Original Article

Inhibition of c-Myc by 10058-F4 induces growth arrest and chemosensitivity in pancreatic ductal adenocarcinoma

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A B S T R A C T

Pancreatic ductal adenocarcinoma (PDAC) is a formidable medical challenge due to its malignancies and the absence of effective treatment. c-Myc, as an important transcription factor, plays crucial roles in cell cycle progression, apoptosis and cellular transformation. The c-Myc inhibitor, 10058-F4, has been reported act as a tumor suppressor in several different tumors. In current study, the tumor-suppressive roles of 10058-F4 was observed in human pancreatic cancer cells in vitro as demonstrated by decreased cell viability, cell cycle arrest at the G1/S transition and increased caspase3/7 activity. And tumor responses to gemcitabine were also significantly enhanced by 10058-F4 in Panc-1 and SW1990 cells. In a subcutaneous xenograft model, however, 10058-F4 showed no significant influence on pancreatic tumorigenesis. When combined with gemcitabine, tumorigenesis was drastically attenuated compared with gemcitabine group or 10058-F4 group; this synergistic effect was accompanied with decreased PCNA-positive cells and reduced TUNEL-positive cells in the combined treated group. Subsequent studies revealed that decreased glycolysis may be involved in the inhibitory effect of 10058-F4 on PDAC. Taken together, this study demonstrates the roles of 10058-F4 in PDAC and provides evidence that 10058-F4 in combination with gemcitabine showed significant clinical benefit over the usage of gemcitabine alone.

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1. Introduction

Pancreatic cancer is one of the most lethal human malignancies with a 5-year survival of 5% and a median survival of 6 months when all stages are combined [1]. Among all currently available treatments, surgical resection is the option with most potential for cure. However, 80–85% of PDAC patients are unresectable by the time of diagnosis due to aggressive growth, early local invasion and tumor metastasis [2]. Gemcitabine (2’-Deoxy-2’-2’-difluorocytidine; GEM) based chemotherapy has been used as first-line treatment for patients with locally advanced or metastatic disease [3]. However, given that the tumor response to single agent GEM is becoming less effectively due to high resistance and many combined treatments failed to improve the overall survival of PDAC patients, it is urgent to develop novel and more effective chemotherapeutic strategies.

The proto-oncogene c-Myc encodes a basic loop-helix-loop zipper (bHLHZip) transcription factor that plays crucial roles in cell proliferation, apoptosis, differentiation and metabolism [4]. The bHLHZip domain of c-Myc is necessary for the dimerization with Max to bind the specific DNA sequences in the promoters of targeted genes, while the N-terminal domain interacts with other co-factors to regulate transcription [5]. Dysregulation of c-Myc as seen in several types of human neoplasias is also a common event in PDAC. Previous studies have demonstrated that dysregulated c-Myc is involved in the development and progression of PDAC [6]. The potential of targeting c-Myc has been reported in several different tumors [7–11]. One of the most studied small molecule c-Myc inhibitor targeting c-Myc-Max dimerization, 10058-F4, has been proved promotes apoptosis, inhibits cell proliferation and confers chemosensitivity [12]. Meanwhile, it has been recognized that cancer cells exhibit a unique metabolic phenotype characterized by enhanced glycolysis even in the presence of oxygen. Data from proteomic analyses have demonstrated that expression of glycolytic enzymes is increased in pancreatic cancer and these changes favors PDAC progression [13]. Given that c-Myc regulates the expression of several glycolytic enzymes including...
hexokinase 2 (HK2) and lactate dehydrogenase A (LDHA), we hypothesize that whether 10058-F4 enhances GEM chemosensitivity in PDAC through regulating glycolytic enzymes expression and glucose utilization.

In the present study, we firstly observed the anti-cancer effect of 10058-F4 in PDAC cell lines, PANCl and SW1990; treatment with 10058-F4 sensitizes PANCl and SW1990 cells to GEM. Through the use of a subcutaneous xenograft model and in vitro assays, we demonstrated that the anti-cancer effect of GEM was more significant when combined with 10058-F4. Mechanistically, this effect may be due, in part, to the decreased glycolysis induced by 10058-F4.

2. Materials and methods

2.1. Preparation of reagents and cell culture

10058-F4 (Selleck Chemicals, Houston, TX, USA) was dissolved in dimethylsulfoxide (DMSO) and further diluted to preferable concentrations in culture medium before use. Gemcitabine (Selleck Chemicals, Houston, TX, USA) was dissolved in 0.1 M hydrochloric acid. The final concentrations of solvents in the media were less than 0.1%. Human PDAC cell lines PANCl and SW1990 were all obtained from American Type Culture Collection. Cells were cultured in DMEM media supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotics at 37 °C in a humidified incubator with 5% CO2.

2.2. Immunohistochemistry and TUNEL staining

Tumor tissue sections were deparaffinized and rehydrated. After neutralization of endogenous peroxidase and preincubated in blocking serum, slides were then incubated overnight at 4 °C with primary antibody (PCNA, Proteintech). After washing in phosphate-buffered saline (PBS) for three times, the sections were labeled by HRP (rabbit) secondary antibody for 1 hour and again washed three times with PBS. The reaction products were visualized with 3, 3′-diaminobenzidine tetrahydrochloride and counterstained by hematoxylin. Then, the positive cells in six fields at random were calculated for further analysis. The TUNEL staining was performed according to manufacturer’s protocols (Roche, Applied Biosystems) and the TUNEL-positive cells were manifested by eosin and calculated as described above.

2.3. Quantitative real-time PCR

Total RNA from PANCl and SW1990 cell lines or frozen tissue samples were harvested using Trizol reagent (Takara, Japan) and reverse transcription were performed using the PrimeScript RT-PCR kit (Takara, Japan). The cDNA was amplified and quantified using an ABI Prism 7500 Sequence Detection System with SYBR Green Master Mix (Takara, Japan). Specific primer sequences used were as follows: H2K2: forward: 5′-TTGACCGAGGATGTACAGG-3′, reverse: 5′-CAACGCGATCAGACCTCA-3′; LDHA: forward: 5′-ATGCACCTCTAAGATGCAC-3′, reverse: 5′-CACCCTCAACACTGTTACT-3′; β-actin: forward: 5′-CATGTAAGTTGTCTAT-CAGGC-3′, reverse: 5′-CTCCTTTAATGCAGGAGAT-3′.

2.4. Cell viability, cell cycle, caspase-3/7 activity assay

For cell viability assay, cells (3 × 105) were seeded into a 96-well plate per well supplemented in the presence of 10% FBS (v/v) and cultured overnight. After treatment with 10058-F4 or GEM for 24, 48, 72, 96 and 120 hours, cell viability was evaluated by Cell Counting Kit-8 (CCK8, Dojindo, Japan) following the manufacturer’s protocols. The absorbance of each sample was measured at 450 nm using a Power Wave XS microplate reader (BIO-TEK). The cell viability was calculated by the percentage of treated cells relative to that of solvent controls. For cell cycle analysis, cells were cultured for 48 hours after 10058-F4 treatment and then fixed into 70% ethanol at −20 °C for 24 hours. The distribution of cell cycle was monitored by flow cytometric analysis of the DNA content of cell populations stained with propidium iodide. The percentage of cells within G1/S and G2, and M phases was determined by using CELLQUEST software (Becton Dickinson). The caspase 3/7 activity assay was performed according to manufacturer’s instructions (Promega).

2.5. Tumorigenicity in a mouse xenograft model

Each aliquot of approximately 5 × 106 of PANCl cells were implanted subcutaneously into the right flank of BALB/c athymic mice. After 3 weeks, the mice were randomly grouped into four groups with six animals in each group. Mice were intraperitoneally administered either PBS (control), 10058-F4 (15 mg/kg), or GEM (50 mg/kg) alone or a combination of both 10058-F4 and GEM every 3 days. Tumor sizes were measured every 3 days and mice were sacrificed for analysis of tumor burden after 30 days. All procedures were performed in accordance with The Animal Care and Use Committee of Hebei Medical University, Shijiazhuang, China.

2.6. Statistical analysis

Data were presented as the means ± standard error of the mean (SEM). Statistical analyses and graphical representations were performed with SPSS 16.0 (SPSS Inc., Chicago, USA) and GraphPad Prism 5 (San Diego, CA) software. The student’s t test was used for comparison between groups. Values of P < 0.05 were considered statistically significant.

3. Results

3.1. c-Myc inhibitor, 10058-F4, inhibits cell proliferation, induces cell apoptosis and enhances chemosensitivity of PDAC cells to GEM

To observe the potential inhibition effect of 10058-F4 in PDAC, we first measured the IC50 value of 10058-F4 in PANCl and SW1990 cells by CCK8 assay. As shown in Fig. 1A, the IC50 value of 10058-F4 were 25 μM for PANCl and 17 μM for SW1990 cells at 48 hours, respectively. Then, cell viability of PANCl and SW1990 cells were detected after treatment with 10 μM 10058-F4. Consistent with the effect of 10058-F4 in ovarian cancer or hepatocellular carcinoma [9,14], the cell viability of both cancer cell lines was reduced after exposure to 10058-F4 for 5 days (Fig. 1B). To examine whether the reduced cell viability by 10058-F4 was caused by growth inhibition or by apoptosis, we performed cell cycle and cell apoptosis assay. The activity of caspase 3/7, which plays key effector roles in apoptosis in mammalian cells, was increased after 10058-F4 treatment (Fig. 1C). And 10058-F4 treatment also resulted in a significant decrease in the percentage of cell population in G0/G1 phase and a sharp increase in S phase (Fig. 1D). As patients with PDAC have an intense desmoplasia which makes them unable to receive high doses of chemotherapy, we investigated whether 10058-F4 sensitizes PDAC cells to GEM. After treatment with 5 μM 10058-F4, which shows no cytotoxicity to PDAC cells, for 8 hours, PANCl or SW1990 cells were treated with different concentrations of GEM as shown in Fig. 1E. The sensitivity of PANCl-1 or SW1990 cells to GEM was remarkably enhanced by 10058-F4, indicating that c-Myc inhibitor not only inhibits cell proliferation and induces cell apoptosis when used alone but also proved to be a potential target for adjuvant therapy.
3.2. 10058-F4 treatment enhances chemosensitivity to GEM in vivo

To further investigate the effect of 10058-F4 in regulating the sensitivity of PDAC cells to GEM, a subcutaneous xenograft model was used to examine the in vivo role of 10058-F4 on PDAC growth. As shown in Fig. 2A, 3 weeks after inoculation, mice were treated as described in methods and tumor volume was measured every 3 days. The results showed that GEM whether used alone or combined with 10058-F4 significantly inhibited tumor growth and the combined treated group exhibited an advantage over the GEM treated group. However, inconsistent with previous findings in cell lines, 10058-F4 alone did not show an implication on tumor growth. Besides, cells in proliferation or face apoptosis were detected by PCNA and TUNEL staining, respectively. Compared with control group, there are decreased PCNA-positive cells and increased TUNEL-positive cells in GEM and GEM + 10058-F4 treated group, but not in the 10058-F4 treated group (Fig. 2B–D). Meanwhile, GEM + 10058-F4 treated group showed a faint advantage over GEM treated group in the inhibitory effect of tumor growth (Fig. 2D).

3.3. The alternations in glucose metabolism induced by 10058-F4 inhibits cell proliferation and enhances chemosensitivity to GEM

Increased glucose and glutamine consumption and enhanced metabolic activity of PDAC have been linked to its aggressiveness [15,16]. The c-Myc oncogene regulates many cellular metabolism including glucose metabolism, glutamine metabolism...
and mitochondrial metabolism through the regulation of related genes [17]. Here, we detected the expression of two glycolytic enzymes, HK2 and LDHA in the treated group from cell lines (Fig. 3A and B) and tissues from subcutaneous xenograft model (Fig. 3C). Expectedly, the expression of HK2 and LDHA were significantly reduced in GEM + 10058-F4 and GEM + 10058-F4 treated group compared with GEM treated group or the control group. Furthermore, no significant difference was found between GEM treated group and the control group. Given the alternation in glycolytic enzymes, we hypothesized whether the glycolysis associates with the chemosensitivity. To test this hypothesis, the glucose in the medium was replaced by galactose to inhibit glycolysis. Indeed, the decreased cell viability and increased chemosensitivity induced by 10058-F4 was completely abolished by galactose treatment (Fig. 3D and E). Collectively, these data above indicate that the changes happened in glucose metabolism may account for the tumor suppressive roles of 10058-F4 in PDAC.

4. Discussion

Amplification of c-Myc gene is evident in the majority of previously established PDAC cell lines and dysregulated c-Myc may lead to a poorer outcome in several types of cancer. Accumulating evidence suggests that c-Myc inhibitor exhibits potent anticancer activities due to its involvement in cell proliferation, apoptosis, differentiation and metabolism [18]. Consideration of the promising experience in c-Myc targeting strategies, a small molecule inhibitor of c-Myc, 10058-F4, was used to examine its effect in PDAC.

Consistent with previous reports [6,7,9], inhibition of c-Myc activities by 10058-F4 inhibited cell proliferation, promoted cell apoptosis and enhanced chemosensitivity in vitro (Fig. 1). However, tumor burden was not affected by 10058-F4 treatment and the PCNA-positive cells or TUNEL-positive cells in 10058-F4 treated group also did not show a significant difference compared with the control group (Fig. 2). The efficacy, pharmacokinetics, tissue distribution and metabolism of 10058-F4 was analyzed in a prostate cancer xenografts previously [19] and no significant anticancer effect was found after treatment of mice with either 20 or 30 mg/kg 10058-F4 due to its rapid metabolism and low concentration in tumors. Dense fibrotic stromal matrix (desmoplasia), which is mainly composed of extracellular matrix, activated fibroblasts and inflammatory cells, is one histological feature of PDAC and together can comprise up to 90% of the tumor volume [20]. Given the poorer vascular and blood perfusion induced by desmoplasia in PDAC, it is reasonable to explain the negative effect of 10058-F4 in vivo.
Intracellular ATP levels are a pivotal determinant of chemoresistance in cancer cells [21]. A broad spectrum of metabolic genes including GLUT1, HK2, phosphofructokinase (PFKM), enolase 1 and LDHA were regulated by c-Myc, implying the important role of c-Myc in regulating glycolysis, a process with rapid ATP production [22]. Here, we demonstrated that the discrepancies of HK2 and LDHA expression between GEM + 10058-F4 treated group and GEM treated group. And this discrepancy ultimately resulted in decreased glycolysis, a process to support anabolism, which enhances the toxicity of GEM. In agreement with a study focused on ovarian cancer, therapeutic stress induced by 10058-F4 resulted in enhanced chemosensitivity of PDAC to GEM. Inconsistent with a report that c-Myc induces cisplatin chemosensitization which is mediated by suppression of cyclin D1 expression and nuclear factor–κB activity in PDAC [23], however, our results advocate the combination of GEM with 10058-F4 for the treatment of PDAC.

Taken together, our study identified that c-Myc inhibitor induces tumor growth arrest and enhances PDAC chemosensitivity of PDAC to GEM through regulating glucose metabolism. Importantly, our work highlights 10058-F4 as a potential anticancer drug in adjuvant therapy of PDAC.

**Disclosure of interest**

The authors declare that they have no conflicts of interest concerning this article.
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References