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The therapeutic potential of targeting HDAC6 with Tubastatin A in TFK-1 and EGI-1 cholangiocarcinoma cells

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Abstract

Cholangiocarcinoma (CCA) is a highly aggressive and invasive malignancy with a poor diagnosis because of the resistance, relapse and limited therapy. Histone deacetylases (HDAC) are a class of enzyme that have important roles in epigenetic modulations. These enzymes are intensely studied and HDAC inhibitors are considered as potent anticancer agents in both solid tumors and hematological malignancies. HDAC inhibitors can affect and induce different mechanisms such as cell cycle arrest, differentiation, and cell death. In this study, we aim to investigate the cytotoxic effect of Tubastatin A, which is a selective HDAC6 inhibitor, on cholangiocarcinoma cell lines, TFK-1 and EGI-1, by MTT assay. Besides, it was aimed to examine the impact on colony formation potential of the cells. The effect of the inhibitor on cell cycle distribution was also examined by using flow cytometry. Tubastatin A has significantly decreased the colony formation and changed cell cycle progression. Taken together, our results suggest that Tubastatin A could be a potent inhibitor against cholangiocarcinoma. On the basis of these results, further mechanistic studies are required to elucidate the antineoplastic activity of Tubastatin A.

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1. Introduction

Cholangiocarcinoma is a malignancy that is originating from biliary duct. It stands for the 10-20% of all liver cancers and it is the second most seen hepatic malignancy [1]. The process of development of CCA involves various mechanisms including alterations in oncogenic signaling pathways, genetic changes, epigenetic mechanisms and chromosomal abnormalities. [2].

Epigenetic modifications have crucial roles in tumor progression. These epigenetic changes include DNA methylation and histone modifications, such as acetylation, and regulates important events such as chromatin structure remodeling and regulation of gene expression [3]. In particular, the enzyme histone deacetylase (HDAC), which removes the acetyl group from histone proteins surrounding DNA, makes DNA less reachable to transcription factors. By doing so, it is considered as an important epigenetic regulator for chromatin remodeling and gene expression [4]. Therefore, any dysregulation in the function of HDACs in cancer cells can lead to suppression of genes involved in the regulation of important cellular events such as cell differentiation, angiogenesis, apoptosis and cell proliferation [5]. Histone deacetylases are highly expressed in both normal cholangiocytes and cholangiocarcinoma cells [6].

There are different members of HDAC family of enzymes [7]. HDAC6 is a unique cytoplasmic enzyme that was shown to interact with and deacetylate tubulin, and then in turn having a regulatory role in cell migration [8]. The overexpression of HDAC6 has been identified in a variety of other cancer cell lines and mouse tumor models and induce oncogenic cell transformation [9]. HDAC6 is an isoform of HDACs that is considered as enhancing CCA cell growth [10].

Selective HDAC6 inhibitors, such as ACY1215 and tubastatin-A, significantly inhibited CCA cell growth both in vitro and in vivo [11,12]. Tubastatin A is a selective HDAC6 inhibitor that has shown promising effects for cancer therapy in many studies. Tubastatin A reduced cell migration and colony-forming capacity of cells in glioblastoma cells and showed a reversing

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effect of epithelial-to-mesenchymal transition [13]. Tubastatin A was investigated on CCA in the aspect of primary cilia formation in malignant transformation and CCA animal model was used to demonstrate the decrease in tumor growth. Different CCA cell lines were used to demonstrate the decrease in cell proliferation upon pharmacological inhibiton of HDAC6 with Tubastatin A [11].

In the current study, it is aimed to investigate the effects of HDAC6 inhibition in cholangiocarcinoma cells by using a selective inhibitor, Tubastatin A. TFK-1 and EGI-1 CCA cell lines were investigated against Tubastatin A for the first time for its anti-cancer properties.

2. Materials and Methods

2.1. Chemicals

MTT (M2128-500MG) reagent and the HDAC inhibitor Tubastatin A (SML0044-5MG) was purchased from Sigma. A 5 mM stock of Tubastatin A solution in DMSO (dimethylsulfoxide) was prepared according to the recommendations of the supplier and the main stocks were stored at -20°C. The RPMI 1640, fetal bovine serum, penicillin/streptomycin, and PBS (Phosphate Saline Buffer) were obtained from Euro Clone, Biological Industries, Euro Clone, and Gibco, respectively. The crystal violet that was used to stain the colonies was purchased from Serva chemicals. RNAse enzyme (R5503) and the propidium iodide dye (P4170) were also obtained from Sigma for use in cell cycle analysis.

2.2. Cell lines and maintenance

Human CCA cell lines, TFK-1 and EGI-1, were obtained from German National Resource Center for Biological Material (DSMZ). Cells were cultured in RPMI medium supplemented with 10% FBS and 100 U/mL penicillin/streptomycin and maintained at 37°C in 5% CO_2.

2.3. Cell proliferation

Antiproliferative effects of Tubastatin A were determined by MTT cell proliferation assay on the indicated cell lines. This test is based on the principle that metabolically active cells convert the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) molecule into insoluble formazan salts and measuring the resulting color at 570 nm [14]. Briefly, 96-well plate were seeded with 10000 cells/well containing 200 µl of growth medium in the absence and presence of increasing concentrations of Tubastatin A. After 48 hours of incubation, 10 µl of MTT reagent was added to each well and incubated for extra 4 hours. The 96-well plates were centrifuged at 1800 rpm for 10 minutes and the formed formazan crystals was dissolved with 100 µl of DMSO. Formazan intensities was read in the spectrophotometer at a wavelength of 570 nm by Varioskan™ LUX multimode microplate reader (Thermo Scientific™). The proliferation graphs were plotted and the IC_{50} (drug concentration that inhibits cell growth by 50%) concentration calculated for Tubastatin A.

2.4. Colony formation assay

Cholangiocarcinoma cell lines, TFK-1 and EGI-1, were seeded in triplicates into 6-well plates (2000 and 1000 cells/well, respectively) and treated with Tubastatin A (10 µM and 50 µM). The cells were cultured in a humidified incubator at 37°C with 5% CO_2 for 14 days. By the end of 14 days, the medium was removed and the colonies were fixed with 4% paraformaldehyde and then stained with 0.1% crystal violet for 30 minutes at room temperature. After the staining, excess dye was removed using distilled water to improve the visualization of the colonies. After the digital images of the colonies were obtained with the camera, colony counts were made using ImageJ software.

2.5. Cell cycle analysis

Cell cycle analyzes of TFK-1 and EGI-1 cells treated with Tubastatin A were performed by flow cytometry and $1x10^6$ cells were incubated with the inhibitor for 48 hours. Cells were washed with 1 ml of cold PBS (pH=7.4) and centrifuged. Then, 4 ml of 70% ethanol was added to the cells and the cells were kept in a -20°C for at least 24 hours. Afterwards, cell pellet was homogenized in 5 ml of cold PBS, washed and centrifuged. Cell pellets were then washed with 1 ml of PBS/Triton X-100, followed by the addition of 100 µl of RNase-A and incubated at 37°C for 30 minutes. Finally, 100 µl of propidium iodide was added and left at room temperature for 10 minutes. Cell cycle analysis was performed by fluorescence-activated cell sorting (FACS).

2.6. Statistical analysis

The results are presented as mean ± standard deviation (SD). The statistical significance was detected using a one-way analysis of variance (ANOVA) for Dunnett's assay compared to the untreated controls. Statistical analysis was performed using GraphPad Prism 8.0.2 program.

3. Results

3.1 Antiproliferative effect of Tubastatin A on TFK-1 and EGI-1 cholangiocarcinoma cells

The cytotoxic effect of Tubastatin A was examined on TFK-1 (Figure 1A) and EGI-1 (Figure 1B) cholangiocarcinoma cells. The cells were treated with concentrations varying between 0.0025 - 10 µM for TFK-1 cells and 0.1-50 µM for EGI-1 cells for 48 hours. Tubastatin A decreased cell viability in a time and concentration dependent manner when compared to the untreated control ($P<0.001$). The IC_{50} value for Tubastatin A, which is the drug concentration that inhibits cell growth by 50%, was calculated as 15 µM and 20 µM for TFK-1 and EGI-1 cells, respectively.

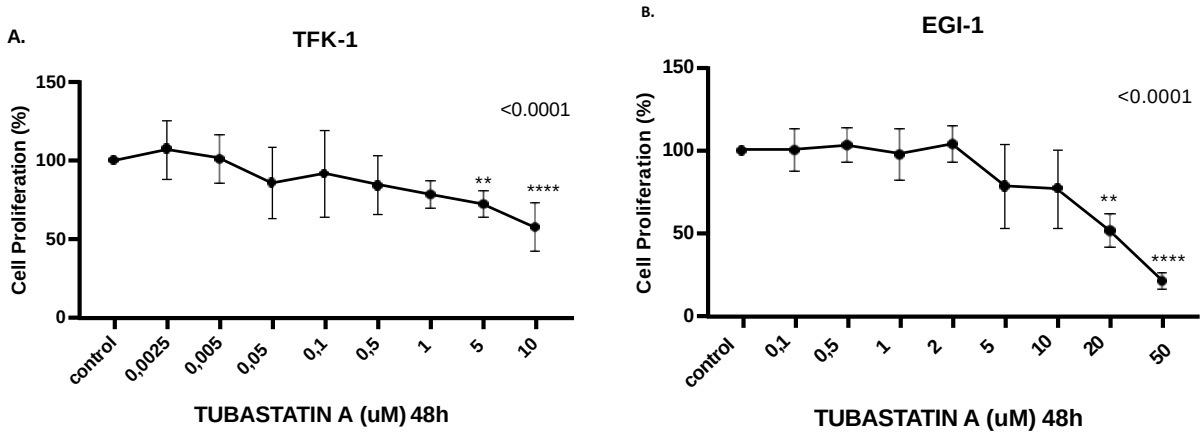

Figure 1. The cytotoxic effect of Tubastatin A on TFK-1 (A) and EGI-1 (B) cells with increasing concentrations for 48 hours. The standard deviation was done on the number of replicates, which is 3 wells per treatment and the experiment was done three times (n=3). (**= $P \leq 0.01$, ****= $P \leq 0.0001$)

3.2 The effect of Tubastatin A on the clonogenicity of cholangiocarcinoma cells

Clonogenic potential of TFK-1 and EGI-1 cholangiocarcinoma cells has been evaluated for 14 days after using two different Tubastatin A concentrations. Tubastatin A treatment caused a decrease in the number of colonies when compared to untreated or DMSO treated control cells (Figure 2).

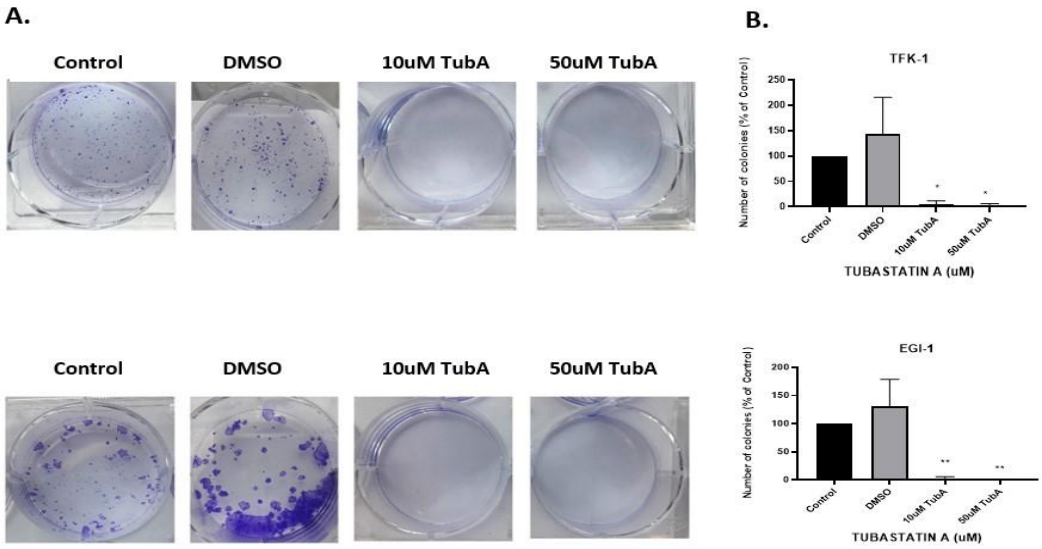

Figure 2. The impact of Tubastatin A on the clonogenicity of cholangiocarcinoma cells. (A) Representative images of clonogenic assays in TFK-1 (upper panel) and EGI-1 (lower panel) cells with different concentrations of Tubastatin A (TubA). (B) The effect of the different treatments on the clonogenic effect on TFK-1 and EGI-1 cells. (*= $P \leq 0.05$, **= $P \leq 0.01$)

3.3 The impact of Tubastatin A on cell cycle distribution

TFK-1 and EGI-1 cells were treated with 10 μM and 50 μM Tubastatin A in complete medium for 48 hours and cell cycle progression was analyzed by fluorescence-activated cell sorting (FACS) analysis (Figure 3). For EGI-1 cells, Tubastatin A treatment demonstrated cell cycle arrest in G2/M phase and the cell population in G0/G1 phase was also decreased by 9 % and 12 % in 10 μM and 50 μM Tubastatin A treatment, respectively. In TFK-1 cells, Tubastatin A did not show a significant effect on cell cycle progression when compared to untreated control cells.

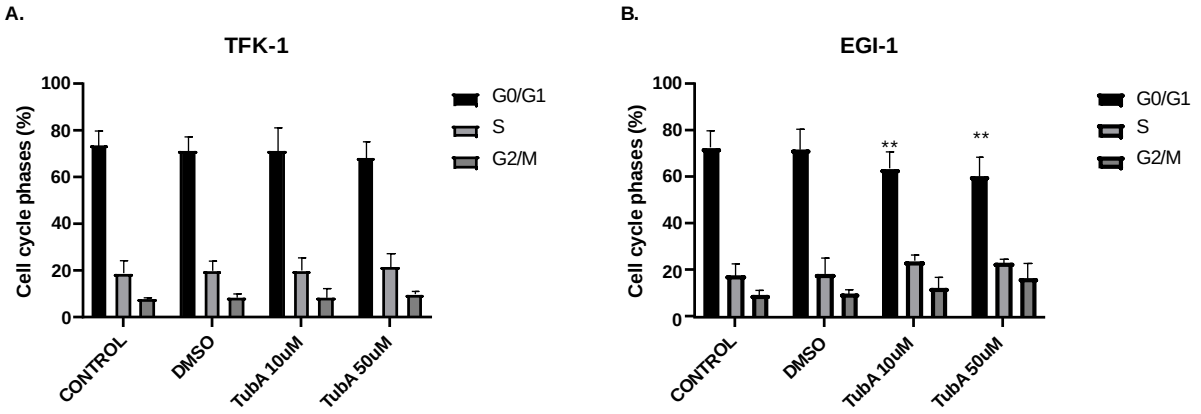

Figure 3. The effect of Tubastatin A on the cell cycle progression. The TFK-1 (A) and EGI-1 (B) cells were treated for 48 hours with 10 μM and 50 μM Tubastatin A (TubA), the cells were harvested, fixed, and stained with propidium iodide. Then the cells were analyzed by flow cytometer. Two independent experiments were performed, combined and analyzed. ** indicates that there is a significant (** = $P \leq 0.01$) difference between control and Tubastatin A administrations.

4. Discussion

In this study, we aimed to investigate the effects of selective HDAC6 inhibitor, Tubastatin A, on TFK-1 and EGI-1 CCA cell lines. Antiproliferative effects of Tubastatin A on CCA cells using MTT assay were determined and a dose dependent cell proliferation decrease was detected. Furthermore, the colony formation assay revealed a significant reduction in colony number depending on the Tubastatin A concentration. Further insight for the cytotoxic mechanism of Tubastatin A, we examined the cell cycle progression by flow cytometry. The cells were shown to be arrested in G2/M phase for EGI-1 cells but there was no significant change in TFK-1 cells.

Cholangiocarcinoma is a highly aggressive and metastatic form of adenocarcinoma with a poor prognosis and mortality rate because of heterogeneity, limited therapy options and developing resistance. It is the second most common type of liver cancer with a rate of 10-20% among all liver cancer types. The chemotherapy options of CCA are mostly gemcitabine and cisplatin, which are considered as a first-line therapy [15]. Some combinational therapies are also done but the overall survival is increased mildly ameliorated [16] and there is a requirement for novel treatment strategies for CCA.

Epigenetics changes involve the DNA and histone modifications which results in changes of genetic expression without changing the DNA sequence. The posttranslational modifications of histone proteins that are tightly bound to the DNA is a significant epigenetic mechanism [4]. These modifications involve acetylation and methylation of certain lysine residues on histone proteins. The histone acetylation causes a change in transcription activity, loosening the compactly packed nucleosome and this process results in the increase in gene expression in many cases [17]. This effect is reversed by histone deacetylation and this reaction is catalyzed by histone deacetylase (HDAC) enzymes. The involvement of epigenetic regulations in various types of diseases, like cancer, got the attention on developing novel strategies targeting epigenetic modifications that has been studied widely [18,19]. HDAC inhibitors show promising effects by causing hyperacetylation and inhibiting the removal of acetyl groups from the lysine residues [20]. Their potential as anticancer agents lead the FDA approval of HDAC inhibitors. HDAC6 is a class II inhibitor that induces cell proliferation in CAA cells and is over expressed in CCA patients [11,12]. HDAC inhibition caused the downregulation of the malignant properties of CCA cells. Many studies demonstrated and suggested the potency of selective HDAC6 inhibition not only for

CCA but also in various types of malignancies like gastric cancer [21], glioblastoma [13], pancreatic cancer [22], colorectal cancers [23]. These selective inhibitors exert anticancer effect by different mechanisms. It has been suggested that Tubastain A enhances temozolomide-induced apoptosis in glioblastoma cells, while treatment affects Hedgehog signaling, thereby reducing glioblastoma progression. [13]. In another study, CCA was used as a model and it was shown that the primary cilia were reduced upon HDAC6 overexpression. The inhibition of HDAC6 and restoration of cilia formation resulted in inhibition of tumor growth [11].

Colony forming capability of cancer cells is a valuable indicator of reproductive potential of living single cancer cells. Tubastatin A was reported to decrease the 2D colony number by 50% in glioblastoma cells [13] and inhibited colony formation in triple negative human breast epithelial/cancer cell lines [24]. Similarly, in this study, decreasing colony formation was also observed in cells treated with Tubastatin A.

HDAC inhibitors are potential anti-cancer agents, and this effect sometimes manifests as cell cycle progression and sometimes cell cycle arrest. This cytoplasmic enzyme also has a nonhistone target, such as tubulin, which plays an important role in cell division and microtubule formation [25]. HDAC6 inhibition was shown to have similar variable effects on cell cycle in three different melanoma cell lines. HDAC6 inhibitors, including Tubastatin A, caused G1 arrest accompanied by changes of cell population in G2 fraction. Although Tubastatin A and other HDAC6 inhibitors were applied to same cancer type but different cell lines, variable effects were observed [26]. In this current study, Tubastatin A induced G2/M arrest for EGI-1 cells and decreased the cell population in G0/G1. On the contrary, the cell population in S phase increased compared to the control. Interestingly, Tubastatin A administration did not have a significant effect on cell cycle distribution. The cell cycle results in the present study is in line with the results that is observed in this previously discussed study [26] that the inhibitor caused different impacts on cell cycle in different CCA cell lines. These observations suggest that Tubastatin A may alter different cell cycle regulatory proteins depending on the type of CCA cell line.

In conclusion, our study demonstrated that the selective inhibitor Tubastatin A exhibits an antiproliferative effect towards TFK-1 and EGI-1 cholangiocarcinoma cells. Tubastatin A's antiproliferative activity is thought to be mediated by its impact and inhibition on clonogenicity of these cells. In addition, the EGI-1 cells were arrested in the G2/M phase upon increasing concentrations of Tubastatin A. Collectively our results show that HDAC6 inhibition with Tubastatin A leads a decrease in cell proliferation, colony formation and cell cycle arrest. Clearly it is important to evaluate the effect of the inhibitor mechanistically. These results provide a starting point for further studies. The focus of future work might be combinational treatments of Tubastatin with different inhibitors that target different pathways. After the validation of conclusions that would be brought, it could be a subject of a multiple inhibitor therapy for CCA. Although there is a need for follow-up studies and further investigation, our results suggest that HDAC6 could be potential therapeutic targets for treating cholangiocarcinoma, and administration of selective inhibitor, Tubastatin A, could be a potential therapeutic approach for cholangiocarcinoma.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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