Desipramine induces apoptosis in hepatocellular carcinoma cells

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Received January 12, 2017; Accepted May 31, 2017

DOI: 10.3892/or.2017.5723

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Key words: desipramine, antidepressants, hepatocellular carcinoma, apoptosis, Hep3B cells

Abstract. Antitumor effects of antidepressants have been reported in many cancer cell lines. However, anti-proliferative effects of desipramine, a tricyclic antidepressant, in hepatocellular carcinoma are currently unknown. In this study, we examined the effects of desipramine in human hepatoma Hep3B cells. To evaluate anti-proliferative effects of desipramine in Hep3B cells, we determined cell viability, reactive oxygen species (ROS) production, mitochondrial membrane potential (MMP), mitogen-activated protein kinase (MAPK) activity, and intracellular Ca²⁺ levels after desipramine treatment. Desipramine reduced cell viability, increased ROS production, and decreased MMP activity in Hep3B cells. In addition, desipramine activated MAPKs (ERK 1/2, JNK, and p38) and increased intracellular Ca²⁺ levels. Pro-apoptotic effects of desipramine were abolished after MAPK inhibitors (PD98059, SB203580, and SP600125) or N-acetyl-L-cysteine (NAC), as a ROS scavenger, treatments. These findings suggest that desipramine shows anti-proliferative effects in Hep3B cells mediated by promotion of apoptosis, activation of MAPK signaling, and increase in intracellular Ca²⁺ levels.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor in the world, especially in Southeast Asia and Africa (1). Hepatitis B and C viral infections, chronic alcohol consumption and aflatoxin B1 exposure are predominant risk factors for HCC development (2). Although understanding of HCC pathogenesis and clinical therapies for HCC treatment have been achieved, HCC incidence and survival of HCC patients are still unsatisfactory (3). Therefore, effective prognostic indicators and therapeutic strategy for HCC are needed.

Materials and methods

Cell culture and reagents. Hep3B cells were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea) and grown in Dulbecco’s modified Eagle’s medium nutrient mixture F-12 HAM (DMEM F-12 HAM) supplemented with 10% fetal bovine serum, 1% antibiotics, 5 mM L-glutamine. Desipramine, 4’,6-diamidino-2-phenylindole (DAPI) and 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethyl benzimidazolyl-carbocyanine iodide (JC-1) was purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA). 2’,7’-Dichlorodihydrofluorescin diacetate (DCFH-DA) was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Cell viability assay. Cell viability assay was measured by using Cell Counting Kit-8 (CCK-8; Enzo Life Sciences). Briefly, Hep3B cells were seeded into 96-well plates (5000 cells/well) and cultured for 24 h in DMEM F-12 HAM containing 10% fetal bovine serum. After treatment of desipramine (30-500 µM) for 24 h, the CCK-8 reagent was added to each well, and the cells were incubated at 37°C for an additional 2 h. Absorbance was measured at 450 nm using
a spectrophotometer (Spectra Max M5; Molecular Devices, Sunnyvale, CA, USA). For the treatment of N-acetylcysteine (NAC), cells were treated with desipramine (3-100 µM) with or without 10 mM NAC for 24 h. Cells were then applied to CCK-8 assay for cell viability assay.

**LDH (lactic dehydrogenase) activity measurement.** LDH activity was measured by a chemical colorimetric method using cytotoxic detection kit (Takara Bio Inc., Shiga, Japan). Briefly, after 48 h incubation of the Hep3B cells in 12-well plates (1x10^4 cells/well), the cells were treated with desipramine (10-500 µM). After 24 h incubation, culture media were collected and centrifuged at 10,000 x g for 10 min. Optical density values were measured at 490 nm using a spectrophotometer (Spectra Max M5; Molecular Devices).

**Measurement of intracellular reactive oxygen species (ROS) production.** Intracellular ROS can oxidize the non-fluorescent DCF to the highly fluorescent DCF. Thus, intracellular ROS can be measured by fluorescence intensity of DCF by a fluorometer. Briefly, after 48 h incubation of Hep3B cells in 12-well plates (1x10^4 cells/well), cells were treated with 10-500 µM desipramine for 24 h. After 24 h incubation, culture media were collected and centrifuged at 10,000 x g for 10 min. Optical density values were measured at 490 nm using a spectrophotometer (Spectra Max M5; Molecular Devices).
desipramine (3-100 µM) for 24 h. Hep3B cells were then treated with desipramine (3-100 µM) for 24 h. Cells were harvested and lysed in RIPA buffer containing protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Protein homogenates were separated on SDS-PAGE gels and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). After blocking for 1 h with 5% non-fat dry milk, the membranes were incubated overnight at 4°C with antibodies against total or phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), c-JUN N-terminal kinase (JNK), p38, or β-actin (Cell Signaling Technology, Danvers, MA, USA). Next, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies (Cell Signaling Technology) for 1 h and the bands were detected using enhanced chemiluminescence. The blots were scanned by a Bio-Rad ChemiDoc XRS and the intensity of each protein was quantified by Quantity One 4.5.0 software (Bio-Rad).

**Western blot analysis of MAPKs.** Hep3B cells were treated with desipramine (100 µM) at each time point (1, 2, 4, and 8 h, respectively). In addition, cells were treated with desipramine (3-10 µM) with/without pre-incubation of MAPK inhibitors (PD98059, SB203580, and SP600125) for 24 h. Cells were seeded in 12-well plates (1x10⁴ cells/well) on laminin-coated coverglass. Cells were then incubated with 10 µg/ml JC-1 for 10 min. JC-1-labelled cells were observed under a fluorescence microscope (IX-81; Olympus Corp.). JC-1 fluorescence was measured using spectrophotometer with 550 nm excitation/600 nm emission and 485 nm excitation/535 nm emission wavelengths for red and green fluorescence, respectively. Data are expressed as means ± SEM. Differences between the groups were analyzed using a one-way ANOVA followed by Bonferroni’s post-hoc test. **P**<0.001 vs. control. Cont, control; Desi, desipramine; NS, not significant.

**Ca²⁺ measurement.** Intracellular Ca²⁺ concentration was measured by using the fluorescent dye, Fura-2/AM (Thermo Fisher Scientific). Hep3B cells were then treated with desipramine (3-100 µM) for 24 h. Cells were harvested and lysed in RIPA buffer containing protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Protein homogenates were separated on SDS-PAGE gels and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). After blocking for 1 h with 5% non-fat dry milk, the membranes were incubated overnight at 4°C with antibodies against total or phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), c-JUN N-terminal kinase (JNK), p38, or β-actin (Cell Signaling Technology, Danvers, MA, USA). Next, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies (Cell Signaling Technology) for 1 h and the bands were detected using enhanced chemiluminescence. The blots were scanned by a Bio-Rad ChemiDoc XRS and the intensity of each protein was quantified by Quantity One 4.5.0 software (Bio-Rad).

**Statistical analysis.** The data are reported as the mean ± SEM. Statistical significance was analyzed by using one-way analysis of variance (ANOVA) with Bonferroni’s post-hoc test (Prism 5.0.3, GraphPad Software Inc., San Diego, CA, USA). **P**<0.05 was considered to indicate a statistically significant difference.

**Results**

**Effects of desipramine on cell viability of Hep3B cells.** In order to determine the anti-proliferative potential of desipramine in hepatocellular carcinoma cells, viability of Hep3B cells was investigated using the CCK-8 assay after treatment with 1, 3, 10, 30, 100, 300, and 500 µM desipramine for 24 h. As shown in Fig. 1A, desipramine induced cell death in a dose-dependent manner. Treatment of Hep3B cells with 1 µM desipramine did not affect cell viability, whereas 3-500 µM desipramine induced a significant decrease in cell viability (90.2±2.9, 87.4±3.0, 69.1±2.1, 45.9±1.1, 36.1±0.7, and 27.8±0.2% at 3, 10, 30, 100, 300, and 500 µM, vs. control cells, respectively) (Fig. 1A). As LDH is used as an indicator of cytotoxicity because of LDH release upon loss of cell membrane integrity, we also determined LDH activity after desipramine treatment (10-500 µM). Desipramine treatment markedly increased LDH activity in a dose-dependent manner (105.7±8.0, 109.8±1.1, 150.3±8.4, 227.7±12.1, and 255.7±40.7% at 10, 30, 100, 300, and 500 µM, vs. control cells, respectively). Collectively, these results demonstrate that desipramine inhibits the proliferation of Hep3B cells (Fig. 1B).
Effects of desipramine on ROS production in Hep3B cells. As ROS production activates pro-apoptotic signaling pathways, we analyzed the involvement of ROS in desipramine-induced apoptosis in Hep3B cells. ROS levels were assessed using DCFH-DA. Desipramine treatment significantly increased intracellular ROS generation in a dose-dependent manner (158.8±10.5, 158.1±15.7, 167.6±3.8, and 205.3±7.6% at 3, 10, 30, and 100 µM vs. control cells, respectively) (Fig. 2A and B). In addition, treatment with the ROS scavenger NAC (10 mM) significantly attenuated desipramine-induced cell death, according to the CCK-8 assay (Fig. 2C).

Effects of desipramine on MMP in Hep3B cells. In order to analyze mitochondrial activity in the presence of desipramine, we observed the effect of desipramine on MMP using JC-1, an MMP-sensing dye. During apoptosis, mitochondrial depolarization occurs, altering the fluorescence of JC-1 from red (aggregates) to green (monomers). Desipramine significantly reduced MMP in Hep3B cells in a dose-dependent manner, indicated by a shift from red to green JC-1 fluorescence (Fig. 3A). To corroborate these results, we monitored the fluorescence intensity of JC-1 using spectrophotometry. As concentration of desipramine increased, the ratio of red/green
fluorescence intensity markedly decreased (85.4±1.2, 76.6±2.9, 74.8±2.0, and 67.9±1.7% at 3, 10, 30, and 100 µM vs. control cells, respectively) (Fig. 3B).

Effect of desipramine on the MAPK signaling in Hep3B cells. In order to identify molecular mechanisms underlying anti-proliferative effects of desipramine in Hep3B cells, we determined protein expression patterns of MAPKs (ERK1/2, JNK, and p38) involved in pro-apoptotic signaling pathways. Results showed that phospho-ERK levels were significantly increased after 100 µM desipramine treatment for 1, 2, and 4 h compared with control cells (185.2±10.1, 151.5±9.8, and 138.5±6.1% at 1, 2, and 4 h vs. control cells, respectively). In addition, phospho-p38 and JNK expression levels were significantly increased after 100 µM desipramine treatment for 1 and 2 h compared with control cells (135.1±4.8 and 116.4±2.3% increase in p-38, 118.9±2.3 and 113.9±2.8% increase in p-JNK vs. control cells, respectively) (Fig. 4A and B). Pre-incubation with 20 µM PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), or SP600125 (JNK inhibitor) followed by exposure to desipramine (3-100 µM) revealed that all three inhibitors abolished the anti-proliferative effect of desipramine in Hep3B cells (Fig. 4C).

Effects of desipramine on [Ca²⁺]i in Hep3B cells. Since abnormal [Ca²⁺]i rise may lead to interruption of ion flux, protein dysfunctions, or apoptosis (16), we analyzed [Ca²⁺]i after desipramine treatment (30, 100, and 500 µM) in Ca²⁺-free or 1 mM Ca²⁺-buffer using Fura-2/AM, a fluorescent Ca²⁺-sensitive dye. Desipramine induced [Ca²⁺]i increase in a concentration-dependent manner in 1 mM Ca²⁺-containing buffer (Fig. 5A). Similarly, [Ca²⁺]i increase occurred after 100 and 500 µM desipramine treatment in Ca²⁺-free buffer (Fig. 5B).

Discussion

In the present study, we demonstrated that desipramine inhibits proliferation of Hep3B cells by inducing apoptosis, which suggests the antitumor potential of this drug in hepatocellular carcinoma. Some antidepressants (e.g., paroxetine, fluvoxamine and sertraline) have potent anticancer properties against various cancer cell lines (17,18). In contrast, these drugs were also reported to stimulate or not affect proliferation of tumor cells (19,20). Cytotoxic effects of desipramine were demonstrated in several cancer cell lines, including prostate cancer, colon, renal tubular, and glialoma cells (21-23). Consistent with previous observations, we found that viability of Hep3B cells was decreased after desipramine treatment in a dose-dependent manner (Fig. 1A). Furthermore, increased LDH release confirmed the cell damage caused by desipramine (Fig. 1B).

ROS play an important role in oxidative stress, which is primarily generated in mitochondria. In tumor cells, increased ROS generation promotes proliferation, altered metabolism, and angiogenesis, and is controlled by the oxidant/anti-oxidant balance system (24). When this system is impaired, excessive amounts of ROS eventually lead to tumor cell death. Our results demonstrated that desipramine treatment markedly induced ROS production in Hep3B cells, an effect prevented by treatment with NAC, a ROS scavenger (Fig. 2). In addition, desipramine decreased MMP in Hep3B cells (Fig. 3). Overproduction of ROS causes mitochondria damage, loss of MMP, and eventually, apoptosis via mitochondria-mediated cell death pathway (25).

MAPK signaling pathways are known regulators of cell survival, proliferation, and stress response and are responsible for the apoptotic cascade in a number of cancer cell lines. Many anticancer agents activate MAPK pathways in various cell types (26). We examined the effects of desipramine on the expression of three major MAPK proteins (ERK1/2, JNK, and p38) in Hep3B cells. Our results suggest that desipramine significantly inhibits the phosphorylation of ERK1/2, JNK, and p38. Furthermore, exposure to MAPK inhibitors suppressed desipramine-induced cell death (Fig. 4). These results indicate that MAPK signaling pathways play an important role in desipramine-induced cell death in Hep3B cells.

Intracellular Ca²⁺ is closely linked to ROS production with accumulation leading to apoptotic cell death (27). Ca²⁺ overload triggers the opening of the permeability transition pore, which is associated with mitochondrial cell death pathways of apoptosis (28). Increase in intracellular Ca²⁺ has been associated with apoptosis in tumor cells (9,29). In this study, [Ca²⁺]i increased in response to desipramine treatment in Ca²⁺-free and 1 mM Ca²⁺ buffer (Fig. 5). These results indicate that
desipramine causes Ca\(^{2+}\) influx or release of Ca\(^{2+}\) from endoplasmic reticulum. Consequently, increase in intracellular Ca\(^{2+}\) participates in desipramine-induced apoptosis of Hep3B cells. In conclusion, the current study provides new evidence that desipramine induces apoptosis of hepatocellular carcinoma cells by increasing ROS production, reducing MMP, promoting accumulation of intracellular Ca\(^{2+}\), and increasing the activity of MAPK proteins (ERK1/2, JNK, and p38). We further propose desipramine as a potential anticancer agent against HCC.

Acknowledgements

This study was supported by the research funds of Korean Ministry of Science (2011-0013872) and the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (2016R1A2B1010904).

References

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