Chemico-Biological Interactions 199 (2012) 38–48

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Anticancer effect of tert-butyl-2(4,5-dihydrogen-4,4,5,5-tetramethyl-3-O-1H-imidazole-3-cationic-1-oxyl-2)-pyrrolidine-1-carboxylic ester on human hepatoma HepG2 cell line

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ARTICLE INFO

Article history:
Received 7 April 2012
Received in revised form 1 June 2012
Accepted 4 June 2012
Available online 13 June 2012

Keywords:
Nitroxyl nitroxide radical
Cytotoxicity
Reactive oxygen species
Oxidative damage
Anticancer effect

ABSTRACT

Tert-butyl-2(4,5-dihydrogen-4,4,5,5-tetramethyl-3-O-1H-imidazole-3-cationic-1-oxyl-2)-pyrrolidine-1-carboxylic ester (L-NNP) is a stable nitroxyl nitroxide radical, which have displayed cytotoxicity on human breast cancer MCF-7 and MDA-MB-231 cell lines. In the present study, we investigated the selective cytotoxicity of L-NNP on isogenetic human hepatoma HepG2 and normal L-02 cell lines. Cell growth inhibition, intracellular reactive oxygen species production, the mitochondrial membrane potential loss, malondialdehyde generation and glutathione levels were analyzed. The expression of Bax, Bcl-2 and NF-κBp65 proteins was also examined. The anticancer activity was evaluated in a HepG2 cell xenograft nude mice model. The results showed that 10, 20, 40 μg/ml L-NNP exposure for 48 h caused 52%, 82% and 91% cell growth inhibition of HepG2 cells, compared with 5%, 10% and 15% that of L-02 cells ($p < 0.01$). Concentrations of 10, 20, 40 μg/ml L-NNP induced cell death by increasing the generation of intracellular reactive oxygen species and MDA, by depolarizing the mitochondrial membrane potential, and by decreasing intracellular GSH levels in HepG2 cells. Western blot assay showed that Bax, Bcl-2 and NF-κBp65 might be implicated in L-NNP-induced selective HepG2 cell death. L-NNP was also found to inhibit HepG2 hepatoma growth and extend the life span of nude mice model ($p < 0.01$). The pretreatment and co-treatment of 10 mM N-acetyl-cysteine alleviated L-NNP exposure induced intracellular reactive oxygen species increase and cell growth inhibition demonstrated that L-NNP exhibited neoplasm-selective cytotoxicity and pro-apoptotic activities via reactive oxygen species mediated oxidative damage in HepG2 cells. It might be promising for developing a new class of anticancer agent for liver cancer.

1. Introduction

Nitroxyl nitroxide radicals are stable nitroxide, and have the characteristics of low molecular weight, permeability into biological membranes, and water solubility. They are widely used in electron paramagnetic resonance spectroscopy [1]. Many studies were performed on these compounds after it was demonstrated that they have antioxidant activity. They were shown to protect cardiomyocytes by repressing the formation of oxygen-derived free radicals [2], and some neuroprotective effects in central and peripheral nervous system models of Parkinson's disease were also observed [3]. Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) is a stable nitroxide that promotes the metabolism of many reactive oxygen species (ROS) and improves nitric oxide bioavailability. It has been studied extensively in animal models of oxidative stress and shown to protect mitochondria against oxidative damage and to improve tissue oxygenation. Tempol has only been used in human subjects as a topical agent to prevent radiation-induced alopecia [4]. Although nitroxides are generally considered non-toxic to mammalian cells following short-term exposure [5], it was suggested that they may be useful as anticancer agents by inhibiting the growth of various tumor cell types. It was reported that Tempol activated a program of apoptotic cell death in breast adenocarcinoma MCF-7 cells, possibly by inducing oxidative stress, and this was dependent on free radical-mediated mechanisms. A similar phenomenon was seen in the human promyelocytic leukemia HL60 cell line [6,7]. Furthermore, Tempol was found to have different radioprotective and cytotoxic effects on neoplastic cell

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http://dx.doi.org/10.1016/j.cbi.2012.06.001

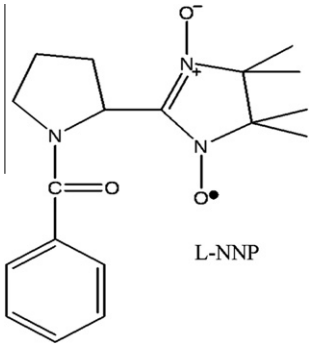

Fig. 1. The chemical constitution of Tert-butyl-2(4,5-dihydrogen-4,4,5,5-tetramethyl-3-O-1H-imidazole-3-cationic-1-oxyl-2)-pyrrolidine-1-carboxylic ester, i.e. L-NNP.

lines compared to non-neoplastic cell lines. The differences in radioprotection might result from enhanced intra-tumor bioreduction of Tempol to its non-radioprotective hydroxylamine analogue. Thus, the nitroxides as a class of compounds might provide a means to exploit the redox differences between normal tissues and tumors [8]. Tert-butyl-2(4,5-dihydrogen-4,4,5,5-tetramethyl-3-O-1H-imidazole-3-cationic-1-oxyl-2)-pyrrolidine-1-carboxylic ester (L-NNP) is a cyclical nitroxyl nitroxide radical, which chemical constitution is partially similar to Tempol (Fig 1). L-NNP is stable, water-soluble and cell membrane-permeable. A recent study by our group showed that L-NNP exhibited cytotoxicity in human breast adenocarcinoma cancer MCF-7 and MDA-MB-231 cell lines via ROS-mediated oxidative damage [9]. Tempol was found to have a selective cytotoxic effect on many cancer cell lines, even though all the tested neoplastic and non-neoplastic cell lines from breast, liver, and ovary, cell lines tested were treated equally [6]. The aim of this study was to investigate whether L-NNP exerted selective antiproliferative effects on isogenetic human hepatoma HepG2 and normal L-02 cell line, and to evaluate the possible molecular mechanism for the induction of cytotoxicity.

2. Materials and methods

2.1. Ethics statement

The Ethics Committee for Animal Experiments of the Fourth Military Medical University approved all animal work (Permit number: 10010) and the experimental protocols strictly complied with Institutional Animal Care and Use Committee guidelines of the Experiment Animal center of the Fourth Military Medical University. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

2.2. Chemicals and reagents

L-NNP was provided by Xiangyang Qin, Department of Chemistry, Fourth Military Medical University. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) were purchased from GIBCOTM Invitrogen Co., Beijing, China. Bicinchoninic acid (BCA) assay kit, reactive oxygen species assay kit and RIPA lysis buffer were purchased from Beyotime Institute of Biotechnology, Jiangsu, China. The MDA assay kit and GSH assay kit were purchased from Nanjing jiancheng Institute of Biotechnology, Xi'an, China. Anti-NF-κBp65, anti-Bcl-2, anti-Bax, anti-β-actin antibodies, goat anti-rabbit IgG and goat anti-mouse IgG conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology, Santa Cruz, USA. Annexin-V-FITC and PI apoptosis detection kit was purchased from BD PharMingen Co., CA, USA. 5-fluorouracil (5-FU) was purchased from XiJing hospital affiliated Fourth Military Medical University. All other chemicals were of analytical purity made in China.

2.3. Cell cultures and treatment

Human normal liver L-02, human hepatoma HepG2, human intestine epithelial HIEC, human colonic cancer SW620, human gastric epithelial GES and human gastric cancer MKN45 cell lines were obtained from the cell bank of Shanghai Institute of Cell Biology, Shanghai, China. Cells were cultured in DMEM medium supplemented with L-glutamine and 10% FBS at 37 °C/5% CO_2 in a humidified incubator. Cells were seeded for 24 h before treatments. L-NNP was dissolved in distilled water at a serial concentration and stored in 4 °C. The serial dilution of L-NNP were carried out (shortly before in use) with DMEM media. The serial dilution of L-NNP with DMEM media in cultured plates yielded final concentrations of 5, 10, 20, 40, 80, 160, 200 μg/ml. Untreated cells were used in all experiments as controls. Treatment times and the concentrations of L-NNP for each experiment were determined by prior optimization.

2.4. Cytotoxicity measurements by MTT assay and Annexin-V/propidium iodide staining

The MTT assay was performed as previously described [10]. After treatment of 5, 10, 20, 40 μg/ml L-NNP for 48 h on HepG2, SW620, and MKN45 cell lines, and 10, 20, 40, 80, 160, 200 μg/ml L-NNP for 48 h on L-02, HIEC, and GES cell lines. Cells were cultured for another 72 h in fresh DMEM, then treated with MTT (1 mg/ml in Phosphate Buffered Saline) and incubated for 4 h at 37 °C. The absorbance was read at 490 nm (reference 630 nm). The experiments were performed at least six times. HepG2 and L-02 cells were co-treated with the 10 mM ROS scavenger N-acetyl-cysteine (NAC) and 40 μg/ml L-NNP for 48 h, and then the MTT assay was performed as previous described.

Annexin-V/propidium iodide staining was used as a method to measure L-NNP induced cell death. HepG2 and L-02 cells were incubated with 10, 20, 40 μg/ml L-NNP for 24 h and 48 h. The Annexin-V was labeled with fluorescein isothiocyanate (FITC) to detect apoptotic cells, and PI was used to detect necrotic cells, the total cell death was calculated by the sum of apoptotic and necrotic cells. The assay was performed according to the manufacturer's instructions. Briefly, approximately 1×10^7 treated cells were washed in PBS, resuspended in Annexin-V/PI staining solution and then analyzed immediately by flow cytometric assay (BD FACS Aria). The experiments were performed at least six times.

2.5. Measurement of intracellular reactive oxygen species (ROS) level

The assay is based on the incorporation of DCFH-DA (2′,7′-dichlorofluorescein diacetate) into the cell. Reactive oxygen species are able to oxidize the cleaved DCFH to DCF, which is highly fluorescent at 530 nm [11]. To measure 10, 20, 40 μg/ml L-NNP induced ROS generation, HepG2 and L-02 cells were harvested after 24 h and 48 h exposure and washed twice with PBS, and then fresh medium containing 10 μM DCFH-DA was added to previously treated cells. After 15 min incubation, cells were washed again and then analyzed immediately by flow cytometric assay (BD FACS Aria). HepG2 and L-02 cells were pretreated for 4 h with the 10 mM ROS scavenger N-acetyl-cysteine (NAC) before 24 h of exposure to 40 μg/ml L-NNP. These treated cells were trypsinized, resuspended in PBS, incubated with fresh medium containing 10 μM DCFH-DA for 15 min, and then subjected to flow cytometric analysis. The experiments were performed at least three times.