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Nortriptyline induces mitochondria and death receptor-mediated apoptosis in bladder cancer cells and inhibits bladder tumor growth *in vivo*

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3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium Bromide (MTT) (PubChem CID: 64965)
DMSO (PubChem CID: 679)
Propidium iodide (PubChem CID:104981)
Tween 20 (PubChem CID:443314)
JC-1 (PubChem CID:5492929)
Dihydroethidium (DHE) (PubChem CID:128682)
N-acetyl-L-cysteine (NAC) (PubChem CID:12035)
Glutathione (GSH) (PubChem CID:124886)

ABSTRACT

Nortriptyline (NTP), an antidepressant, has antitumor effects on some human cancer cells, but its effect on human bladder cancer cells is not known. In this study, we used a cell viability assay to demonstrate that NTP is cytotoxic to human TCCSUP and mouse MBT-2 bladder cancer cells in a concentration and time-dependent manner. We also performed cell cycle analysis, annexin V and mitochondrial membrane potential assays, and Western blot analysis to show that NTP inhibits cell growth in these cells by inducing both mitochondria-mediated and death receptor-mediated apoptosis. Specifically, NTP increases the expression of Fas, FasL, FADD, Bax, Bak, and cleaved forms of caspase-3, caspase-8, caspase-9, and poly(ADP-ribose) polymerase. In addition, NTP decreases the expression of Bcl-2, Bcl-xL, BH3 interacting domain death agonist, X-linked inhibitor of apoptosis protein, and survivin. Furthermore, NTP-induced apoptosis is associated with reactive oxygen species (ROS) production, which can be reduced by antioxidants, such as N-acetyl-L-cysteine. Finally, we showed that NTP suppresses tumor growth in mice inoculated with MBT-2 cells. Collectively, our results suggest that NTP induces both intrinsic and extrinsic apoptosis in human and mouse bladder cancer cells and that it may be a clinically useful chemotherapeutic agent for bladder cancer in humans.

1. Introduction

Recently, the incidence and prevalence of bladder cancer (BCa) has been rising. It is the fourth and fifth most commonly diagnosed malignancy in men in Europe and the United States, respectively (Rosser et al., 2009; van den Bosch and Alfred Witjes, 2011), and the seventh most common malignant neoplasm of the urinary tract in Taiwan (Peng et al., 2006; Yuan et al., 2011). Approximately 75% of all newly diagnosed cases of bladder cancer are non-muscle-invasive BCa (NMIBC), while the remaining cases are muscle-invasive BCa (MIBC) (van den Bosch and Alfred Witjes, 2011). The standard therapies for NMIBC include transurethral tumor resection and intravesical chemotherapy and immunotherapy; however, about 30% of all tumors are refractory and about 50% recur within five years (Carradori et al., 2012; Leliveld et al., 2011). Similarly, MIBC cases that are treated with

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neoadjuvant chemotherapy plus radical cystectomy also tend to have poor prognosis and a mortality rate of about 50% (Dhawan et al., 2008). As a result, novel therapeutics for BCa are needed.

Tricyclic antidepressants (TCAs), such as amitriptyline and desipramine, which are commonly used to treat depression and chronic pain (Kirino and Gitoh, 2011; Dell and Butrick, 2006), also have antineoplastic activity in a wide variety of cancer cells, such as human colon cancer HT-29 cells, human osteosarcoma MG63 cells, human prostate cancer PC3 cells, rat glioma C6 cells, mouse skin squamous carcinoma (Ca3/7), and human multiple myeloma MM cells (Kabolizadeh et al., 2012; Lu. et al., 2009; Chang et al., 2008; Ma et al., 2011; Kinjo et al., 2010; Mao et al., 2011). Other TCAs, such as imipramine and clomipramine, induce apoptosis in HL-60 human acute myeloid leukemia cells by increasing production of reactive oxygen species (ROS), activating caspase 3, and disrupting the mitochondrial membrane potential (Xia et al., 1999a, 1999b). Similarly, nortriptyline (NTP; Fig. 1a), also exhibits anticancer activity in several different types of cells. For example, in human cutaneous melanoma cells, NTP has a half maximal inhibitory concentration (IC_{50}) of 9 μM compared with 27 μM and 33 μM for clomipramine and amitriptyline, respectively (Parker et al., 2012). In addition, NTP is cytotoxic to human osteosarcoma cells ($IC_{50} \approx 35$ μM) and induces apoptosis in PC3 cells ($IC_{50} > 50$ μM) by Ca^{2+}-mediated mechanisms (Hsu et al., 2004; Chih-Chuan et al., 2010). However, the antitumour effects of NTP in bladder cancer and their underlying mechanisms are not known. Therefore, in this study, we determined the antitumor effects of NTP in both mouse MBT-2 and human TCCSUP bladder cancer cells. We also investigated the mechanisms responsible for these effects.

2. Materials and methods

2.1. Cell culture and reagents

Human TCCSUP and mouse MBT-2 bladder cancer cell lines were cultured in RPMI medium supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA) and 1% antibiotic antimycotic solution (Gibco, Grand Island, New York). Cells were incubated at 37 °C in a 5% CO_2 atmosphere. NTP (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 100 mM and stored at −20 °C until use. 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium Bromide (MTT) powder, Propidium Iodide, N-acetyl-L-cysteine (NAC) and glutathione (GSH) were obtained from sigma company (St. Louis, MO, USA), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanineiodide (JC-1) and Dihydroethidium (DHE) were purchased from Invitrogen company(Carlsbad, CA, USA) and Setareh Biotech company (LLC, Eugene, OR, USA), respectively.

2.2. Animals

Adult male C3H/HeN mice (25–30 g body weight; 2-3 months of age) were obtained from the animal center of National Cheng Kung University. The animals were maintained in an air conditioned procedures were conducted to the guidelines of the Committee of Ethics in Research of Taichung Veterans General of Hospital. To create a mouse model of bladder cancer, we subcutaneously injected MBT-2 cells (1×10^7) into the right flank of 16-week-old male C3H/HeN mice. Ten days later, fifteen tumor-bearing mice (mean tumor volume = 50 mm^3) were divided into three groups of five and treated with either NTP (10 or 20 mg/kg) or vehicle control for three weeks. Every day, treated mice and control mice were injected intraperitoneally with 100 μl of NTP in 0.1% DMSO or vehicle only, respectively. Tumor volume was calculated as 0.5 × length × width × thickness.

2.3. Cell viability assays

The effect of NTP on the viability of TCCSUP and MBT-2 cells was determined visually by phase contrast microscopy and quantitatively by the 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium Bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA). First, TCCSUP and MBT-2 cells (2×10^4) were seeded onto 24-well plates and treated with NTP at concentrations of 6.25, 12.5, 25, 50 and 100 μM or vehicle alone at 37 °C in a CO_2 incubator for 24 h. Then, the RPMI culture medium with drug or vehicle control was removed and 200 μl from 1 mg/ml MTT solution was added to each well. Four hours later, the MTT solution was aspirated and the formazan product was solubilized in 600 μl of DMSO. Finally, a 150 μl aliquot was analyzed by using a microplate autoreader (PerkinElmer L225-0137, Taiwan) to measure the absorbance at 540 nm. All analyses were performed in triplicate. IC_{50} values were linearly interpolated from dose-response curves.

2.4. Cell cycle analysis

Propidium iodide (PI) staining and flow cytometry were used to perform cell cycle analysis. First, TCCSUP and MBT-2 cells (1×10^6) were plated on 10-cm dishes and incubated with either NTP or 0.1% DMSO as the vehicle control for 24 h. Specifically, TCCSUP cells were treated with 25 μM, 50 μM, or 100 μM NTP, while MBT-2 cells were treated with 12.5 μM, 25 μM, or 50 μM NTP. Subsequently, the floating cells and attaching cells, which were trypsinized, were combined, centrifuged at 450xg, washed with ice-cold phosphate-buffered saline (PBS) two times, and fixed with 70% ethanol at −20 °C overnight. The next day, the cells were washed with ice-cold PBS and then incubated with PI staining solution (0.2 mg/ml ribonuclease, 20 μg/ml propidium iodide, and 0.1% Triton X-100) for 30 min at room temperature in the dark. Finally, the cells were counted with a flow cytometer (BD, FACSCalibur San Jose, CA 95131, USA) and data were analyzed with WinMDI software (version 2.9). All experiments were performed in triplicate and 10,000 events were counted for each sample.

2.5. Annexin V assay

Apoptosis was measured by an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BioVision, Milpitas, CA, USA). The manufacturer's protocol was followed. Briefly, TCCSUP and MBT-2 cells (1×10^6) were seeded onto 10-cm dishes for 24 h, and then TCCSUP cells were treated with 0 μM (vehicle only), 25 μM, 50 μM or 100 μM NTP for 24 h and MBT-2 cells were treated with 0 μM (vehicle only), 12.5 μM, 25 μM or 50 μM NTP for the same period of time. Subsequently, the floating cells were collected and the attaching cells were harvested by trypsinization. Both floating and attaching cells were combined and centrifuged, washed twice with PBS, and resuspended in 500 μl of binding buffer. Cell suspensions were then incubated with 5 μl of annexin V-FITC and 5 μl of PI for 10 min at room temperature in the dark. Finally, the cells were analyzed by flow cytometry.

2.6. Western blot analysis

Western blotting was used to analyze protein expression. Specifically, cell lysates with equal amounts of protein, as determined by a Bradford assay (Biorad, Alfred Nobel Dr Hercules, CA, USA), were separated by 10–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then, proteins were electrophoretically transferred to polyvinylidene fluoride membranes, which were blocked with 5% nonfat milk in TBST buffer (20 mM Tris–HCl, 120 mM NaCl, and 0.1% Tween 20) for 1 h. Subsequently, the membranes were incubated with primary antibodies against