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Original article

Niclosamide induces apoptosis through mitochondrial intrinsic pathway and inhibits migration and invasion in human thyroid cancer in vitro

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ABSTRACT

The morbidity of thyroid cancer has been rising obviously throughout the world during the past years. Classic treatment procedure is generally curable for low risk differentiated thyroid cancer, but may lead to many postoperative complications. And low-level of thyroid stimulating hormone after surgery has side effects on both cardiovascular system and skeletal system. Furthermore effective treatment approaches for more aggressive differentiated thyroid cancer, poorly differentiated thyroid cancer and anaplastic thyroid cancer are absent, thus new candidates that can inhibit tumor growth and metastasis are urgently needed. In this study, niclosamide, an FDA approved anthelminthic drug, was evaluated for its anti-thyroid cancer activity in vitro. Niclosamide potently inhibited cell proliferation and induced apoptosis in human papillary thyroid cancer cell lines TPC-1 and BCPAP, as well as anaplastic thyroid cancer cell line ACT-1. In addition, the occurrence of TPC-1 apoptosis was correlated with activation of Bax and cleaved caspases-3, and inhibition of Bcl-2 and the mitochondrial membrane potential (ΔYm), indicating that niclosamide may induce apoptosis through a mitochondria-mediated intrinsic apoptotic pathway. Moreover, niclosamide markedly impaired TPC-1 cells and ACT-1 cells invasion. And we further found the inhibitory effect of TPC-1 was closely related with down-regulating of matrix metalloproteinase (MMP)-2 and −9 and up-regulating of tissue inhibitor of metalloproteinase (TIMP)-2. Taken together, these results demonstrated that niclosamide could be a potential agent for inhibiting the growth and metastasis of thyroid cancer.

1. Introduction

In recent decades, as the most common endocrine malignancy, the morbidity of thyroid cancer(TC) has rapidly increased in global [1,2]. Papillary thyroid cancer (PTC), the most common subtype of thyroid cancer, accounts for almost 80–90% of all thyroid malignances and carries the best overall prognosis [3,4]. But overtreatment problems for low risk disease arise frequently. And both postoperative complications and side effects of low TSH indicated after surgery leave challenges for surgeons and physicians [5]. Moreover, although surgical resection, with or without radio therapy and thyroid stimulating hormone (TSH) suppression shows good prognosis, the recurrence rates among PTC patients are approximately 20% at 10yr of follow-up [6,7].

Abbreviations: PTC, papillary thyroid carcinoma; ATC, anaplastic thyroid carcinoma; TC, thyroid cancer; TSH, thyroid stimulating hormone; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; DMSO, dimethyl sulfoxide; Rh123, 2-(6-Amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; CC-3, cleaved caspase-3; FBS, fetal bovine serum; PBS, phosphate-buffered saline; FCM, flow cytometry; ΔΨm, mitochondrial transmembrane potential; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; SD, standard deviation; Apaf-1, apoptotic protease activating factor 1; ECM, extracellular matrix.

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Anaplastic thyroid cancer(ATC) is the most aggressive form of thyroid cancer with the poorest prognosis, and the benefits of present treatments are very limited [8]. Therefore, new candidates for thyroid cancer that exert potential antitumor activity are urgently needed. RET/PTC rearrangements and BRAF mutations are the top two alternative genetic alterations in the progress of PTC, respectively accounting for 20% and 48%, and are two major events in pathogenesis [9–12]. Cancers with BRAF mutations have proven to lose the affinity to radioiodine therapy, a common post-operative approach of PTC, and to harbor high-risk clinicopathological characteristics [12].

Niclosamide, a kind of salicylamide derivative, whose chemical name is 2′,5-dichloro-4′-nitrosalicylanilide, is an FDA approved oral anthelminthic drug that has been used for treating tapeworm infections for nearly 50 years [13,14]. In recent years, antitumor activity of niclosamide has been recognized gradually. Niclosamide is cytotoxic, and is an attractive candidate of cancer treatment for its potent inhibitory effects on a panel of tumor cell lines [15]. It was reported that niclosamide could induce apoptosis of breast cancer, acute myeloid leukemia and renal cell carcinoma cells, interfere with cancer-driving signaling cascades in glioblastoma, and inhibite colon cancer metastasis, etc. [14,16–20]. However, the therapeutic effects of niclosamide on thyroid cancers remain to be determined, and further researches to elucidate the mechanisms of niclosamide are needed.

The aim of this study was to provide evidences for wide application of niclosamide in TC prevention and treatment in vitro.

2. Materials and methods

2.1. Chemicals and reagents

Niclosamide was purchased from (Heowns Biochem LLC, Tianjin, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), dimethyl sulfoxide (DMSO), 2-(6-Amino-3-imino-3H-xanthen-9-yl) benzoic acid methyl ester (Rh123) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Hoechst 33258 was purchased from Beyotime (Beijing, China). The Annexin V-FITC apoptosis detection kit was purchased from KeyGen Biotech (Nanjing, China). The primary antibodies against cleaved caspase-3 (CC-3), Bax, tissue inhibitor of metalloproteinase-2 (TIMP-2), matrix metalloproteinase-9 (MMP-9) and β-actin were purchased from Cell Signaling Technology Co. (Beverly, MA, USA); Bcl-2 and matrix metalloproteinase-2 (MMP-2) were from Abcam (Cambridge, MA, USA), and secondary antibodies were from ZSGB-BIO Co. (Beijing, China).

Niclosamide was prepared initially as a 20 mM stock solution in DMSO and stored at −20 °C. Then the stock solution diluted in the relevant assay medium, and 0.1% DMSO served as a vehicle control.

2.2. Cell culture

The TPC-1 cell line, BCPAP cell line, derived from human papillary thyroid carcinoma, separately harboring RET-PTC rearrangement and BRAF mutation, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and propagated in RPIM 1640 medium (Gibco, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Cao Yuan Lv Ye Bio-engineering, Hohhot, China) and 1% antibiotics (penicillin and streptomycin) in 5% CO_2 at 37 °C. ACT-1 cell line, originated from anaplastic thyroid cancer, was kindly provided by Stem Cell Bank, Chinese Academy of Sciences and propagated in DMEM medium (Gibco, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Cao Yuan Lv Ye Bio-engineering, Hohhot, China) and 1% antibiotics (penicillin and streptomycin) in 5% CO_2 at 37 °C.

2.3. Cell viability assay

The cell viability of niclosamide-treated thyroid cancer cells was assessed by MTT assay [21]. Briefly, the exponentially growing cells (3000–5000 cells/well) were seeded in 96-well plates. After 24 h incubation, the cells were treated with various concentrations of niclosamide (0, 1.25, 2.5, 5, 10, 20 μM). After treatment for 24 h, 48 h and 72 h, respectively, the 20 μL of a 5 mg/mL MTT was added to each well for 2 ∼ 4 h incubation at 37 °C. The medium was subsequently removed, and 150 μL of DMSO per well was added to dissolve the formazen. The absorbance was recorded at 570 nm using a Spectra MAX M5 microplate spectrophotometer (Molecular Devices, CA, USA) and the median inhibitory concentration (IC50) values were calculated by Excel. Taking NORMSVIN (% viability) + 5 on Y axis, and log10 (concentration) on X axis, then plotting a curve, and the curve formula(F) was obtained by regression analysis. $X_{IC50} = F_{(Y=5)}$. Each experiment was replicated at least 3 times.

2.4. Colony formation assay

Colony formation assay was measured as previously described [22]. Briefly, TPC-1 cells (2000/well), BCPAP cells (2000 cells/well), and ACT-1 cells(3000/well) were seeded in 6-well plates, respectively. After 24 h incubation, the cells were treated with designed concentrations of niclosamide (0, 1.25, 2.5, 5, 10, 20 μM) and cultured for additional 12 days. Then, the cells were washed with cold phosphate-buffered saline (PBS), and the colonies were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet staining solution (Beyotime, Beijing, China) for about 15 min and the colonies (>50 cells) were counted under microscope. The data shown represents the average of three independent experiments.

2.5. Apoptosis analysis by hoechst staining

Apoptotic cells have unique morphologic characteristics: cell body shrinkage, chromatin condensation and margination as well as apoptotic bodies [23]. To identify the apoptosis-inducing effects of niclosamide, we analyzed the apoptotic cells by Hoechst 33258 staining. In brief, TPC-1 cells (2×10^5 cells/well) were plated onto 18-mm cover glass in 6-well plates for 24 h. After treatment with different concentrations (0, 1.25, 2.5, 5, 10, 20 μM) of niclosamide for following 48 h, the cells were washed with cold PBS twice and fixed in methanol for 15 min. The cells were stained with Hoechst 33258 solution according to manufacturer's instructions. Then the cells were photographed under a fluorescence microscopy (Olympus, BX53, Japan).

2.6. Apoptosis analysis by flow cytometry (FCM)

To further confirm the apoptosis induction effects of niclosamide, AnnexinV-FITC apoptosis detection kit was used [24]. Briefly, cells (2×10^5 cells/well) were seeded in 6-well plates and treated with niclosamide (0, 1.25, 2.5, 5, 10, 20 μM) respectively for 48 h. Then the cells were harvested and washed twice with cold PBS. The apoptosis levels were detected by FCM using the apoptosis detection kit and the data were analyzed with FlowJo software. The data shown represents the average of three independent experiments.

2.7. Detection of mitochondrial membrane potential ($\Delta\Psi m$) and reactive oxygen species (ROS)

Mitochondrial membrane potential assay was performed as previously described and detected by FCM using Rh123 staining