

Article

LOW-DOSE CISPLATIN EXPOSURE AND SNAIL, VIMENTIN, E-CADHERIN EXPRESSION IN HEPG2 CELL LINE

Exposición a dosis bajas de cisplatino y expresión de SNAIL, Vimentina, E-cadherina en línea celular HEPG2

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ABSTRACT

Cisplatin, the first platinum compound approved for cancer treatment, is widely used in the treatment of various cancers including hepatocellular carcinoma (HCC). HCC incidence rates rise globally. Epithelial mesenchymal transition (EMT) is implicated in cancer invasion and metastasis, which are associated with increased mortality. Cisplatin dose might influence cancer invasion and metastatic behavior of the cells. The aim of the study was to investigate the effect of low-dose cisplatin treatment on EMT-related changes in HepG2 cells. Following treatment with 4 µM

cisplatin, HepG2 cells were evaluated morphologically. Gene expression of E-cadherin, Vimentin, Snail1 was assessed by quantitative PCR. Immunofluorescence analyses of NA-K ATPase were performed. Although the low-dose cisplatin treated cells exhibited a more stretched morphology, no statistical difference was detected in gene expression of E-cadherin, Vimentin, Snail1 and immunofluorescence of NA-K ATPase. Findings on low-dose cisplatin effects in HepG2 might contribute to the knowledge of antineoplastic inefficacy by further understanding the molecular mechanisms of drug action.

Keywords: Cisplatin, SNAIL, Vimentin, E-cadherin, Epithelial mesenchymal transition

1. Introduction

Cisplatin was first synthesized nearly 100 years ago in 1944 by Peyrone. Later in 1960s, cell division inhibiting properties were observed by Rosenberg (Kauffman, *et al.*, 2010; Rosenberg, *et al.*, 1969). In 1978, it was approved by FDA as the first platinum compound for cancer treatment. Cisplatin, as a cytotoxic drug that damages DNA, inhibits DNA synthesis and mitosis, and induces apoptotic cell death (Dasari & Tchounwou, 2014), is one of the most effective anticancer agents widely used in the treatment of various cancers including hepatocellular carcinoma (HCC).

HCC is the most common form of liver cancer with a high mortality, which continues to increase globally (Kim, *et al.*, 2017). Unfortunately, systemic tyrosine kinase inhibitors and immune checkpoint inhibitors prolonged survival duration only for a limited time in advanced HCC. Cisplatin is used in the treatment of sorafenib-resistant HCC via hepatic intraarterial administration or in combination with other systemic anticancer agents to increase the efficacy (Laface, *et al.*, 2021). Inadequate efficacy and resistance to cisplatin chemotherapy are important pharmacological concerns. Determining the optimal dose and the time interval between doses is still challenging.

Epithelial mesenchymal transition (EMT) is characterized by the loss of epithelial cell markers, and acquisition of mesenchymal markers. EMT is a complex process that takes place during wound healing, but it is also implicated in cancer invasion and metastasis (Gurzu, *et al.*, 2019; Jou & Diehl 2010). E-cadherin, a cell adhesion protein, is a classical marker of epithelial cells (Niknami, *et al.*, 2020). Vimentin is a mesenchymal marker with the potential of regulating several different physiological functions (Satelli & Li, 2011). Snail1 is a transcription factor that mediates EMT by inducing downregulation of E-cadherin and upregulation of Vimentin (Myong, 2012). Snail1 was shown to be expressed in approximately half of HCCs and its expression correlates with disruption of adherence junctions in epithelial cells and a worse prognosis (Jou & Diehl, 2010). NA-K ATPase was also demonstrated in the loss of the epithelial phenotype and reported to be associated with the progression of EMT through transforming growth factor-beta (TGF- β) (Rajasekaran, *et al.*, 2010).

Cisplatin was shown to induce the expression cancer stem cells in a previous study. The effect was obtained with low-dose cisplatin treatment (Zhang, *et al.*, 2014). Furthermore, Donmez Cakil *et al* presented increased expression of CSC markers including CD44 and CD90 after treatment with low dose cisplatin (Donmez Cakil, *et al.*, 2021). EMT pathways are often driven via the activation of cancer stem cells (CSCs) that might also induce drug resistance.

There is still a need for more efficacious HCC pharmacotherapy. Understanding the molecular mechanisms of drug action is important to define new targets and develop strategies to improve HCC treatment. Low-dose cisplatin treatment may also cause EMT-related changes. The aim of the study is to evaluate the effects of low dose cisplatin on gene expression of E-cadherin, Vimentin, Snail1 and immunofluorescence of NA-K ATPase.

2. Materials and methods

Cell culture

HepG2 cells (American Type Culture Collection, USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Biosera LM-T1720/100, France) containing 1% antibiotics (10 mg/ml streptomycin and 10.000 U/ml penicillin, PAN-Biotech GmbH, Germany) and 10% fetal bovine serum (PAN-Biotech GmbH, Germany) in an incubator with 5% CO₂ at 37°C. Live cell images were taken with a Zeiss Primovert Compact inverted microscope (Germany).

Drugs

Cells were treated with 4 µM cisplatin (Glentham Life Sciences, UK) in DMEM for 72 hours. The dose determination was based on a previous study of our group, which demonstrated 84.5% viability in HepG2 cells in response to 4 µM cisplatin exposure for 72 h (Donmez Cakil, *et al.*, 2021).

Total RNA extraction, cDNA synthesis, and quantitative PCR (qPCR)

Total RNA isolation was performed with GeneJET RNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's instructions. RNA integrity was evaluated using UV absorbance (Synergy Microplate Reader). 2 µg total RNA, oligo dT primers and RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) were used for cDNA synthesis. QPCR reaction was carried out in a LightCycler® 96 instrument (Roche Diagnostics International, Switzerland) using Ampliqon RealQ Plus Master Mix Green Without ROX (Denmark) and gene specific primers for E-cadherin, Vimentin, Snail1 (BM Lab, Turkey) and GAPDH (Thermo Scientific). QPCR products were quantified using delta delta Ct ($2^{-\Delta\Delta Ct}$) relative quantitation method.

Confocal laser scanning microscopy

HepG2 cells were fixed with ice-cold methanol for 10 min. Following 1% BSA incubation for 30 min to block nonspecific binding, the cells were incubated with NA-K ATPase antibody (Abcam, ab7671, 1:100) for 30 min at room temperature (RT). Secondary antibody incubation was performed with goat anti-Mouse IgG (H+L) secondary antibody, DyLight 550 (Thermo Scientific, 84540, 1:200) for 30 min at 4°C at dark. Antibody incubations were performed in the presence of 1% BSA. Hoechst 33342 (Thermo Scientific, 1:1000) was used to stain the nuclei. The coverslips were wet-mounted on microscope slides and observed under a Zeiss LSM 700 confocal laser scanning microscope (Germany).

Statistical Analysis

The data are expressed as mean ± standard error of the mean (SEM). The Student's t-test was used for comparison between two groups. p<0.05 was considered significant.

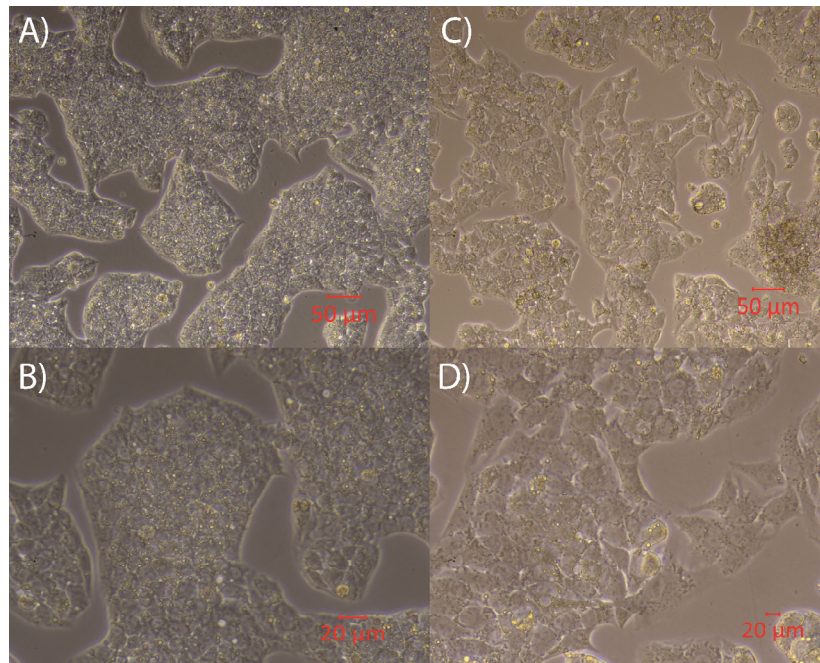
3. Results

Morphological analysis

Live cells images of the untreated control and cisplatin treated cells were taken to compare the cellular morphology of the groups. While control group cells tend to form aggregates and identification of individual cells is difficult, cisplatin treated cells exhibited a more stretched and fibroblast like morphology (Figure 1A-B).

Figure 1:

Live images of HepG2 cells. Low-dose cisplatin treatment was performed for 72 hours. 20X images of the untreated control and treated cells (A and C) and 40X images of the untreated control and treated cells (B, D).

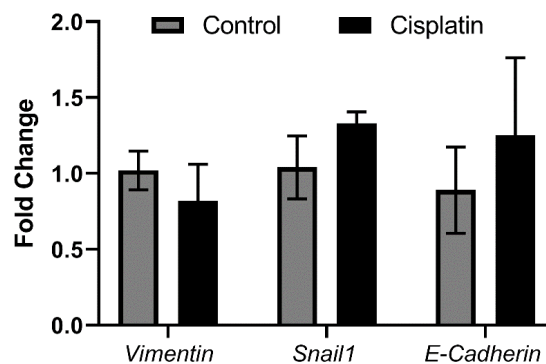


E-cadherin, Vimentin and Snail1 gene expression levels after treatment with low-dose cisplatin

HepG2 cells were treated with 4 µM cisplatin to investigate the effects of low-dose cisplatin treatment on the expression of the EMT-related genes. QPCR analysis did not demonstrate a significant change in the expression of E-cadherin, Vimentin and Snail1 between the untreated control and low dose Cisplatin treated groups as depicted in Figure 2 ($p=0.5718$; $p=0.5044$; $p=0.2588$, respectively).

Figure 2:

Expression levels of EMT-related markers, Vimentin, Snail1, and E-cadherin in response to 4µM cisplatin for 72h. The data are expressed as mean \pm standard error of the mean (SEM). The Student's t-test was used for comparison between two groups.

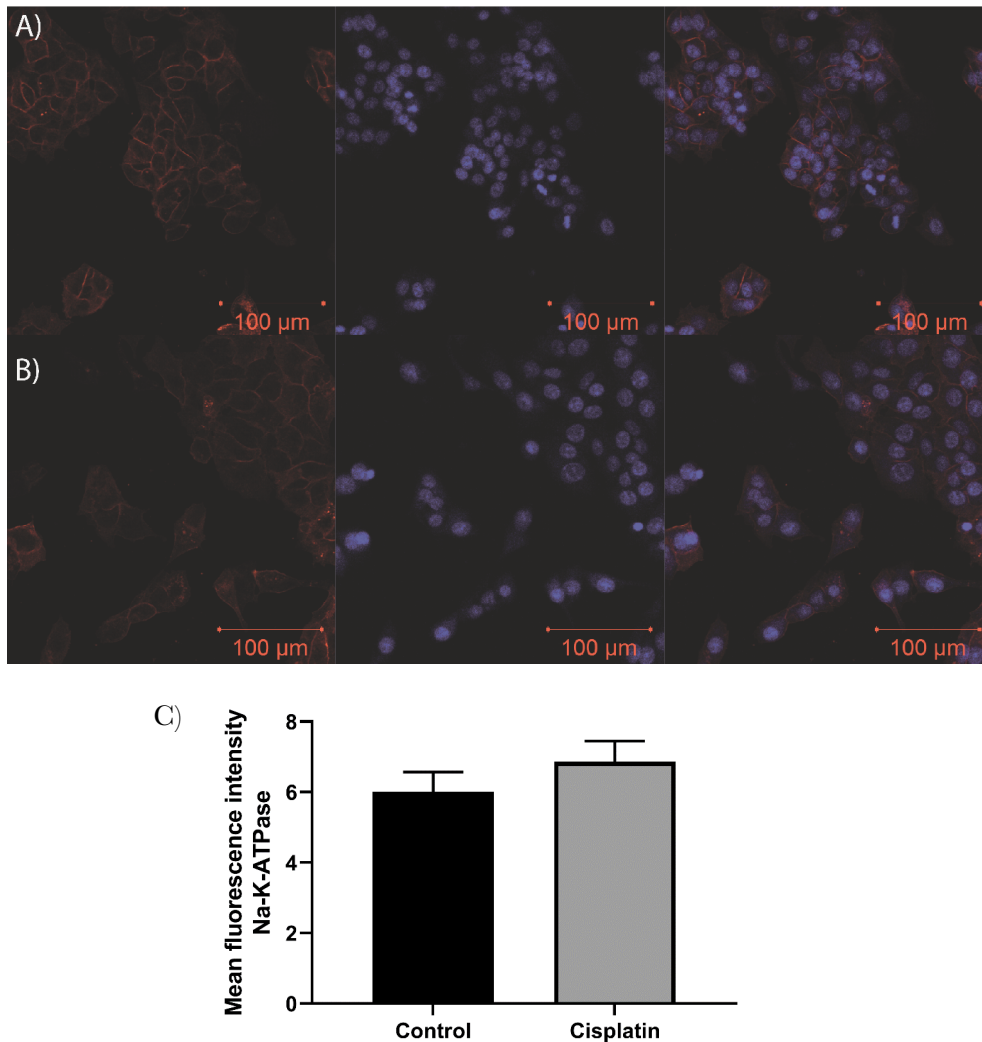


Immunofluorescence analysis

Immunofluorescence analysis were employed to investigate the expression of Na-K ATPase in HepG2 cells (Figure 3A, B). No significant difference in mean fluorescence intensity was obtained when the untreated control and cisplatin treated groups were compared ($p= 0.3170$, Figure 3C). The morphological differences obtained with live cell imaging were observed also with the confocal laser scanning microscope.

Figure 3:

Images of Na-K ATPase immunofluorescence analysis in control (A) and low dose cisplatin treated cells (B). Mean fluorescence intensity was plotted and the Student's t-test was used for comparison between two groups (C).



4. Discussion

Cisplatin is readily used in the treatment of various cancers. However, drug inefficacy and acquired resistance are major limitations. EMT process is thought to be among the underlying mechanisms for non-response cisplatin treatment leading to invasion of tumor cells (Ashrafizadeh, *et al.*, 2020). In a previous study, our group demonstrated increased expression of CSC markers CD44 and CD90

following low-dose cisplatin treatment (starting from 7.1 μM), (Donmez Cakil, *et al.*, 2021). Another study in HepG2 cell line showed that, treatment with 1-4 $\mu\text{g}/\text{mL}$ cisplatin increased the percentage of ALDH1+ or CD133+ cells (Zhang, *et al.*, 2014). In line with the previous findings, and based on the linkage between CSCs and EMT (Tanabe, *et al.*, 2020), this study aims to investigate if there are any potential EMT-related changes in HepG2 cells after low-dose cisplatin exposure. Previously, we demonstrated that 4 μM cisplatin treatment results in a high cell proliferation (84.5%). In the current study, we observed that 4 μM cisplatin treated cells exhibited a more stretched and fibroblast like morphology. On the other hand, control group cells tended to form aggregates. However, we did not obtain any alterations in the expression of EMT-related genes and NA-K ATPase.

During EMT, epithelial cells lose their epithelial morphology and acquire a mesenchymal-like phenotype (Gurzu, *et al.*, 2019; Jou & Diehl 2010; Yang, *et al.*, 2020). EMT is characterized with a decrease in E-cadherin, and an increase in expression of Vimentin and Snail1 (Dang, *et al.*, 2011; Myong, 2012; Seo, *et al.*, 2021). The expression levels of EMT markers including E-cadherin, Snail and Vimentin were reported to correlate with the expression levels of CSC markers and the metastatic behaviour of the cells (Babaei, *et al.*, 2021; Choi, *et al.*, 2017). A previous study by Latifi *et al* reported that cisplatin caused morphological changes in primary and metastatic epithelial ovarian carcinomas in a dose dependent manner between 1 and 10 $\mu\text{g}/\text{ml}$. The researchers demonstrated reduced E-cadherin, and increased Vimentin and Snail expression together with increased cell surface expression of CSC-like markers such as CD44 (Latifi, *et al.*, 2011). Furthermore, in another study in nasopharyngeal carcinoma, EMT process was reported to promote cisplatin-resistance (Su, *et al.*, 2017).

While E-cadherin is a key gene implicated in EMT, its loss was shown not as a prerequisite for c-erbB2-induced EMT (Nilsson, *et al.*, 2014). Moreover, loss of E-cadherin might not be required for metastasis and the cells can maintain their epithelial morphology during tumor cell movement (Liu, *et al.*, 2014). Similarly, in a previous study in osteosarcoma, cisplatin was shown to promote mesenchymal-like characteristics without any changes in E-cadherin and Vimentin expressions. However, an increase in N-cadherin expression was obtained and also knockdown of Snail reversed cisplatin-induced EMT (Fang, *et al.*, 2016). Snail 1 is among the EMT transcription factors inducing EMT (Myong, 2012). An *in vitro* study by Haslehurst *et al* reported that the cisplatin-resistant A2780 ovarian adenocarcinoma cell line grown in the presence of 1 μM cisplatin displays a mesenchymal phenotype. Importantly, the researchers knocked down Snail and Slug and reversed the EMT phenotype and cisplatin resistance (Haslehurst, *et al.*, 2012). In our study, we explored the effects of low-dose / short-term cisplatin treatment on EMT in HepG2 cells. While the cells displayed a change in morphology, the expression of E-cadherin, Vimentin and Snail1 did not alter. Type of cancer, the dose of the drug, the duration of treatment might lead to different outcomes. Also, Snail expression is not altered in each of clinical HCC sample (Woo, *et al.*, 2011).

NA-K ATPase is mentioned as a target for cisplatin. Previously, cisplatin has been shown to inhibit the pump (Kubala, *et al.*, 2014). Cysteiny residues on the cytoplasmic part of NA-K ATPase were demonstrated to interact with cisplatin (Šeflová, *et al.*, 2018). Moreover, in another study, lower expression of NA-K ATPase $\alpha(1)$ subunit was demonstrated in cisplatin resistant ovarian adenocarcinoma cells (Schneider, *et al.*, 2013). However, no alteration in expression was obtained following low-dose cisplatin exposure for 72 hours in our study.

Recently, Yang *et al* on behalf of the EMT International Association (TEMTIA) published guidelines and definitions for research on EMT due to the increasing body of research and increasing need for a consensus in this topic (Yang, *et al.*, 2020). Accordingly, there is a great diversity of EMT

phenotypic changes and multiple alternative paths can be involved in the process (Ashrafizadeh, *et al.*, 2020; Yang, *et al.*, 2020). Therefore, a large number of molecular markers are required to assess the EMT status of the cells. Cisplatin with low doses caused morphological changes. These changes are not sufficient to support EMT but also lack of difference in EMT-related expression levels might not exclude EMT-related changes.

Antineoplastic inefficacy is a complex challenge. The data on molecular mechanisms of drug action and antineoplastic inefficacy are valuable to identify better targets for developing drugs with an improved efficacy and develop strategies for sensitization of cancer cells to chemotherapeutic agents.

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RESUMEN

El cisplatino, el primer compuesto de platino aprobado para el tratamiento del cáncer, es ampliamente utilizado en el tratamiento de varios tipos de cáncer, incluido el carcinoma hepatocelular (CHC). Las tasas de incidencia de CHC aumentan a nivel mundial. La transición mesenquimal epitelial (EMT) está implicada en la invasión del cáncer y la metástasis, que se asocian con un aumento de la mortalidad. La dosis de cisplatino podría influir en la invasión del cáncer y el comportamiento metastásico de las células. El objetivo del estudio fue investigar el efecto del tratamiento con dosis bajas de cisplatino en los cambios relacionados con la EMT en las células HepG2. Tras el tratamiento con cisplatino de 4 μM, se evaluaron morfológicamente las células HepG2. La expresión génica de E-cadherina, vimentina, caracol1 se evaluó mediante PCR cuantitativa. Se realizaron análisis de inmunofluorescencia de NA-K ATPasa. Aunque las células tratadas con cisplatino en dosis bajas exhibieron una morfología más estirada, no se detectaron diferencias estadísticas en la expresión génica de E-cadherina, vimentina, Snail1 e inmunofluorescencia de NA-K ATPasa. Los hallazgos sobre los efectos del cisplatino en dosis bajas en HepG2 podrían contribuir al conocimiento de la ineficacia antineoplásica al comprender mejor los mecanismos moleculares de la acción del fármaco.

Palabras clave: Cisplatino, Snail, Vimentina, E-cadherina, Transición epitelio mesenquima
