Cancer Letters 409 (2017) 9–19

Contents lists available at ScienceDirect

Cancer Letters

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

Original Article

A novel chemoradiation targeting stem and nonstem pancreatic cancer cells by repurposing disulfiram

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ARTICLE INFO

Article history:
Received 21 May 2017
Received in revised form
18 August 2017
Accepted 21 August 2017

Keywords:
PDAC
Stem cells
Chemoradiation
Disulfiram
Chemoradiotherapy-resistance

ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) has a 5-year relative survival rate of 8% and is projected to be the second leading cause of cancer death by 2030, underscoring the urgency to develop new strategies to improve current therapeutic modalities for PDAC. Targeting pancreatic cancer stem cells (PCSCs), which are resistant to radiation and chemotherapy, is a promising strategy. A novel approach which can be readily clinically translated is to repurpose disulfiram (DSF), a drug for treating alcoholism, to target PCSCs. Chemoradiation or the combination of chemotherapy agents FOLFIRINOX, currently standard care for PDAC, can increase stemness in some established or primary PDAC cell lines. However, DSF in the presence of exogenously or endogenously supplied copper (Cu), when combined with chemotherapy or chemoradiation, targets both PCSCs and nonstem PDAC cells. Previously, we demonstrated that DSF/Cu effectively targets breast cancer stem cells in the context of fractionated radiation (FIR) by inhibiting the NF-κB–stemness gene pathway. Therefore, the hypothesis that PCSCs can be effectively targeted by incorporating DSF/Cu into the standard chemoradiation regimen consisting of 5-FU and FIR was investigated and found to be effective *in vitro* in targeting PCSCs, identified as either $ALDH^{bright}$ or CD24+/CD44+/ESA+ or sphere-forming cells, as well as nonstem PDAC cells. *In vivo*, the combination of IR+5-FU+DSF/Cu was more effective (72.46%) than either IR+5-FU (30.32%) or IR+FOLFIRINOX therapy (43.04%) in inhibiting growth of the mouse Panc02 tumor. These encouraging results provide a solid foundation for clinical trials to improve the outcomes of the current standard chemoradiation therapy regimen for PDAC.

Introduction

PDAC is rarely curable and has a 5-year relative survival rate of 8%, for all stages combined, due to difficulty of early diagnosis and its high resistance to chemotherapy and radiation [1]. Patients with resectable, borderline and locally advanced PDAC often undergo in varying sequences, an operation, chemotherapy, and chemoradiation. Chemoradiation consisting of fractionated irradiation therapy (FIR) and concurrent radiosensitization with Fluorouracil (5-FU) is a common treatment for PDAC [2,3]. It is known that pancreatic cancer stem cells (PCSCs), considered responsible for tumor recurrence, progression and metastasis, are highly chemo- and radiation-resistant [4,5]. Therefore, effective targeting of PCSCs

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http://dx.doi.org/10.1016/j.canlet.2017.08.028

Abbreviations

5-FU	Fluorouracil
ALDH	Aldehyde dehydrogenase
Cu	Copper
DSF	Disulfiram
DSF/Cu	DSF-Cu complexes
FIR	Fractionated radiation
Gy	Gray
i.p.	Intraperitoneally
PCSCs	Pancreatic cancer stem cells
PDAC	Pancreatic ductal adenocarcinoma
PE	Plating efficiency
s.c.	Subcutaneously
SF	Surviving fraction

in combination with chemo, radiation or chemoradiation should be considered for clinical trials aiming at significantly improving the efficacy of the standard care for PDAC patients.

Previously, we reported that radiation-induced breast cancer stemness was blocked by targeting the NF-κB-stemness gene pathway with disulfiram (DSF) and Copper (Cu2+). DSF is an aldehyde dehydrogenase (ALDH) inhibitor and an FDA-approved drug for treating alcoholism. DSF binds to Cu2+ to form DSF-Cu complexes (DSF/Cu), a potent proteasome inhibitor which, in turn, inhibits NF-κB activation [6]. This groundwork prompted testing whether DSF/Cu depletes PCSCs in combination with chemoradiation. Recent convincing evidence shows that CSCs and relatively differentiated nonstem cancer cells coexist in dynamic equilibrium and are subject to bidirectional conversion [7]. Thus, any successful therapeutic strategy needs to target preexisting PCSCs and block formation of therapy–induced PCSCs from nonstem PDAC cells [6]. Therefore, the effect of DSF/Cu as a novel chemoradiation sensitizer for PDAC cells was investigated by comparing DSF/Cu+FIR + 5-FU to FIR + 5-FU in their ability to target PCSCs, defined either as $ALDH^{bright}$ or CD24+/CD44+/ESA+ or sphere-forming cells, and nonstem bulk cancer cells and to reduce growth of human PDAC cell lines *in vitro* and mouse PDAC cells *in vivo*.

Materials and methods

Cell culture

The human PDAC cell lines, PANC-1 (ATCC) and PDAC2, PDAC3, PDAC6 (each established respectively from the ascites of patients with metastatic PDAC in C. Ferrone's lab) [8] were used in this study. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals). The mouse PDAC cell line Panc02 [9] (kindly provided by Dr. M. T. Lotze, The University of Pittsburgh Cancer Institute) was cultured in RPMI 1640 medium (Mediatech) supplemented with 10% fetal calf serum (FCS; Atlanta Biologicals) (complete medium). All cells were cultured at 37 °C in a 5% CO_2 atmosphere.

Chemical reagents and antibodies

Tetraethylthiuram disulfide (disulfiram, DSF), Copper Chloride or Copper(II) D-gluconate (Cu), and NF-κB inhibitor IMD-0354 were purchased from Sigma-Aldrich. DSF and IMD-0354 were reconstituted in DMSO for all *in vitro* experiments. DSF was reconstituted in olive oil for *in vivo* experiments. Cu was reconstituted in distilled water for all experiments. 5-fluorouracil (5-FU) was purchased from Teva Parenteral Medicines or Sigma. Oxaliplatin and Irinotecan were purchased from Abcam and Leucovorin was purchased from Fisher Scientific.

Antibodies and dilutions used for Western blotting were: human HER2/ERBB2 (#2242) (1:1000 dilution)-, human c-myc (#9402) (1:1000 dilution), human PARP (# 9542) (1:1000 dilution) cleaved PARP (#9541) (1:1000 dilution), and human β-actin (#4970) (1:2000 dilution)- specific rabbit monoclonal antibodies (mAbs) and goat anti-rabbit IgG, HRP-linked antibody (#7074) (1:2000) were purchased from Cell Signaling Technology. Human SOX9-specific rabbit antibody (Ab) (ab26414) (1 μg/mL) was purchased from Abcam. All antibodies were diluted in Tris Buffered Saline with 0.1% Tween® 20 (TBST) containing 5% nonfat dry milk plus 2% bovine serum albumin (BSA). All dilutions were prepared immediately before use.

Antibodies and dilutions used for cell surface staining were: human CD133/2 (Miltenyi Biotec Inc.) (clone: 293C3) (1:11 dilution), CD24 (BD Biosciences, CD24 APC-H7 Clone ML5) (1:20 dilution), CD44 (BD Biosciences, PE Mouse Anti-Human CD44 Clone G44-26) (1:5 dilution), and ESA (STEMCELL Technologies, FITC- ESA Antibody, Clone 5E11) (1:20 dilution).

Animals

C57BL/6 female mice, 6–8 weeks old were obtained from Taconic. All animal studies were approved by the Institutional Animal Care and Use Committee.

Flow cytometry

Cells were collected and were then incubated with ALDEFLUOR® reagent (Stem Cell Technologies), with or without the ALDH inhibitor DEAB according to the manufacturer's instructions. CD133+ cells were stained with the primary CD133/2 antibody and the secondary R-Phycoerythrin F(ab')2 Fragment Goat anti-mouse IgG (H + L) (Jackson Immunoresearch) (1:100dilution). CD24+/CD44+/ESA+ cells were stained with the APC-CD24, PE-CD44, FITC-ESA antibodies. Dead cells were excluded by 7-AAD staining (BD Biosciences, 1:20 dilution). Stained cells were analyzed by BD Accuri™ C6 Flow Cytometer with BD CSampler Software (version 1.0.264.21; Dako-Accuri Cytometers, Inc).

Irradiation (IR) treatment

In vitro fractionated IR (FIR) consisting of 3 gray (Gy) daily for 5 d or single doses of IR (0–6Gy) was performed on cells seeded in 6-well plates at an indicated density/well in 2 mL of complete medium. *In vivo,* a single dose of 5 or 8 Gy was delivered locally to each mouse tumor while the rest of body was protected from IR with lead shields. The X-RAD 320 Biological Irradiator (Precision X-ray, Inc, North Branford, CT) was used for all IR experiments in this study.

Clonogenic assay

Cells were seeded in 6-well plates at a density of 1×10^5. After 24 h, cells were treated with 5-FU (2.5 μM) and DSF/Cu (0.2/1 μM) for 20 h before exposure to 0 Gy, 2 Gy, 4 Gy, or 6 Gy of X-ray irradiation. Immediately after IR, the drugs were completely removed and treated cells were reseeded in triplicate in 6-well plates in complete medium. After 10 d, media was removed from wells, colonies were washed with PBS twice, before stained with 0.5% crystal violet. The number of colonies consisting of at least 50 cells was recorded. The plating efficiency (PE) was calculated as the number of colonies observed/number of cells plated. Surviving fraction (SF) is the colonies counted divided by the number of colonies plated with a correction for the plating efficiency (Corrected SF = [colonies counted]/[cells seeded × PE] × 100%). All surviving fraction data was multiplicity corrected (Final SF = 1-(1-corrected SF)^(1/cell multiplicity)).

Western blotting analysis

Cells were plated in 6-well plates at a density of 1×10^5 cells/well (PANC-1) or 7×10^4 cells/well (PDAC3) in 2 mL of culture medium and were treated as indicated. Cells were collected and lysed in lysis buffer (10 mM Tris–HCl (pH 8.2), 1% NP40, 1 mM EDTA, 0.1% BSA, 150 mM NaCl) containing 1/50 (vol/vol) protease inhibitor cocktail (Calbiochem). Western blotting for proteins of stemness genes was carried out as described [6].

Sphere formation assay

Cells were plated in 6-well plates at a density of 1×10^5 cells/well in 2 mL of complete medium followed immediately by treatment with 5-FU and FIR. Twenty-four h after irradiation, cells were treated with DSF/Cu (2.5 μM/1 μM) or 1 μM NF-κB inhibitor IMD0354 for an additional 24 h. Sphere formation was performed by seeding the cells (3000 cells/well) in a 24-well ultra-low adherent plate (Corning Incorporated) in 500 μL of mixed medium containing 32% MethoCult medium, 20% MammoCult basal human medium with a final concentration of 2% MammoCult proliferation supplements (STEMCELL Technologies), and 48% DMEM supplemented with final concentrations of 100 pg/mL EGF, 50 ng/mL bFGF, 5 ng/mL stem cell factor, 1×10^{-6} M hydrocortisone, and 5 μg/mL insulin. The cells were cultured at 37 °C in a 1% O_2 and 5% CO_2 humidified atmosphere for 5 or 14 d. Sphere formation was performed using the cells from a single cell suspension collected from a dis-aggregated (with Collagenase IV) (1 mg/mL PBS) mouse tumor.