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DBeQ-mediated pharmacological modulation of the retrotranslocation step of ERAD may exhibit a potent therapeutic approach against colorectal cancer

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ABSTRACT

The incidence of colorectal cancer is 30% higher in men compared to women. Although there are different treatment options for colorectal cancer, including chemotherapy and immunotherapy, acquired drug resistance and some specific mutations substantially restrict the treatment options. N(2), N(4)-dibenzylquinazoline-2,4-diamine (DBeQ) is a selective and ATP-competitive inhibitor molecule of p97/Valosin-containing protein (VCP) protein. p97/VCP is a well-conserved and abundant hexameric type II ATPases associated with diverse cellular activities (AAA+) type ATPases protein. It functions as an ATP-dependent segregase and plays a role in various cellular processes, such as autophagy and endoplasmic reticulum-associated degradation (ERAD). Herein, we evaluated the therapeutic potential of DBeQ on colorectal cancer cells, Caco-2 and HT-29. Our data indicated that DBeQ treatment strongly reduced the proliferative capacity, colonial growth and anchorage-independent growth of colorectal cancer cells. Moreover, DBeQ strongly increased cytochrome-c and CCAAT-enhancer-binding protein homologous protein (CHOP) protein levels and also induced cleaved caspase-3 and caspase-7 levels. Present findings suggest that DBeQ may offer a potential therapeutic effect for colorectal cancer treatment.

Keywords: Anti-tumorigenic, colon cancer, DBeQ, ER-associated degradation, p97/VCP

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INTRODUCTION

Colorectal cancer begins to spread from the large intestine or the rectum. It is called colon or rectal cancer, depending on where it starts[1]. It can be caused by inherited or acquired mutations and certain types of diets that may lead to activating oncogenes or turning off tumor suppressor genes. The incidence rates of colorectal cancer are 30% higher in men than in women. The lifetime risk of developing colorectal cancer is about 4.3% for men and 4% for women. Although there are different treatment options for colorectal cancer, including radiation therapy, surgery, chemotherapy, targeted therapy and immunotherapy, acquired drug resistance and some specific mutations substantially restrict the treatment options for colorectal cancer [2, 3]. Therefore, there is a need to improve our understanding of the molecular mechanisms underlying colorectal cancer and the development of effective new treatment approaches.

N(2), N(4)-dibenzylquinazoline-2,4-diamine (DBeQ) is a selective, reversible and ATP-competitive inhibitor molecule of p97/Valosin-containing protein (VCP)[4]. DBeQ debilitates autophagy and the destruction of the endoplasmic reticulum-associated degradation (ERAD) substrates[5]. ERAD is a main quality-control mechanism responsible for targeting unfolded, misfolded or improper oligomerized endoplasmic reticulum (ER) proteins for proteasomal degradation[6]. Recent studies highlighted that mammalian ERAD has been associated with the progression of carcinogenesis, including prostate cancer[7]. ERAD is a sophisticatedly controlled physiological mechanism consisting of multiple steps, including recognition and ubiquitination of substrate molecules, retrotranslocation from ER lumen to cytosol and proteasomal-mediated degradation. A large number of proteins responsible for the functioning of these steps work simultaneously in a synchronized manner[5,6]. The retrotranslocation step, which is responsible for the transfer of substrate molecules from the ER lumen to the cytosol, is extremely critical and this function is mainly coordinated by the p97/VCP in yeast and mammalian[8]. p97/VCP is a well-conserved, ubiquitously localized and abundant expressed hexameric type II AAA+ (ATPases associated with diverse cellular activities) type ATPases protein[8]. It functions as an ATP-dependent segregase and also plays a crucial role in cellular processes, including transcriptional control, cell-cycle regulation, endosomal differentiation, homotypic membrane fusion, autophagy and ERAD[8,9].

In recent studies, it has been better understood that protein quality control mechanisms, such as ERAD supported the carcinogenesis process by exhibiting varying activities in many cancer types, such as breast and prostate cancer.

In the present study, we aimed to investigate the therapeutic potential of phar-

macological inhibition of p97/VCP on colorectal cancer cells by DBeQ. For this purpose, we used human epithelial colorectal adenocarcinoma cells Caco-2 and HT-29, which mimic colorectal cancer well *in vitro*. Our findings indicated that DBeQ treatment strongly reduced the proliferative capacity of colorectal cancer cells. Also, colony formation and anchorage-independent growth of colorectal cancer cells were significantly reduced by DBeQ in a dose-dependent manner. Lastly, we analyzed some cell death-related protein levels, including cytochrome-c, CHOP (CCAAT-enhancer-binding protein homologous protein), full- and cleaved caspase-3 and -7 levels by immunoblotting to understand the mechanism of anti-cancer effect of DBeQ on colorectal cancer cells at the molecular level. Our data indicated that DBeQ treatment remarkably increased the expression levels of cytochrome-c and CHOP and also induced cleavage of caspase-3 and caspase-7 proteins. These findings suggest that pharmacological modulation of protein quality control components may offer a potent therapeutic approach against colorectal carcinoma.

METHODOLOGY

Materials

Cell culture materials, including L-Glutamine, fetal bovine serum (FBS) and other cell culture grade requirements were obtained from Capricorn Scientific (Capricorn Scientific GmbH, Ebsdorfergrund, Germany). McCoy's 5a Medium and Eagle's Minimum Essential Medium (EMEM) were purchased from Biological Industries (Biological Industries, USA). DBeQ (sc-499943) was purchased from Santacruz Biotechnology (Santa Cruz Biotechnology, Inc. California, USA). Rabbit polyclonal antibodies caspase-3 (#9662)(1:1500), caspase-7 (#12827)(1:1500), cytochrome c (#11940)(1:2000) and mouse polyclonal CHOP (#2895)(1:2000) were obtained from Cell Signaling Technology (Cell Signaling Technology Inc., Danvers, Massachusetts, USA). Monoclonal mouse beta-actin antibody (#A5316)(1:10000) was obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA). HRP-conjugated secondary goat anti-mouse antibody (#31430)(1:5000) and goat anti-rabbit antibody (#31460)(1:5000) were purchased from Thermo Scientific (Thermo-Scientific Pierce, Fisher Scientific, Dublin, Ireland). High pure Dimethyl Sulfoxide (DMSO) was obtained from Serva (SERVA Electrophoresis GmbH, Heidelberg, Germany).

Cell Culture

Human epithelial colorectal adenocarcinoma cell lines Caco-2 (HTB-37™) and HT-29 (HTB-38™) were obtained from American Type Culture Collection (ATCC) (ATCC, Rockville, MD). Caco-2 and HT-29 cells were routinely pro-

pagated in EMEM and McCoy's 5a Medium enriched with 10% FBS and 2 mM L-glutamine in a conventional cell culture condition, the humidified atmosphere of 5% CO_2 and 95% air at a constant temperature of 37°C, respectively. Identification of mycoplasma infection was evaluated by EZ-PCR Mycoplasma Test kit (Biological Industries, USA).

Cell viability test

The cell viability was measured by WST-1 assay according to the manufacturer's instructions (TaKaRa, Mountain View, CA, USA). The cells were seeded in 96-well plate (7500 cells/well) and growth in regular media for 24 h. Following cells were treated with DBeQ as indicated doses for 48 h. To determine the cell proliferation, 20 µL WST-1 was added per well and cells were incubated for 2 h under the conventional cell culture conditions at 37°. The absorbance values were read at 450nm, with 600nm set as the reference wavelength by using microplate reader (BioTek, Epoch 2).

Western-blotting

Immunoblotting analysis was performed as described before[7]. Cells were lysate by radioimmunoprecipitation assay (RIPA) buffer and then centrifugated at 14.000 rpm for 20 min at 4°C. The insoluble phase was removed and supernatant was collected. The total concentration was determined by bicinchoninic acid assay (BCA) kit (TaKaRa, Mountain View, CA, USA). Protein samples were mixed with 4x Laemmli buffer and boiled at 70°C for 15 min. Typically, 30 µg of total protein was loaded in the hand-cast sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to an Immun-blot® polyvinylidene fluoride (PVDF) membrane for 24 h (Bio-Rad, Hercules, CA, USA). Target proteins were marked by specific primary antibodies and HRP-conjugated secondary antibodies and then visualized by using clarity western enhanced chemiluminescence (ECL) substrate in ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, USA).

Colony formation assay

Cells were seeded in a 6-well cell culture plate (2000 cells/ml) and propagated at conventional cell culture conditions for 24 h and then cells were treated with a compound. Following 72 h, colonies were fixed and stained with %0.05 crystal violet solution (MERCK, Darmstadt, Germany). Quantification of growing colonies were analyzed by using ImageJ software (http://imagej.nih. gov/ij/).

3D cell culture

3D cell culture analysis was performed as described before[7]. Cells were embedded in low melting agar enriched with growth media and treated with compound after 24 h. Cells were propagated under conventional cell culture conditions for 14 days and then the images of growing colonies were taken using Sunny SopTop invert microscope and OD400UHW camera system. % colonial growth was calculated by counting the colonies in the photographs taken from 5 independent areas and comparing them between the groups.

Statistical Analysis

Results are presented as mean ± standard deviation and analyzed by using GraphPad Prism 5 software. The statistical significance of differences between groups was determined by a two-tailed equal variance Student's t-test with a minimum of 95% confidence interval. The significant level was set at 5% ($p<0.05$) for all tests.

RESULTS and DISCUSSION

The evaluation of the effect of DBeQ on cell viability of Caco-2 and HT-29 cells

To evaluate the effect of DBeQ on the cell-viability of Caco-2 and HT-29 cells, cells were treated with various doses of DBeQ, including 1, 2.5, 5, 7.5, 10, 12.5 and 15 μmol for 48 h and then cell viability was measured with WST-1 based cell viability assay. Our results indicated that DBeQ treatment significantly decreased cell viability for both cell lines in a dose-dependent manner (Figure 1). We also determined the IC_{50} value of DBeQ, 9.201 μmol for Caco-2 and 7.625 μmol for HT-29 cells (Figure 1).

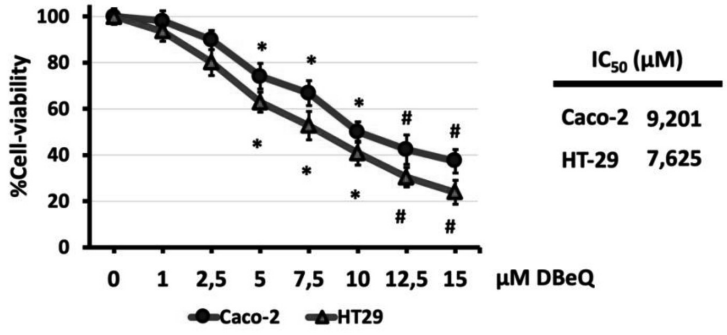

Figure 1. Evaluation of the effect of DBeQ on cell-viability in Caco-2 and HT-29 cells

Cells were treated with a vehicle or 1, 2.5, 5, 7.5, 10, 12.5 and 15 μmol DBeQ for 48 h. Cell viability was analyzed by WST-1 based assay. Data represented as mean ± SE of three independent experiments made in three replicates (*p<0.05, # p<0.001).

DBeQ remarkably reduced the colony formation ability of Caco-2 and HT-29 cells

To evaluate the effects of DBeQ on the tumorigenic capacity of colon cancer cells, we performed the 2D colony formation assay. Caco-2 and HT-29 cells were treated with IC_{50} and $^1/_2$ IC_{50} doses of DBeQ. Our findings indicated that DBeQ administration significantly reduced the colonial growth of both cell lines in a dose-dependent manner (Figure 2a, b). Moreover, we observed that HT-29 cells were more sensitive to the DBeQ than Caco-2 cells.

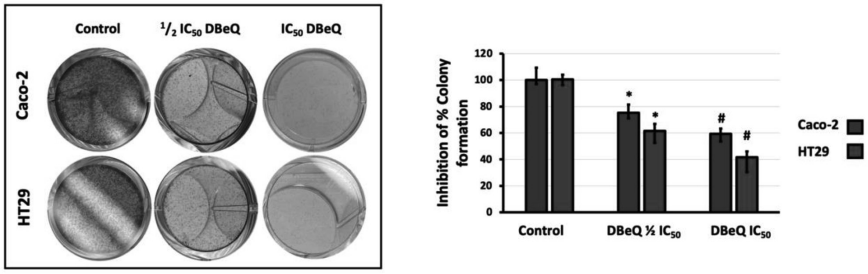

Figure 2. The effect of DBeQ on colony formation of Caco-2 and HT-29 cells

Cells were treated with vehicle or IC_{50} and $^1/_2$ IC_{50} doses of DBeQ for 72 h and the cells were fixed and stained with crystal violet solution. % inhibition of colonial growth was calculated by ImageJ software. Data represented as mean ± SE (n=3) (* p<0.05, # p<0.001).

3D tumor formation of Caco-2 and HT-29 cells is markedly reduced by DBeQ treatment

Anchorage-independent growth is considered one of the tumor hallmarks[13]. Our findings indicated that DBeQ administration significantly limited the 3D tumor formation of Caco-2 and HT-29 cells. Also, our data revealed that tumor volume was remarkably minimized by DBeQ treatment for both cell lines in a dose-dependent manner compared to the control group (Figure 3).

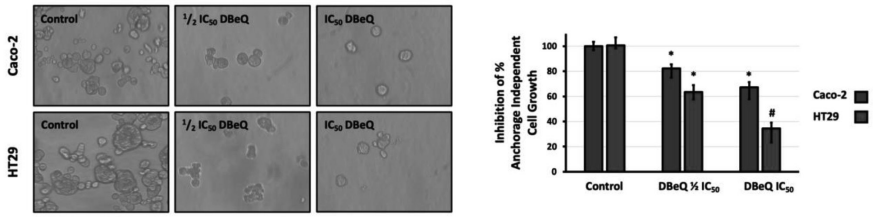

Figure 3. The effect of DBeQ on anchorage-independent cell growth in Caco-2 and HT-29 cells

3D cell culture protocol was performed as explained in the material-method section. Cells were treated with vehicle or IC_{50} and $^{1}/_{2}$ IC_{50} doses of DBeQ. Data represented as mean ± SE of three independent experiments made in three replicates (* $p<0.05$, # $p<0.001$).

Investigation of the effect of DBeQ on cell death-associated proteins in Caco-2 and HT-29 cells

To evaluate the impact of DBeQ on cell death-related proteins in Caco-2 and HT-29 cells, the protein levels of cytochrome-c, CHOP, full and cleaved forms of caspase-3 and caspase-7 were examined by immunoblotting assay. Our data indicated that DBeQ dose-dependently induced cleavage forms of caspase-3 and caspase-7 in both cell types (Figure 4). Although the expression level of CHOP protein was increased in both cell lines depending on the applied doses of DBeQ, it was more strongly induced in HT-29 compared to Caco-2 cells in a dose-dependent manner (Figure 4). Moreover, cytochrome-c levels were gradually increased depending on the dose of DBeQ in both cells.

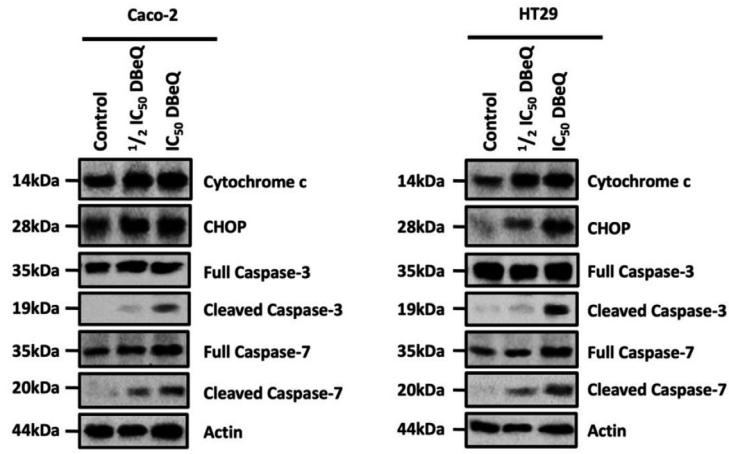

Figure 4. Investigation of the effects of DBeQ on cell death-related protein levels in Caco-2 and HT-29 cells

Cells were treated with vehicle or IC_{50} and $^1/_2$ IC_{50} doses of DBeQ for 24 h and then expression levels of CHOP, cytochrome-c, full and cleaved caspase-3 and caspase-7 were analyzed by immunoblotting assay. Beta-actin was used as a loading control.

Recent studies suggest that ER protein quality-control mechanism, ERAD is a potent therapeutic target for numerous cancer types, such as breast and prostate cancer[7,10]. ERAD is one of the most effective protein degradation systems in mammalian cells, which is specifically targeting the undesirable proteins to the 26S proteasome[10]. It effectively regulates the steady-state level of numerous physiologically crucial proteins, such as cholesterol biosynthesis rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase, tumor suppressor Kangai-1 (KAI1)/Cluster of Differentiation 82 (CD82) and superoxide dismutase 1 (SOD1) and brain and muscle ARNT-like1 (Bmal1)[7,11,14]. Due to the increasing protein quality processes in cancer cells, the pharmacological targeting of ERAD makes it a therapeutically potent target in new treatment approaches.

Herein, we tested the anti-tumorigenic effect of DBeQ on colorectal cancer cells, Caco-2 and HT-29. Firstly, we examined the effect of DBeQ on cell viability and determined the IC_{50} doses for both cell types. Our results indicated that HT-29 cell was more sensitive to DBeQ than Caco-2 cells. IC_{50} value of DBeQ for Caco-2 cells is 9.201 μmol and for HT-29 cells is 7.625 μmol (Figure 1). IC_{50} is expressed as the half-maximum inhibitory concentration and is the most widely used and informative measure of the efficacy of a drug[12]. Therefore, in the next experimental steps, studies were continued with 1x and $^1/_2$ x IC_{50} doses of DBeQ.

Increased invasive and migration ability, colonial growth capacity and anchorage-independent cell growth are essential hallmarks of cancer progression[13]. Therefore, we aimed to evaluate the impact of DBeQ on these tumorigenic characteristics by 2D colony formation and 3D anchorage-independent cell growth assay. Our results indicated that DBeQ administration strongly limited the colonial growth of Caco-2 and HT-29 cells in a dose-dependent manner (Figure 2). 3D tumor formation of Caco-2 and HT-29 cells was significantly reduced by DBeQ administration and also tumor volume was importantly minimized for both cell types in a dose-dependent manner (Figure 2). Collectively these results suggest that DBeQ efficiently restricts the tumor progression of colon cancer cells by reducing the tumorigenic characteristics.

Lastly, we examined the anti-tumorigenic effect of DBeQ with studies at the protein level in colorectal cancer cells. For this aim, we evaluated some programmed cell death-related protein levels, including cytochrome-c, CHOP, full and cleaved caspase-3 and caspase-7. Cytochrome-c is localized in the inner membrane of mitochondria and is known as an electron-transporting protein. Also, it plays a pivotal role in oxidative phosphorylation-mediated ATP synthesis in eukaryotic cells[14]. Alteration in the levels of cytochrome-c is mostly used in investigating apoptotic cell death in cells. Upon apoptotic stimulation, cytochrome-c is released from mitochondria to the cytoplasm and causes activation of caspases[15]. Our data indicated that DBeQ treatment increased the cytochrome-c levels in Caco-2 and HT-29 cells in a dose-dependent manner (Figure 4). Consistent with these results, cleaved forms of caspase-3 and caspase-7 levels were markedly induced depending on the doses of DBeQ (Figure 4). Caspase-3 and caspase-7 are effector caspase proteins liable for cleaving downstream substrates, including Poly (ADP-ribose) polymerase 1 (PARP-1)[16–18]. Our results suggest that DBeQ promotes the programmed cell death of colorectal cancer cells by activating caspase-3 and caspase-7.

Additionally, we examined the effect of DBeQ on CHOP protein expression. CHOP is a pro-apoptotic transcription factor and is mainly induced under certain cellular stresses, including nutrient starvation and prolonged ER stress[19]. Our data indicated that DBeQ administration remarkably induced steady-state levels of CHOP protein in a dose-dependent manner in colorectal cancer cells (Figure 4). Together present findings suggest that DBeQ administration strongly induces programmed cell death in colorectal cancer cells. Considering the expanded metabolic requirements in cancer cells, including increased protein synthesis capacity, DBeQ-mediated disruption of ER-protein quality-control processes in colorectal cancer cells suggests the underlying cause of potent anti-cancer properties of DBeQ.

Herein, we showed that the reversible selective inhibitor of p97/VCP, DBeQ, significantly reduced the cell viability of colorectal cancer cells. Also, we found it remarkably decreased tumorigenic features of colorectal cancer cells, such as colony formation and anchorage-independent growth by inducing programmed cell death. Present findings suggest that pharmacologically targeting the ER protein quality-control mechanism components may present a promising therapeutic approach against colorectal cancer.

STATEMENT OF ETHICS

This study does not require any ethical permission.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Y. ERZURUMLU initiated, directed the project, designed and conducted the experiments, analyzed, interpreted the results and wrote the manuscript. H.K. DOGAN assisted experimental studies. All correspondence and requests for materials should be addressed to Y.E. All authors have read and approved the final version of the manuscript.

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