cytotoxicity assays demonstrated that ABC294640 significantly decreased cell viability in a dose-dependent fashion in HCT-116 cells. In contrast, ABC294640 had marginal effect on cell viability in LS174T and WiDr cells, with less than 10-fold change in viability ($p < 0.001$; Figure 1A). In addition to cytotoxicity, apoptosis was evaluated in the three CRC cell lines following ABC294640 treatment. ABC294640 treated HCT-116 cells displayed higher caspase 3/7 activity relative to the untreated group ($p < 0.001$ and $p < 0.0001$; Figure 1B). However, ABC294640 treatment had no effect on apoptosis in LS174T and WiDr cells (Figure 1B). These results demonstrate that HCT-116 cells are sensitive to ABC294640 treatment whereas LS174T and WiDr cells are more resistant.

Upregulation of BMX in CRC cells associated with ABC294640 sensitivity

To reveal the mechanism underlying drug resistance, the ABC294640-resistant cell line, HCT-116R, was established from HCT-116 cells by continuous passage in the presence of sub-lethal doses of ABC294640. The drug sensitivities of HCT-116R and the parental HCT-116 cells to ABC294640 were determined using the CCK8 cytotoxicity and caspase 3/7 assays. As shown in Figure 2 A, the half maximal inhibitory concentration of ABC294640 treatment in HCT-116 cells was 260 nM, whereas ABC294640 did not affect HCT-116R cell viability ($p < 0.001$). Consistently, ABC294640 increased the level of caspase 3/7 activity in HCT-116 cells $p < 0.001$ and $p < 0.0001$ while showing no effect in HCT-116R cells (Figure 2 B).

To investigate the effect of BMX on CRC resistance to ABC294640, the expression of BMX was evaluated in drug-resistant cells and drug sensitive cells by qPCR and Western blotting. Both protein and mRNA expression levels of BMX were significantly higher in HCT-116R, LS174T, and WiDr compared to HCT-116 cells ($p < 0.0001$; Figure 2 C and D).

Inhibition of BMX augment ABC294640 potency in CRC

To clarify the role of BMX in CRC resistance to ABC294640, HCT-116R, LS174T and WiDr cells were treated with ABC294640 in the presence or absence of siRNA against BMX (siBMX). Co-treatment with siBMX and ABC294640 significantly inhibited the cell survival of CRC compared to each single treatment group ($p < 0.001$ and $p < 0.0001$; Figure 3A). In addition ABC294640 treatment (3 μM) and siBMX induced apoptosis of CRC cells 48 h post-treatment. Combinational treatment of ABC294640 and siBMX dramatically enhanced apoptosis in CRC cells ($p < 0.001$ and $p < 0.0001$; Figure 3B). The results indicate that BMX is resistance molecule during ABC294640 treatment, and specific inhibition of BMX enhanced ABC294640 sensitivity in CRC cells.

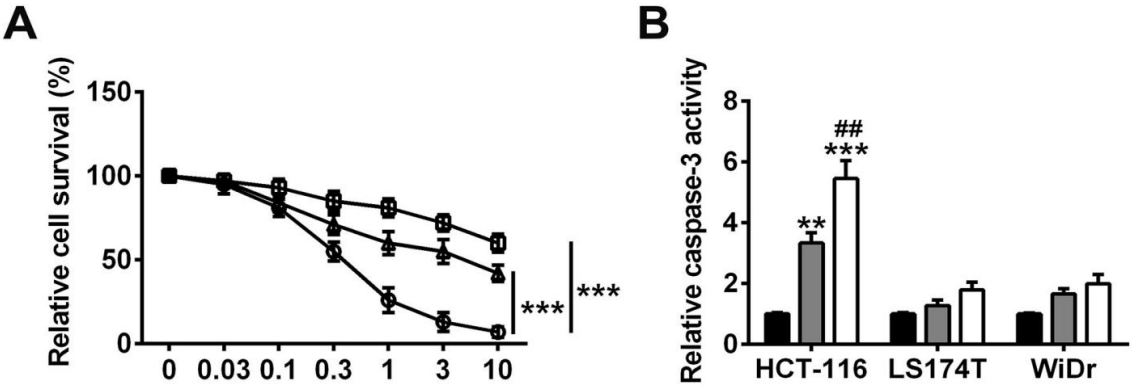

Figure 1: (A) Impact of ABC294640 treatment on CRC cell viability. HCT-116, LS174T, and WiDr cells were treated with ABC294640 (0-10 μM) for 72 h and cell survival was assessed. Relative cell survival rate was represented as a percentage of control group. (B) Effect of ABC294640 on caspase 3/7 activity in CRC cells. Cells were incubated with ABC294640 at 0.3 and 1 μM and caspase 3/7 activity evaluated. DMSO treatment was used as control. ▲: WiDr; ⊟: LS174T; ⊸: HCT-116; ■: DMSO; ▩:0.3 μM; □: 1μM. Data represent the mean ± SD. $^{**}p < 0.01$, $^{\#\#}p < 0.01$, and $^{***}p < 0.001$

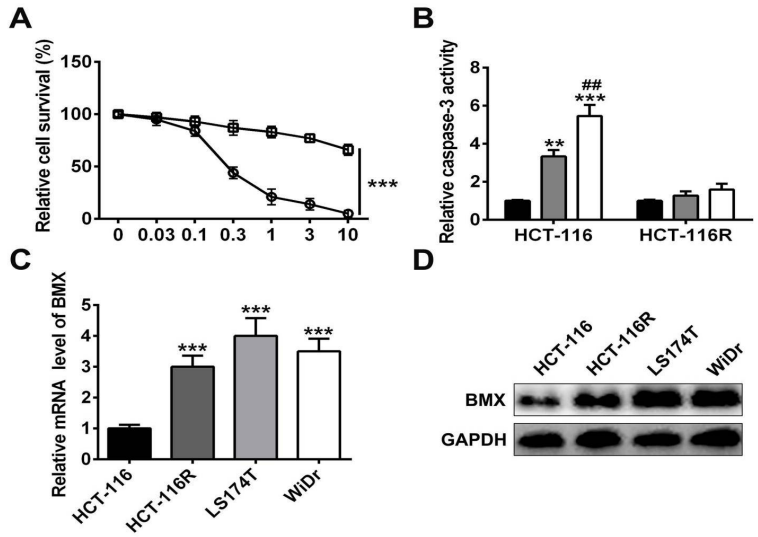

Figure 2: Upregulation of BMX associated with ABC294640 sensitivity in CRC. (A) Effect of ABC294640 on HCT-166 and HCT-166R cell viability. HCT-116 and HCT-166R cells were incubated with ABC294640 (0-10 μM) for 72 h and cell survival was determined. (B) Effect of ABC294640 on caspase 3/7 activity in HCT-166 and HCT-166R. Cells were incubated with ABC294640 at 0.3 and 1 μM and caspase 3/7 activity was evaluated. (C) mRNA levels of BMX in CRC cells were measured by qPCR. (D) BMX protein expression was measured by western blot in CRC cells. ⊟: HCT-166R; ⊖: HCT-116; ■: DMSO; ■:0.3 μM; □: 1μM. Data represent mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{\#\#}p < 0.01$

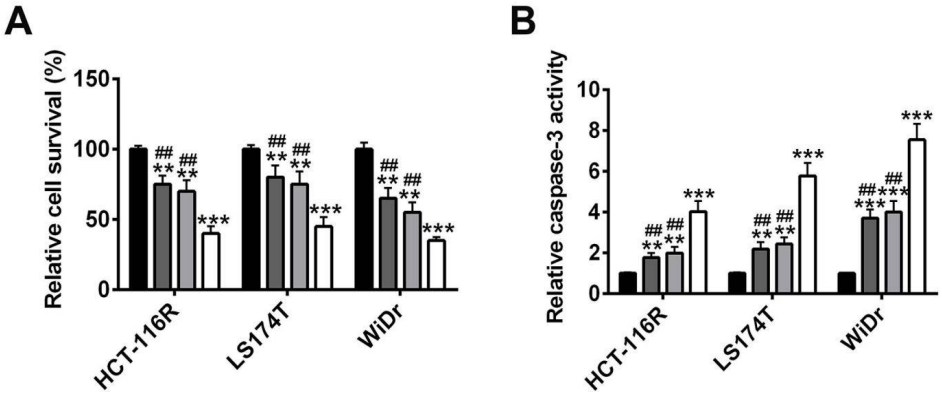

Figure 3: Inhibition of BMX increased ABC294640 potency *in vitro*. CRC cells were incubated with ABC294640 (3 μM) in the absence and presence of BMX siRNA. Cell survival (A) and apoptosis (B) were determined by CCK8 assay and caspase 3/7 activity assay, respectively. ■: DMSO; ■: ABC294640 3 μM; ■: siBMX; □: ABC + siBMX. Data represent mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{\#\#}p < 0.01$

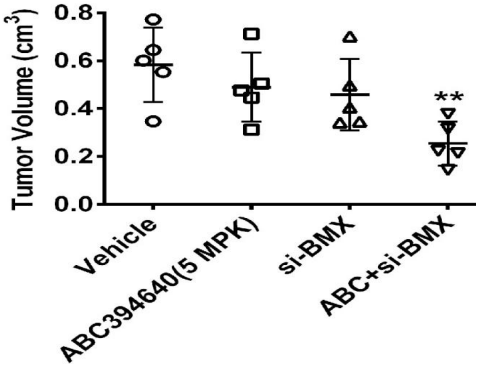

Figure 4: Inhibition of BMX increased ABC294640 potency *in vivo*. HCT-116R cells or HCT-116R in combination with siBMX was injected into the right flanks of nude mice to induce tumors. Tumors were measured with digital calipers to obtain an average size of 100-150 mm^3 followed by randomization into treatment groups. The mice were orally administrated 0.5 % MC (vehicle control) or ABC294640 at 5 mpk, once per day, respectively. Tumors were measured 3 times weekly. Data represent mean ± SD. $^{**}p < 0.01$

BMX inhibition increase ABC294640 potency in CRC *in vivo*

As ABC294640 treatment and knockdown of BMX showed an additive effect *in vitro* in CRC,it was investigated that whether this also occurred *in vivo*. As indicated in Figure 4, neither ABC294640 treatment nor siBMX influenced the HCT-116R CRC xenografts in nude mice when given alone. However, administration of ABC294640 at 5 mpk in combination with siBMX suppressed the growth of HCT-116R tumors ($p < 0.01$).

DISCUSSION

There S1P rheostat determines the fate of tumor cells. Thus far, two isoforms of SK, SK1 and SK2, have been identified in humans [19]. It has been shown that cell proliferation and migration was suppressed by downregulation of SK2, which had a greater affect than SK1 [8]. Recently, phase I clinical data for ABC294640 treatment of solid tumors, a highly specific SK2 inhibitor, have demonstrated variable clinical activity, which may reflect either the lack of biomarkers for patient selection and/or deficiency of knowledge about drug-resistant mechanisms [20]. In the current study, the molecular target that contributes to the limited therapeutic efficacy of ABC294640 in CRC was investigated.

ABC294640 has been extensively studied for the treatment of many human tumor types and animal tumor models including CRC. ABC294640 suppressed cell proliferation and induced cell apoptosis in CRC cells *in vitro*. ABC294640 treatment remarkably inhibited HT-29 tumor growth *in vivo*, suggesting that ABC294640 might be an effective anti-tumor drug in CRC [21]. In the current study, ABC294640 sensitivity in several CRC cell lines was tested, with variable effects among these cell lines, consistent with its clinical activity. This indicates the presence of intrinsic CRC resistance to ABC294640.

Bone marrow kinase in chromosome X is reported to be overexpressed in various tumor types [22]. It also participates in drug resistance as it suppresses the apoptotic regulator BAK activation via phosphorylation, which facilitates the survival of cells subjected to cytotoxic agents [23]. Bone marrow kinase in chromosome X also is suggested to protect prostate cancer cells from photodynamic induced apoptosis [24]. In addition, suppressing BMX expression reduced the chemo-resistance of H69/AR cells [25]. In this study, BMX mRNA and protein expression were increased in ABC294640-resistant CRC cells and silencing BMX down-regulated cell sensitivity to ABC294640 in ABC294640-resistant cells. Further, knockdown of BMX enhanced the effect of ABC294640 treatment on tumor growth both *in vitro* and *in vivo*. These findings suggest that BMX upregulation contributes to the resistance of CRC to ABC294640 therapy.

CONCLUSION

These findings demonstrate that BMX upregulation impedes the antitumor effect of ABC294640 in CRC cells, and knockdown of BM augments ABC294640 ability to suppress the tumor growth of CRC *in vitro* and *in vivo*. Therefore, these data may present a novel therapeutic strategy for the CRC therapy using a combination of ABC294640 treatment and BMX blockade.

DECLARATIONS

Conflict of Interest

The authors declare that no conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zhoufeng Chen designed all the experiments and revised the paper. Ruifang Jin and Haibo Xue performed the experiments, Ruifang Jin and Mengjun Chen wrote the paper.

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