DZNep Does Not Influence Autophagy Gene Expression in the Colon Cancer Cell Line HT-29

DZNep Kolon Kanseri Hücre Hattı HT-29'da Otofaji Gen İfadelerini Etkilememektedir

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ABSTRACT

Objective: Autophagy (AP) is a well-preserved cell survival mechanism that plays a critical role by degrading dysfunctional organelles and misfolded proteins. There are a limited number of studies examining the relationship between 3-Deazaneplanocin A hydrochloride (DZNep) and autophagy. The purpose of this preliminary study was to assess the effect of DZNep on the expression of the AP-related genes Beclin-1, Ulk1, Ulk2 and Uvrag in HT-29 colorectal adenocarcinoma cells.

Material and Methods: HT-29 human colorectal adenocarcinoma cells were plated into growth medium. The next day, 25 µM DZNep, an inhibitor of S-Adenosylmethionine-dependent methyltransferase, was added to the cells. DZNep were removed from the cells after 4 days by refreshing the media. The cells were maintained in a tissue culture incubator. Total RNA isolation was performed from DZNep treated and untreated HT-29 cells 4 days after DZNep application. cDNAs were reverse transcribed from 2 µg of DNase treated RNA. cDNAs were then analyzed by quantitative PCR. The results were presented as relative expression level in HT-29 cells.

Results: DZNep induced extreme cell death on HT-29 colorectal cancer cell lines; more than 50% of the colorectal cancer cells were dead. The expression level of Beclin-1 was the smallest while Ulk1 and Uvrag expression levels were higher than that of Beclin-1, and Ulk2 had the highest expression level in untreated HT-29 colorectal cancer lines.

Conclusion: The autophagy related genes studied here consisting of Beclin-1, Ulk1, Ulk2, and Uvrag were not affected by DZNep application. Based on mRNA expression levels of AP genes, administration of DZNep does not stimulate cell toxicity through autophagy in the HT-29 cells.

Key Words: 3-Deazaneplanocin A, Autophagy, HT-29, Colon carcinoma, Gene expression

ÖZ

Amaç: Otofaji (AP), fonksiyonel olmayan organelleri ve hatalı katlanmış proteinleri degrade ederek, hücrenin hayatta kalmasını sağlayan iyi korunmuş bir mekanizmadır. 3-Deazaneplanocin A hydrochloride (DZNep) ve otofaji arasındaki ilişkiyi inceleyen az sayıda çalışma bulunmaktadır. Bu öncül çalışmanın amacı, DZNep'in kolon kanseri hücre hattı HT-29'da otofaji ile ilişkili genler olan, Beclin-1, Ulk1, Ulk2 ve Uvrag'a olan etkilerini araştırmaktır.

Gereç ve Yöntemler: HT-29 insan kolorektal adenokarsinom hücreleri kültür ortamına ekildi. Ertesi gün, S-Adenosylmethionine bağımlı metiltransferaz inhibitörü olan 25 µmol DZNep, hücre kültürüne ilave edildi. DZNep, dört gün sonra kültür ortamı değiştirilerek hücrelerden uzaklaştırıldı. Hücreler deney süresince doku kültürü inkübatöründe tutuldu. DZNep uygulamasının 4. gününde, DZNep uygulanmış ve uygulanmamış hücrelerden total RNA izolasyonu yapıldı. cDNA'lar, DNaz uygulanmış 2 µg RNA'dan ters transkriptaz ile sentezlendi. cDNA'lar daha sonra kantitatif polimeraz zincir reaksiyonu ile analiz edildi. Sonuçlar, HT-29 hücrelerinde relatif ekspresyon düzeyleri şeklinde sunuldu.

Bulgular: DZNep, HT-29 kolorektal kanser hücrelerinin %50'sinden fazlasını öldürerek ekstrem hücre ölümünü indükledi. Otofaji genlerinin ifadeleri şu şekildeydi; Beclin-1 için hafif, Ulk1 ve Uvrag için orta ve Ulk2 için yüksek.

Sonuç: Burada çalışılan bazı otofaji genleri; Beclin-1, Ulk1, Ulk2, Uvrag, DZNep uygulamasından etkilenmemiştir. DZNep uygulaması sonucunda, HT-29 hücrelerinde meydana gelen hücre ölümü otofaji aracılı değildir.

Anahtar Sözcükler: 3-Deazaneplanocin A, Otofaji, HT-29, Kolon karsinomu, Gen ifadesi

INTRODUCTION

Autophagy is a well-preserved cell survival mechanism that plays a critical role by degrading dysfunctional organelles and misfolded proteins. On the other hand, it is not a simple cell survival mechanism and might play opposite roles depending on the cellular microenvironment. There are four known types of autophagy: macroautophagy, selective autophagy, chaperone-mediated autophagy and microautophagy. Formation of autophagosomes is related to macroautophagy which is controlled by specific genes such as *Ulk1*, *Ulk2*, and *Beclin-1*. Besides, *Uvraq* has also been demonstrated to regulate AP by at least four different mechanisms (1). The role of AP in tumor formation has been well demonstrated in studies (2). As AP is an integral part of cancer cell survival, it also contributes to the escape of tumor cells from chemotherapy and radiotherapy-induced cell death. The influence of AP in drug- and radio-resistance has been established in many cancer cell lines (3-5). AP facilitates chemoresistance development by blocking apoptosis (6).

Colorectal cancer (CRC) remains a significant life-threatening malignancy despite numerous therapeutic approaches,. A relationship between colorectal cancer and authophagy has been demonstrated. The role of AP in the development of colorectal cancer is especially due to dysregulation of the cell death pathways (7).

Resistance against chemotherapy is an essential complication in CRC therapy (8). To increase the response rate, new additional targets that contribute to chemoresistance are still needed. 3-Deazaneplanocin A (DZNep) is an S-adenosylhomocysteine synthesis and histone methyltransferase EZH2 inhibitor that has a wide range of effects in various human cancers. In recent studies, DZNep has also been shown to have an impact on apoptosis in CRC cell cultures (9). However, the relationship of AP-related genes in DZNep-treated CRC cells remains to be elucidated.

The aim of this preliminary study was to assess the effect of DZNep on the expression of the AP-related genes *Beclin-1*, *Ulk1*, *Ulk2* and *Uvrag* in HT-29 CRC cells.

MATERIAL and METHOD

Cell culture and DZNep treatment

HT-29 human colorectal adenocarcinoma cells (ATCC, Manassas, VA, USA) were plated ($3x10^5$ cells/well) into 6-well tissue culture plates (Corning, Corning, NY, USA) with growth medium comprising high glucose DMEM (Gibco, Waltham, MA, USA), 10% FBS (Gibco), 1X L-glutamine (Gibco), 1X NEAA (Gibco), and 1X antibiotic-antimycotic (Gibco). The next day, 25 µM 3-Deazaneplanocin A hydrochloride (DZNep) (Sigma, St Louis, MO, USA), an inhibitor of S-Adenosylmethionine-dependent methyltransferase, was added to the cells. DZNep was removed from the cells after 4 days by refreshing the media. The cells were maintained in a tissue culture incubator at 37°C with 5% CO_2.

MTT (Cell Viability Assay)

HT-29 cells (10^4 cells/well) were plated into 96-well plates. Following the application of 25 µM DZNep, MTT solution (Millipore, MA, USA) (5 mg/ml) was added into each well on day 4. After incubation at 37 °C for 4 hours, the medium was removed and DMSO was added into each well to dissolve the purple formazan. Formazan absorbance was measured at 570 nm. Based on the untreated control cells, the percentage of viable cells was calculated by using the absorbance values. The experiments were conducted with three biological and three technical replicates. Statistical analysis was carried out with Student's t-test on the SPSS 19.0 program. Statistical differences between groups were identified as significant at $P<0.05$.

Quantitative polymerase chain reaction (qPCR)

Total RNA isolation was performed from DZNep-treated and -untreated HT-29 cells 4 days after DZNep application by using a Purelink RNA mini kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions. cDNAs were reverse transcribed from 2 µg of DNase treated RNA by using a Maxima first strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA) as per the manufacturer's directions. cDNAs were then analyzed by quantitative PCR in a Light Cycler 96 system (Roche, Basel, Switzerland). The qPCR mixture was prepared as 20 µL comprising KAPA SYBR FAST qPCR master mix (KAPA Biosystems, Wilmington, MA, USA), forward and reverse primers (Table I), and ~20 ng of cDNA. qPCR conditions were as follows: 95°C for 3 min initial denaturation, 95°C for 10 s denaturation, 60°C for 30 s annealing/extension with 40 cycles performed overall. Relative quantification was calculated with $2^{-\Delta\Delta Ct}$ and normalized to *Beta actin* expression. The results were presented as relative expression level in HT-29 cells.

qPCR experiments were repeated for three times with two technical replicates each time. Statistical analysis was carried out with Student's t-test on the SPSS 19.0 program. Statistical differences between groups were identified as significant at $P<0.05$.

This study is an experimental research study and was carried out in a laboratory environment. No animal material was used. The HT-29 human cell line used here was purchased from ATCC. Therefore, the approval of the ethics committee was not needed. In addition, the work was carried out by the relevant authorities and no informed consent was required.

Table I: Primers used in qPCR.

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'
Beclin-1	TCCGGGCTCCCGAGG	TTCCTCCTGGGTCTCTCCTG
Ulk1	GTTCCAAACACCTCGGTCCT	GGACCTGAAGACAAGGCAGAT
Ulk2	ATTGAGAGAAGACTGTCGGCG	TCCCCTCTTCCTCACGTTCC
Uvrag	GATCGAGATGAGCGCCTCC	TAACAATGTTCCGGGCAGCA
Actb	GCCTCGCCTTTGCCGA	GGAATCCTTCTGACCCATGC

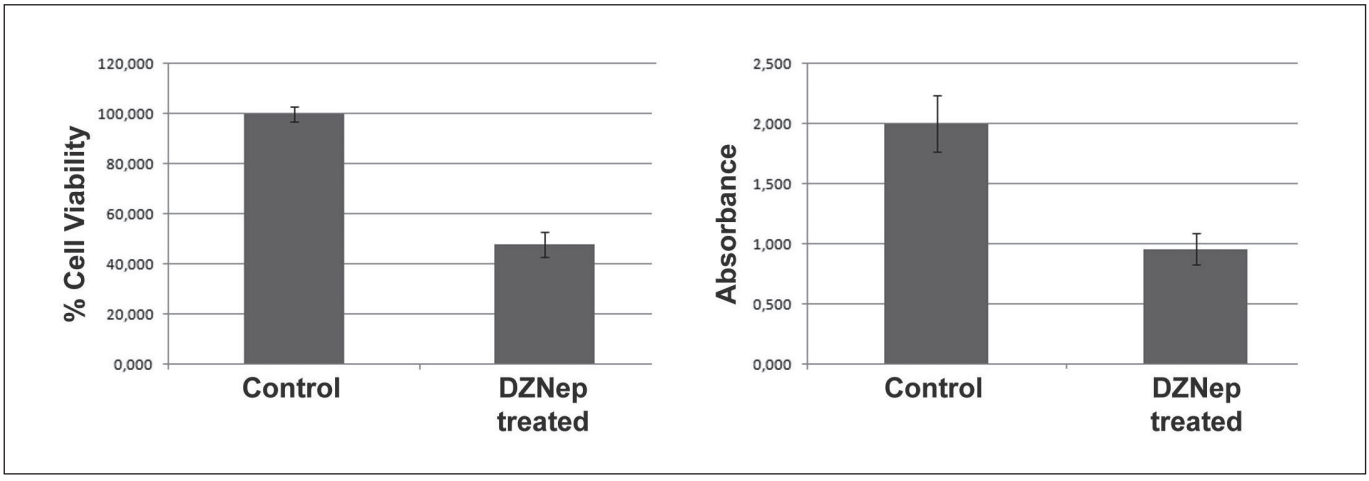

Figure 1: Cell viability assay (MTT). Absorbance of DZNep treated and untreated HT-29 cells (right panel). Percentage of cell viability (left panel) of DZNep treated and untreated HT-29 cells. Data are represented as mean value, (n=3).

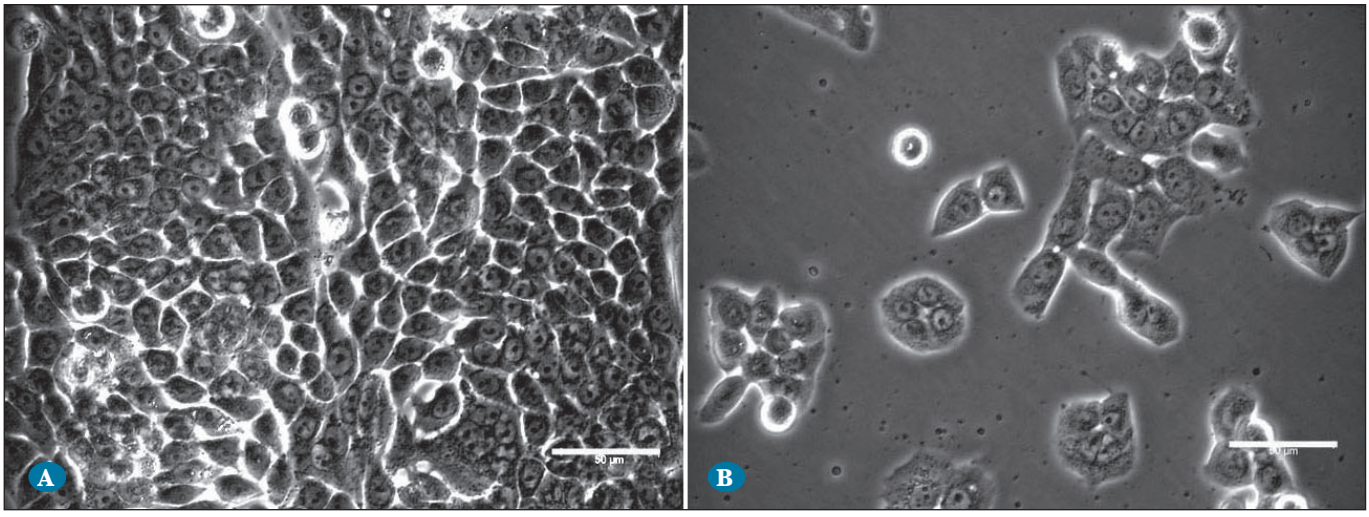

Figure 2: Toxic effect of DZNep on HT-29 cell line. **A)** Control group, HT-29 cells with no DZNep application. **B)** HT-29 cells 4 days after DZNep application. Bar is 50µm.

RESULTS

DZNep application induced extreme cell death on the HT-29 colorectal cancer cell lines and more than 50% of the colorectal cancer cells were dead (Figure 1, 2A,B). DZNep application significantly reduced the cell viability. However, this application had no effect on AP gene expressions. To investigate the relationships between AP and cell death, we investigated the expression level of AP-related genes (such as *Beclin-1*, *Ulk1*, *Ulk2* and *Uvrag*) in DZNep-treated and -untreated HT-29 cell lines by using quantitative real-time PCR. The expression level of *Beclin-1* was the smallest while *Ulk1* and *Uvrag* expression levels were higher than for *Beclin-1*, and *Ulk2* had the highest expression level in untreated HT-29 colorectal cancer lines. We demonstrated that DZNep application did not change the expression levels of *Beclin-1*, *Ulk1* and *Ulk2* ($p>0.05$). However, the expression level of *Uvraq* was increased in DZNep-treated HT-29 cells ($p<0.05$) (Figure 3).

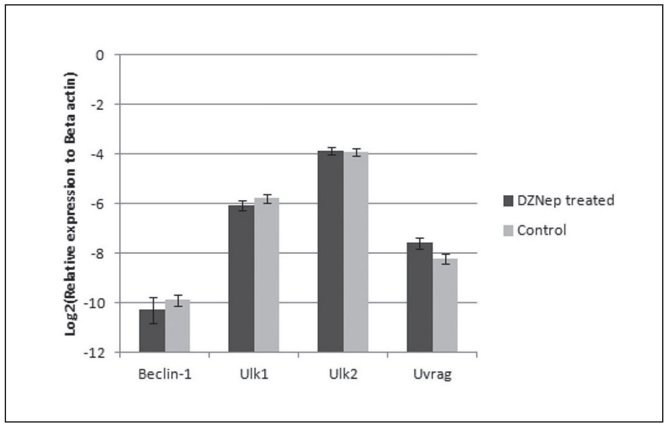

Figure 3: Expression of autophagy genes in HT-29 cell line in the presence of DZNep.

DISCUSSION

AP has a critical role in cancer survival under mild conditions (10, 11). In our study, we showed that *Ulk1* and *Ulk2*, which are essential for AP induction through their kinase activity by phosphorylating *Beclin-1* (12), are already expressed at a moderate level in the HT-29 cell line.

This result suggests that AP is in progress at least partially. Moreover, low level expression of *Beclin-1* suggests that Ulk/Atg1 kinase activity might have other targets for AP activation as Nazarco & Zhong stated (12).

Alterations in DNA methylation, histone modifications and nucleosomal occupancies are silencing tumor-suppressing mechanisms in cancer. DZNep non-selectively inhibits histone methylation and also reactivates silenced genes in cancer cells (13). EZH2, which is the target for DZNep, is associated with tumor proliferation, metastasis, and a poor prognosis. Targeting EZH2 can be an effective therapeutic strategy for colon cancer. Sha et al. demonstrated that DZNep can inhibit the growth and survival of colon cancer HCT116 cells by inducing cellular senescence and apoptosis (9). This study is also consistent with our results in which DZNep showed a toxic activity on HT-29 cell line. Yao et al, showed that decrease of EZH2 also exerts an influence on cell cycle, proliferation and migration of CRC cells (14). Even though Yao et al. demonstrated a relationship between autophagy and EZH2 downregulation, that relationship needs to be fully elucidated in the colorectal cancer cell line HT-29 (14). In our study we investigated the activity of AP in the presence of DZNep. However, although DZNep had a toxic effect (Figure 1, 2) on this colon cancer cell line, we did not see a relation between the AP mechanism and HT-29 cell death.

CONCLUSION

There is a limited number of studies evaluating the DZNep and authophagy relationship. Here we investigated whether AP plays a role in DZNep toxicity on HT-29 cell lines by looking at the expression level of some but not all AP-related genes; *Beclin-1*, *Ulk1*, *Ulk2*, *Uvrag*. These genes, and thereby AP, were demonstrated to be functioning in the HT-29 cell line, whereas DZNep application may not stimulate cell death through AP in this cell line. However, it should be noted that autophagic activity cannot be evaluated solely by the mRNA expression of AP-related genes. Levels of autophagy-related proteins (such as LC3) should also be evaluated in further studies to clarify the relationship between AP and DZNep.

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