Biomedicine & Pharmacotherapy 81 (2016) 482–490

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

A novel small-molecule YLT256 inhibits proliferation and induces apoptosis both *in vitro* and *in vivo* in solid tumors

Hongxia Deng[1], Jun Zeng[1], Yongxia Zhu, Tinghong Ye, Yong Xia, Yaojie Shi, Ningyu Wang, Lidan Zhang, Xuejiao Song, Weiqiong Zuo, Tiantao Gao, Luoting Yu*

State Key Laboratory of Biotherapy/Collaborative Innovation Center for Biotherapy, West China Hospital, West China Medical School, Sichuan University, Chengdu, China

ARTICLE INFO

Article history:
Received 31 December 2015
Received in revised form 19 April 2016
Accepted 19 April 2016

Keywords:
YLT256
Human pancreatic cancer
Apoptosis
Mitochondrial apoptosis pathway

ABSTRACT

Pancreatic carcinoma is a still unsolved health problem all over the world with poor prognosis and high mortality rate. YLT256, a novel synthesized chemical small inhibitor, displays potent antineoplastic activities *via* inducing apoptosis both *in vitro* and *in vivo*. In this study, we found that YLT256 showed growth inhibition against a broad spectrum of human cancer cell lines and pancreatic cancer cell line BxPc-3 was the most sensitive with an IC_{50} of 0.42 μM. We also found YLT256 could induce apoptosis of BxPc-3 cells in a dose-dependent manner. Western blot analysis revealed that the occurrence of its apoptosis was associated with activation of caspases-3 and -9, up-regulation of pro-apoptotic Bak, and down-regulation of anti-apoptotic Bcl-2. Moreover, YLT256-treated resulted in changes of mitochondrial membrane potential ($\Delta\psi m$), and generation of reactive oxygen species (ROS). Furthermore, our data also revealed that YLT256 suppressed the growth of established tumor-bearing xenograft models without obvious side effects. Immunohistochemical analyses and TUNEL assay revealed an increase in cleaved caspase-3-positive cells and TUNEL-positive cells, a decrease in Ki67-positive cells upon YLT256. Together, all the results of present study provided evidence demonstrating that YLT256 could be a promising potential drug candidate for pancreatic cancer therapy.

1. Introduction

Pancreatic cancer is one of the most devastating human malignancies, with a median survival of 6.7 months and a 5-year relative survival rate of only 6% [1,6]. There are about 48,960 cases pancreatic carcinoma diagnosed in 1,658,370 estimated new cancer, and estimated deaths are highly 40,560 cases in the United States in 2015 [2]. Although the lung cancer will still rank the number one cancer killer throughout the entire period, deaths due to pancreatic cancer are projected to increase dramatically to become the second leading cause of cancer-related deaths before 2030 [3]. Due to insensitive to chemotherapy and radiotherapy, surgery is the only potentially strategy for pancreatic cancer treatment but less than 20% of patients are eligible for surgical resection [4]. However, after curative resection of pancreatic cancer, more than 80% of patients have a recurrence [5]. The current drugs for pancreatic cancer treatment included gemcitabine, the pro-drug of gemcitabine, and combinations with paclitaxel [6,7]. However, owing to the onset of chemo-resistance, these drugs resulted in very modest benefit for increasing in median life expectancy of pancreatic cancer [8]. Many years have past, the survival rate of pancreatic cancer has not improved [9]. Therefore, more effective strategies for cancer therapy are urgently needed.

Most chemotherapeutic drugs kill cancer cells by triggering apoptosis, so apoptosis targets are currently being explored for anticancer drug discovery [10–12]. The apoptosis pathway is composed of both upstream regulators and downstream effectors [13]. The extrinsic apoptotic pathway and the mitochondrial-

Abbreviations: Bcl-2, B-cell lymphoma 2; Bak, Bcl-2 antagonist/killer-1; $\Delta\Psi m$, mitochondrial membrane potential; ROS, reactive oxygen species; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-di-phenyl-2*H*-tetrazoliumbromide; PI, propidium iodide; Rh123, rhodamine-123; DCFA-DA, 2′7′-dichlorofluoresein diacetate; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle medium; FCM, flow cytometry; IC_{50}, median inhibitory concentration; RIPA, radioimmunoprecipitation assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; TUNEL, transferase-mediated dUTP nick end-labeling; H&E, hematoxylin and eosin.

* Corresponding author at: State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, 17#3rd Section, Ren Min South Road, Chengdu 610041, China.
E-mail address: yuluot@scu.edu.cn (L. Yu).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.biopha.2016.04.038
0753-3322/

mediated intrinsic pathway make up the upstream regulators. The intrinsic pathway requires disruption of the mitochondrial membrane and the release of mitochondrial proteins, and the regulation by the opposing actions of pro- and anti-apoptotic Bcl-2 family members [14,15]. Anti-apoptotic Bcl-2 protein binds to and suppresses two pro-apoptotic triggering proteins (Bax and Bak), which are embedded in the mitochondrial outer membrane [16]. Once relieved of inhibition by their anti-apoptotic members, the pro-apoptotic members are activated while the anti-apoptotic members are inhibited, then the integrity of the mitochondrial membrane is disrupted, which result in release of cytochrome c [13,16]. Released cytochrome c leads to activate the pro-caspase-9 and subsequent activate the central executor of apoptosis caspase-3 and/or caspase-7, because they can process their substrates leads to morphological changes associated with apoptosis, such as chromatin condensation, and membrane blebbing [17–19].

The cytochrome c released from the inner membrane of mitochondria precedes the loss of mitochondrial membrane potential ($\Delta\Psi$m) in cells [20]. The disruption of $\Delta\Psi$m leads signs of apoptosis and high levels of reactive oxygen species (ROS) [21,22]. In general, cytochrome c would be released from mitochondria and triggers caspase activation, which will induce apoptosis. Mitochondria is the main source of ROS, and persistent with high levels of ROS could lead to apoptotic cell death [22,23].

In this study, our research group has found a novel amino thiadiazoled derivative, 4-(((5-amino-1,3,4-thiadiazol-2-yl)thio) methyl)-*N*-(4-(trifluoromethyl)phenyl)benzamide (YLT256), which displayed anti-proliferative activity *in vitro*. We also demonstrated that YLT256 could induce apoptosis in pancreatic cancer cell *via* the mitochondrial apoptosis pathway *in vitro* and inhibit tumor-bearing xenograft models by inducing apoptosis *in vivo*.

2. Materials and methods

2.1. Drugs and regents

YLT256 (Fig. 1) was synthesized by our group (State Key Laboratory of Biotherapy, Sichuan University, Sichuan, China). And the structure of YLT256 was confirmed by ^{1}H NMR, ^{13}C NMR and HRMS (ESI). YLT256 was dissolved with dimethyl sulfoxide (DMSO) at a stock solution of 40 mM and was stored at −20 °C. For all *in vitro* assays, YLT256 was diluted to the appropriate concentration with cell culture medium at final DMSO concentration of 0.1% (V/V). For *in vivo* experiment, YLT256 was dissolved in ultrapure water and Cremophor EL/ethanol (50:50, Sigma Cremophor EL, 100% ethyl alcohol).

3-(4,5-dimethyl-2-thiazolyl)-2,5-di-phenyl-2*H*-tetrazolium bromide (MTT), propidium iodide (PI), Rhodamine-123 (Rh123), 2′7′-dichlorofluoresein diacetate (DCFA-DA), Hoechst 33342, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co (St. Louis, MO). Annexin V-FITC/PI apoptosis detection kit was obtained from BD Biosciences (San Diego, CA). The antibodies against cleaved caspase-3, cleaved caspase-9, Bcl-2, Bak were purchased from Cell Signaling Technology Company (Beverly, MA). Antibody against β-actin was acquired from Santa Cruz Biotechnology Company (Santa Cruz, CA). Antibodies against Ki67 and CD31 were acquired from Abcam Company.

2.2. Cell culture

The human cancer cell lines BxPc-3, PANC-1, A375 and other cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Bel-7402, SMMC-7721 and H1975 cancer cell lines were acquired from the China Center for Type Culture Collection (CTCCC, Wuhan, China). These cells were cultured in Dulbecco's modified Eagle medium (DMEM) or RPIM 1640 supplemented with 10% fetal bovine serum (FBS; Gibco, Auckland, N.Z.), 100 unit/mL penicillin, and 100 μg/mL streptomycin in a humid chamber at 37 °C under 5% CO_2 in atmosphere.

2.3. Cell proliferation assay

The cell viability of YLT256 treatment were performed by MTT assay. Briefly, the exponentially growing cells were seeded in 96-well plates at a density of 2–5 × 10^3 cells/well and cultured for 24 h. After treatment with various concentrations of YLT256 for 72 h, a volume of 20 μL of 5 mg/mL MTT solution was added to each well and incubation for 2–4 h at 37 °C. 0.1% DMSO was added to the vehicle. The medium was subsequently discarded and 150 μL of DMSO was added to each well to dissolve the formazan crystal produced by living cells. 5 min later, the 96-well plates were read on Spectra MAX M5 microplate spectrophotometer (Molecular Devices) at 570 nm wavelength, and the median inhibitory concentration (IC_{50}) of each cell line was calculated. The results were obtained from at least three independent experiments.

2.4. Colony formation assay

To determine the survival of the BxPc-3 cells treated with YLT256, the cells were seeded in six-well plates at a density of 400–500 cells/well and cultured for 24 h, then treated with various concentrations of YLT256 (0–5 μM) for 72 h. Then the cells were re-fed once every 3 days for another 13 days with fresh cell culture medium and then fixed with 4% paraformaldehyde by 30 min and stained with 0.5% crystal violet.

2.5. Morphological analysis after Hoechst staining

Hoechst 33342 staining is used to detect morphological changes associated with apoptosis. Briefly, the BxPc-3 cells were plated onto 18-mm cover glass in 6-well plates and incubated for 24 h, then treated with YLT256 for 72 h. Cells were rinsed with cold PBS (Phosphate Buffered Saline, PH = 7.4) and then fixed with 4% paraformaldehyde solution for 15 min. The cells were subsequently stained in 5 μg/mL Hoechst 33342 solutions followed by PBS washing. Nuclear morphological changes of the apoptosis cells were observed under fluorescence microscope (Zeiss, Axiovert 200, Germany).

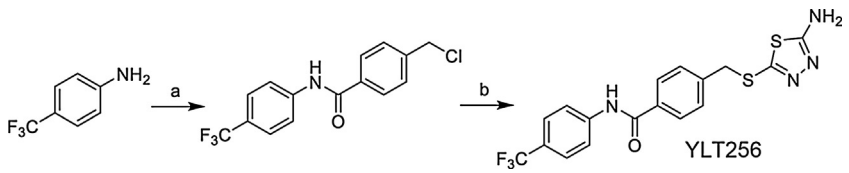

Fig. 1. The synthesis route of YLT256. (a) 4-(chloromethyl)benzoyl chloride, dichloromethane, triethylamine, reflux; (b) ethanol, 2-amino-5-sulfanyl-1,3,4-thiadiazole, NaOH, reflux.