Chapter 10

Enhancement of the antiproliferative activity of gemcitabine by modulation of c-Met pathway in pancreatic cancer

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Abstract
Pancreatic-ductal-adenocarcinoma (PDAC) is amongst the most lethal malignancies, mainly because of its metastatic spread and multifactorial chemoresistance. Since c-Met is a marker of pancreatic-cancer-stem-cells (CSC), playing a key role in metastasis and chemoresistance, this study evaluated the therapeutic potential of the novel c-Met/ALK inhibitor crizotinib against PDAC cells, including the Capan-1-gemcitabine-resistant cells (Capan-1-R).

Crizotinib inhibited PDAC cell-growth with IC50 of 1.5μM in Capan-1-R, and synergistically enhanced the antiproliferative and pro-apoptotic activity of gemcitabine, as detected by sulforhodamine-B-assay, flow cytometry and combination-index method. Capan-1-R had higher expression of the CSC markers CD44+/CD133+/CD326+, but their combined expression was significantly reduced by crizotinib, as detected by quantitative-RT-PCR and FACS-analysis. Similarly, Capan-1-R cells had significantly higher protein-expression of c-Met (~2-fold), and increased migratory activity, which was reduced by crizotinib (e.g., >50% reduction of cell-migration in Capan-1-R after 8-hour exposure, compared to untreated-cells), in association with reduced vimentin expression. Capan-1-R had also significantly higher mRNA expression of the gemcitabine catabolism-enzyme CDA, potentially explaining the higher CDA activity and statistically significant lower levels of gemcitabine-nucleotides in Capan-1-R compared to Capan-1, as detected by Liquid-chromatography-mass-spectrometry. Conversely, crizotinib significantly reduced CDA expression in both Capan-1 and Capan-1-R cells.

In aggregate, these data show the ability of crizotinib to specifically target CSC-like-subpopulations, interfere with cell-proliferation, induce apoptosis, reduce migration and synergistically interact with gemcitabine, supporting further studies on this novel therapeutic approach for PDAC.

Key words: Pancreatic ductal adenocarcinoma, c-Met pathway, cancer stem cells, crizotinib, gemcitabine
Introduction
Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths, with a 5 year survival rate of less than 3%. Despite extensive efforts, the prognosis of this disease is still poor, mostly because of its aggressive behavior and its intrinsic resistance to most chemotherapeutic agents [1-2].

Recently, cancer-stem-cell (CSC) and epithelial-to-mesenchymal transition (EMT) have been associated with highly invasive and tumorigenic behaviors of PDAC as well as with PDAC resistance to conventional chemotherapy [3-6]. In particular, several studies detected specific CSC subpopulations in human pancreatic cancers, including cells expressing the cell surface markers CD44⁺, CD24⁺, and CD326⁺ (or epithelial-specific antigen, ESA⁺) [7-8].

The receptor tyrosine-protein kinase c-Met has also been identified as a marker of human pancreatic CSCs, with a key role in growth and metastasis of pancreatic tumors in mice [8]. This transmembrane receptor is encoded by a gene located at 7q21-31, which has been identified as a proto-oncogene [9-10]. Several studies demonstrated that c-Met plays a pivotal role in the control of tissue homeostasis under normal physiological conditions [11-13], while abnormal stimulation of c-Met signaling pathway, through the binding of hepatocyte growth factor (HGF), mediates the activation of a wide range of different cellular signaling pathways in cancer cells involved in proliferation, motility, migration and invasion [8, 14].

Previous studies showed that c-Met plays a key role in the cancer/stroma interaction [15], and is overexpressed in PDAC and pancreatic-CSCs [8, 16-17]. Induction of this pathway has been shown to stimulate growth and invasion of pancreatic cancer cells [16, 18], which was correlated with poor prognosis/chemoresistance [17, 19].

Therefore, in the present study we evaluated the therapeutic potential of crizotinib, a novel c-Met/ALK (Anaplastic Lymphoma Kinase) inhibitor (Fig. 1A-B). This compound was tested for its ability to suppress the c-Met signaling pathway, in order to overcome the aggressive behavior and chemoresistance of PDAC. To achieve this goal, we evaluated several mechanisms underlying crizotinib-gemcitabine interaction in PANC-1, LPC028 (primary cell culture), Capan-1 and Capan-1-gemcitabine-resistant (Capan-1-R) PDAC cells, before and after treatment with crizotinib, gemcitabine and their combination.

Materials and Methods

Drugs and chemicals
Crizotinib was obtained from Bio-Connect Diagnostics BV (Huissen, The Netherlands), while gemcitabine was kindly provided by Eli Lilly Corporation (Indianapolis, IN). The drugs were dissolved in DMSO and sterile water, respectively. The Roswell Park Memorial Institute medium (RPMI 1640) and Dulbecco's Modified Eagle Medium (DMEM) were obtained from Lonza (Verviers, Belgium), while foetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco (Gaithersburg, MD). All other chemicals were from Sigma (Zwijndrecht, The Netherlands).

Cell culture
PANC-1 (primary pancreatic ductal adenocarcinoma) and Capan-1 (pancreatic metastatic
ductal adenocarcinoma) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The gemcitabine-resistant cell lines Capan-1-R [resistant to 10 μM gemcitabine] were established by Dr. Karl Quint (Institute for Surgical Research, Philipps-University of Marburg, Marburg, Germany), after continuous exposure of Capan-1 cells to gemcitabine and maintained with gemcitabine 10 μM [20]. The primary pancreatic ductal adenocarcinoma cell culture LPC028 was established by Dr. Niccola Funel (Department of Surgery, University of Pisa, Pisa, Italy) from a primary tumor tissue, as described previously [21]. The cell lines were tested for their authentication by PCR profiling using short tandem repeats (STR), which was performed by BaseClear (Leiden, The Netherlands).

PANC-1 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 1% penicillin (50 IU/ml) and streptomycin (50 μg/ml), while LPC028, Capan-1 and Capan-1-R cells were maintained in RPMI-1640 with 20% FBS and 1% penicillin and streptomycin. The cells were kept in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C, and harvested with trypsin-EDTA in their exponentially growing phase.

**Quantitative Real-Time Reverse-Transcriptase Polymerase-Chain-Reaction (qRT-PCR)**

Total RNAs were extracted from cell pellets using the TRI REAGENT LS (Invitrogen, Carlsbad, CA, US), according to the manufacturers’ instruction. The quality and purity of the extracted RNAs were assessed at 260-280nm with the NanoDrop®-1000-Detector (NanoDrop-Technologies, Wilmington, USA). cDNA was synthesized from one μg of RNA using the DyNAmo cDNA Synthesis Kit (Thermo Scientific, Vantaa, Finland), according to

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**Figure 1. c-Met and EML4-ALK signaling pathways and cellular context.** (A) Structure of crizotinib. (B) Representative figure of crizotinib mechanism of action: crizotinib inhibits both c-Met and EML4-ALK signaling pathways causing a reduction of cellular migration/proliferation, and increasing apoptosis.
Modulation of c-Met signalling pathway in pancreatic cancer

qRT-PCR was performed as described previously [22], in 25 μl reaction volume containing TaqMan Universal master mix (Applied Biosystems, Forster City, CA) in duplicate. Primers and probes to specifically amplify CD44, CD326, CD133, hENT1, hCNT1, dCK, CDA, RRM1, RRM2, Vimentin and E-cadherin were obtained from Applied Biosystems TaqMan Gene expression products (Hs00191940_m1, Hs01009250_m1, Hs01009250_m1, Hs01085706_m1, Hs00984403_m1, Hs01040726_m1, Hs00156401_m1, Hs00168784_m1, Hs01072069_g1, Hs00185584_m1 and Hs01023894_m1, respectively). To evaluate the possible modulation of these biomarkers by crizotinib, PCR reactions were also performed in Capan-1 and Capan-1-R cells, treated with crizotinib, gemcitabine or their combination, for 24 or 72 hours.

The samples were amplified using the ABI PRISM 7500 sequence detection system instrument (Applied Biosystems), with the following thermal profile: 50°C for 30 minutes, 95°C for 10 minutes, 45 cycles of denaturation at 95°C for 15 seconds followed by annealing and extension at 60°C for 1 minute. Gene expression values were normalized to β-actin.

Western blot analysis

To evaluate the basal c-Met and phospho-c-Met protein expression, cell pellets were collected from PANC-1, Capan-1, Capan-1-R and LPC028, as well as in Capan-1-R cells treated with crizotinib 10 μM, after 10 minutes exposure to HGF 40 ng/ml, and western blotting was executed as described previously [22]. Briefly, the cells were lysed on ice, using a lysis buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 50 mM β-glycerophosphate, 5 mM EDTA, 10% [v/v] glycerol, 5 mM dithiothreitol [DTT], 1% [v/v] triton X-100, and 4.0% [v/v] of a protease inhibitor cocktail), followed by centrifugation. The supernatant was collected and the total protein content was determined using the Bio-Rad protein assay (Bio-Rad, Hertfordshire, UK). Protein (40 μg per sample) was loaded and separated on a 10% SDS-polyacrylamide gel. The proteins were then transferred onto a PVDF membrane (Immobilion®-FL, Millipore). The membrane was next blocked with Rockland Blocking Buffer (Rockland Inc., Pennsylvania, USA).

The primary antibodies used in this study were: rabbit anti-Met and anti-phospho-Y1003-c-Met (1:1000 diluted in the blocking solution; Santa Cruz Biotechnology, INC.) and mouse anti-β-actin (1:10000; Sigma–Aldrich Chemicals, Zwijndrecht, The Netherlands). The secondary antibodies were goat anti-rabbit-InfraRedDye® 800 Green (1:10000; #926-32211; Westburg, Leusden, The Netherlands) and goat anti-mouse-InfraRedDye® 680 Red (1:10000; #926-32220, Westburg, Leusden, The Netherlands). Fluorescent proteins were detected by an Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, Nebraska USA), at 84 μm resolution using high quality settings. Odyssey version 3.0 software was used for semi-quantitative analysis of the bands.

Immunocytochemistry

The evaluation of c-Met and phospho-c-Met expression was also performed by immunocytochemistry in LPC028 cells. The cells were grown in Chamber Slides System (Lab-Tek, IL, USA) in a humidified incubator at 37°C with 5% CO₂. After 48 hour the cells were fixed with acetic acid and methanol solution (ratio 1:3) at room temperature for 10 min. Immunocytochemistry was performed using the specific monoclonal rabbit anti-human c-Met and anti-phospho-c-Met antibodies (overnight incubation and 1:30 dilution). The
cells were then stained with avidin-biotin-peroxidase complex (UltramarqueTM HRP Detection kit, Greenwood, AR, USA). Negative controls were obtained by replacing the primary antibody with buffer. In order to quantify the results, we used a system based on staining intensity and on the number of stained cells. With regard to the intensity score, if the intensity was comparable to the negative controls, intensity was classified as 0, if the cells stained weakly, the intensity was classified as 1+, if the cells stained moderately, the intensity was classified as 2+, and if the cells stained strongly the intensity was classified as 3+. With regard to the number of cells, if none of the cells were stained the score was 0, if less than 25% of the cells were stained the score was 1+, if 25-50% of the cells were stained the score was +2, and if >50% of the cells were stained, the score was 3+.

**FACScan analysis of CSC markers**

In order to quantify the different cell subpopulations of CSCs markers in Capan-1-R compared to Capan-1 cells, fluorescence-activated cell scan (FACScan) was performed in Capan-1-R compared to Capan-1 cells. Cells were dissociated as single cells and washed two times with washing buffer (PBS containing 0.1% BSA, 0.05% NaN₃), followed by centrifugation at 1500 rpm for 5 minutes. A total of 2×10⁶ cells were resuspended in PBS in a final volume of 50 µl and then incubated for 45 minutes at 4°C with the following antibodies: Fluorescein isothiocyanate (FITC) anti-human CD44 (1:200 dilution), Phycoerythrin (PE) anti-human CD133 (1:100 dilution) and Peridinin-chlorophyll proteins (PerCP)/Cy5.5 anti-human CD326 (EpCAM) (1:50 dilution) (ITK Diagnostics b.v., BioLegend, Uithoorn, The Netherlands).

Flow cytometry was carried out using a Becton Dickinson FACSAria flow cytometer (Becton Dickinson, San Jose’, CA, USA). Analysis was performed using BD FACSDiva software version 6.1.3.

**Analysis of CDA enzymatic activity**

The enzymatic activity of CDA was measured in Capan-1 and Capan-1-R cells using Dionex Ultimate 3000 micro-HPLC system, as described previously [23]. The cells were lysed and the supernatant was used as a cytoplasmic extract containing CDA enzyme. In order to determine the enzyme activity, 20µl of this extract was mixed with 170 µl buffer (50 mM β-mercapto-ethanol in 0.1 M Tris/HCl, pH 8.0) and the substrate gemcitabine (250 µM). The reaction was incubated at 37°C for 15 and 30 minutes to ensure enzyme activity was in a linear range; a reversed phase ion pair HPLC method with Aqua C18 (Phenomenex) column with PIC-B7 (Waters Chromatography, the Netherlands) supplemented with 3.5% acetonitrile, pH 2.8, flow 1ml/min was used for quantification of the product, dFdU. Calibration lines of dFdU were prepared in water in the range of 0.5–200 µM. Peak areas were quantified using Chromeleon 6.7-software (Dionex Gmbh, Germany). The HPLC method was linear (r²>0.99) over the analytical range, and dFdU formation was normalized for protein concentration, measured by Bradford assay (Sigma).

**Liquid chromatography–mass spectrometry (LC-MS/MS)**

Accumulation of total gemcitabine-nucleotides was measured using LC-MS/MS and calculated from the difference before and after alkaline phosphatase treatment, as described previously [24]. Approximately 2×10⁶ cells of Capan-1 and Capan-1-R were seeded into petri dishes, and exposed to 10 µM gemcitabine for 4 hours. Thereafter cells were harvested and pellets were snap frozen. Cell pellets were re-suspended in a known aliquot of phosphate buffer and then precipitated with excess isopropyl alcohol. The supernatant was
removed and evaporated to dryness via freeze-drying. The dry samples were reconstituted in 200 µl of water and 20 µl aliquots were used for LC/MS-MS analysis. The remaining samples were treated quantitatively with alkaline phosphatase (4 units) at 37°C overnight. Chromatography was conducted using a Dionex Ultimate 3000 micro HPLC system coupled via a Turbo spray ionization source to an Applied Biosciences SCIEX API 3000 mass spectrometer (Applied). Data analysis was performed with version 1.52 Analyst software (AB Sciex, Nieuwerkerk aan den Ijssel, The Netherlands) controlled by Dionex Mass Spectrometry Link software version 2.8, combined with Chromeleon management software modules (Thermo Scientific).

**Analysis of cell growth inhibition**

The cell growth inhibitory effects of crizotinib, gemcitabine and their combination, were investigated by sulforhodamine B (SRB) assay in PANC-1, Capan-1, Capan-1-R and LPC028 cells. The cells were plated in 96-well plates at density of 5×10^3 cells/well and maintained at 37°C and 5% CO2 for 24 hours. Then, the cells were treated for 72 hours with crizotinib (0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 µM), gemcitabine (0.01, 0.1, 0.5, 1, 5, 10, 50, 100 nM) or their simultaneous combination. After 72 hours, plates were processed for the SRB assay, as described previously [25-26]. Optical density was measured at 540 nm using the Tecan SpectraFluor (Tecan, San Diego). The 50% inhibitory concentration of cell growth (IC50) values of the drugs were expressed as the percentage of surviving cells after treatment relative to untreated controls, and calculated by non-linear least squares curve fitting (GraphPad PRISM, Intuitive Software for Science, San Diego, CA, USA).

**Evaluation of pharmacological interaction of crizotinib with gemcitabine**

To evaluate the pharmacological interaction of crizotinib and gemcitabine, the cell growth inhibition of each drug alone was compared with the cell growth inhibition of the combination, using the combination index (CI), where CI<0.9, CI=0.9-1.1, and CI>1.1 indicated synergistic, additive and antagonistic effects, respectively. Combination studies were focused on simultaneous treatment, testing at least six different concentrations of gemcitabine, using a fixed concentration of crizotinib (10 µM). The median-drug effect analysis method was employed for evaluating the drug interaction of crizotinib with gemcitabine [27]. Growth inhibition lower than 50% was not considered as relevant. CI values at fraction affected (FA) of 0.5, 0.75 and 0.9 were averaged for each experiment, and these values were used to calculate the mean between experiments [28]. CalcuSyn software version 2.0 was employed to analysis of the data (Biosoft, Oxford, UK).

**Analysis of cell cycle and measurement of cell death**

To explore the perturbation of cell cycle and cell death after 72-hour drug exposure, Capan-1 and Capan-1-R were treated with crizotinib, gemcitabine and crizotinib/gemcitabine combination. Cells were stained by Propidium Iodide and cell cycle perturbations were evaluated using a FACScan (Becton Dickinson, San José, CA), equipped with the CELLQuest software for data analysis. Crizotinib, gemcitabine and their combination were also characterized for their ability to induce cell death and apoptosis, which was detected after 72-hour drug exposure. In particular, we evaluated both the sub-G1 region of the previous FACS analysis, and the cells displaying apoptotic features by fluorescence microscopy analysis with bisbenzimide staining, as described previously [22, 29].
Wound healing assay
In order to assess the ability of cells to migrate into the wound area after drug treatment, Capan-1 and Capan-1-R cells were plated at a density of $3 \times 10^4$ cells/well onto 96 wells plates. After 24 hours, artificial wound tracks were created by scraping with a specific scratcher within the confluent monolayers. The cells were gently washed with PBS for removal of the detached cells and exposed to 10 μM crizotinib, gemcitabine at IC$_{50}$, or their combination. Images were taken at the beginning of the exposure (time 0), and after 4, 8, 12, 16, 20 and 24 hours. Migration was evaluated using the LeicaDMI300B (Leica) migration station integrated with the Scratch Assay 6.1 software (Digital Cell Imaging Labs, Keerbergen, Belgium).

Statistical analysis
The experiments were performed in triplicate and were repeated at least twice. Data were expressed as mean values±S.E. and analyzed by Student’s t-test or ANOVA followed by the Tukey’s multiple comparison test. The level of significance was set at P<0.05.

Results
Overexpression of CSC markers and phospho-c-Met in Capan-1-R cells
The expression of the PDAC CSC markers CD44, CD133, and CD326, were detected by qRT-PCR and FACS analysis. The PCR studies showed that CD44, CD133 and CD326 were increased in Capan-1-R compared to Capan-1 cells (+31, +41 and +18%, respectively, Fig. 2A). These markers were also studied at the protein level, and the combined analysis showed a significant increase in Capan-1-R with respect to Capan-1 cells (Fig. 2B).

Since c-Met has recently been identified as a marker of CSCs [8], we also evaluated the expression of c-Met and phospho-c-Met using Western-blotting which showed approximately 2-fold-increase in Capan-1-R cells compared to Capan-1 (Fig. 2C). Furthermore we performed immunocytochemistry of the LPC028 primary cells (Fig. 2D), showing similar results to the originator PDAC tissue (data not shown).

Modulation of gemcitabine determinants in Capan-1-R cells
Since previous studies showed that alterations in the expression or function of gemcitabine metabolizing enzymes can lead to resistance (e.g., decreased deoxycytidine kinase [dCK] or increased cytidine deaminase [CDA] expression or activity [30-33]), we evaluated the expression/activity of several gemcitabine determinants in Capan-1 and Capan-1-R cells. As shown in Fig. 3A, the mRNA expression of the gemcitabine catabolism-enzyme CDA was significantly increased (approximately 3.5-fold) in Capan-1-R versus Capan-1 cells, while gene expression of hCNT1, dCK, RRM1 and RRM2 were decreased compared to Capan-1. However, the mRNA levels of hENT1 were similar. Moreover, the enzymatic activity of CDA was significantly increased in Capan-1-R cells (525 nmol/hr/mg) compared to Capan-1 (216 nmol/hr/mg, Fig. 3B). These results explain why the accumulation of gemcitabine-nucleotides was significantly reduced in Capan-1-R (0.26 pmol/μg protein) compared to Capan-1 cells (7.6 pmol/μg protein), as detected by LC-MS/MS (Fig. 3C).
Figure 2. Expression of CSC markers and c-Met/phospho-c-Met in PDAC cells. (A) Relative mRNA expression of CD44, CD326 and CD133 in Capan-1-R (black columns) versus to Capan-1 (white columns), as measured by qRT-PCR; (B) representative results of CSC markers expression at protein level in Capan-1-R (black columns) and Capan-1 (white columns), as detected by FACS analysis; (C) Protein expression of c-Met and phospho-c-Met in Capan-1, Capan-1-R and PANC-1 cells, using Western-blotting; (D) representative pictures of immunocytochemistry of c-Met and phospho-c-Met in LPC028 cells, compared to control. Columns, mean values obtained from three independent experiments; bars, SEM. *Significantly different from Capan-1 cells (P<0.05).

**Crizotinib synergistically enhances the antiproliferative activity of gemcitabine**

Crizotinib caused a concentration-dependent inhibition of cell proliferation in all the PDAC cells. Gemcitabine also inhibited cell growth, with IC$_{50}$ values ranging from 7.2±2.3 nM (Capan-1) to 80.5±6.5 μM (Capan-1-R), as described previously [20-22]. The IC$_{50}$ values of crizotinib and gemcitabine are reported in the Table 1.

Representative growth inhibition curves for PANC-1 cells are shown in Fig. 4A. Median drug-effect analysis revealed strong synergism in the Capan-1 and PANC-1 cells, and slightly synergistic or nearly additive interaction in LPC028 cells.

<table>
<thead>
<tr>
<th>Table 1. Sensitivity to gemcitabine and crizotinib</th>
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<tbody>
<tr>
<td><strong>Cells</strong></td>
</tr>
<tr>
<td><strong>Drugs</strong></td>
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<tr>
<td>---------------------------------------------------</td>
</tr>
<tr>
<td>PANC-1</td>
</tr>
<tr>
<td>Capan-1-R</td>
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</table>
The average CI values for all the combinations in the PDAC cells are summarized in Fig. 4B. To explore the mechanisms underlying these synergistic drug interactions, we performed several cellular and molecular analyses, as detailed below.

**Crizotinib and its combination with gemcitabine significantly induces apoptosis**

Crizotinib, gemcitabine and their combination affected the cell cycle of PANC-1, Capan-1, Capan-1-R and LPC028 cells (Table 2). In particular, crizotinib blocked PANC-1 cells in the G1-S boundary, increasing the percentage of PANC-1 cells in the G0/G1 phase from 55 to 65%, while gemcitabine increased the cells in G2/M phase from 27 to 36% (P<0.05). Conversely the drug combination significantly increased the percentage of cells in the S-phase. Similar perturbations of the cell cycle were observed after exposure to crizotinib in Capan-1, Capan-1-R and LPC028 cells (e.g., increases of +10%, +9% and +7% in the G0/G1 phase, respectively).
Table 2. Effects of gemcitabine, crizotinib and their combination on cell cycle distribution and apoptotic index

<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatment</th>
<th>G0/G1 Phase (%)</th>
<th>S Phase (%)</th>
<th>G2/M Phase (%)</th>
<th>Apoptotic</th>
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<tr>
<td>PANC-1</td>
<td>Control</td>
<td>55.2±1</td>
<td>17.7±1.3</td>
<td>27.2±2.2</td>
<td>3.2±0.4</td>
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<td></td>
<td>Gemcitabine</td>
<td>46.4±2.8</td>
<td>17.8±2.8</td>
<td>35.9±5.6</td>
<td>8.7±1.2*</td>
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<td></td>
<td>Crizotinib</td>
<td>65.2±2.2</td>
<td>14.4±0.2</td>
<td>20.4±5.1</td>
<td>19.0±3.3*</td>
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<tr>
<td></td>
<td>Crizotinib+ Gemcitabine</td>
<td>50.0±1.9</td>
<td>28.5±2.7</td>
<td>21.5±1.3</td>
<td>39.5±5.6**</td>
</tr>
<tr>
<td>Capan-1</td>
<td>Control</td>
<td>34.8±2.3</td>
<td>23.7±1.5</td>
<td>37.6±1.8</td>
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<td></td>
<td>Gemcitabine</td>
<td>34.3±0.9</td>
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<td>35.0±1.2</td>
<td>9.4±1.3*</td>
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<td></td>
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<td>43.3±2.1</td>
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<td>23.9±2.7*</td>
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<td>30.8±1.5</td>
<td>35.3±1.0</td>
<td>33.9±0.7</td>
<td>43.4±3.8**</td>
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<td>Capan-1R</td>
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<td>30.1±2.8</td>
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<td>19.3±2.0</td>
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<td>21.9±1.4*</td>
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<td>LPC028</td>
<td>Control</td>
<td>45.5±2.2</td>
<td>10.5±1.7</td>
<td>44.0±0.9</td>
<td>2.1±0.6</td>
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<td></td>
<td>Gemcitabine</td>
<td>39.3±3.5</td>
<td>20.2±1.1</td>
<td>39.9±3.9</td>
<td>7.8±0.9*</td>
</tr>
<tr>
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<td>34.3±0.9</td>
<td>23.5±1.8*</td>
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<td>Crizotinib+ Gemcitabine</td>
<td>32.5±1.4</td>
<td>22.3±1.1</td>
<td>45.2±0.7</td>
<td>33.3±4.7**</td>
</tr>
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</table>

Cells were exposed to IC50s values of gemcitabine, 10 µM crizotinib and their combination #Cells growing with gemcitabine 10 µM; *P<0.05 with respect to control cells; **P<0.05 with respect to gemcitabine

However, in the Capan-1 and LPC028 cells gemcitabine increased the percentage of cells in the S-phase, which was additionally enhanced by the combination. Analysis of the sub-G1 region demonstrated that drug treatments significantly enhanced cell death compared to control. In particular, the Capan-1 cells treated with the combination exhibited the largest sub-G1 signal (e.g., 43.4%).
Further analysis with fluorescence microscopy showed that cells exposed to crizotinib, gemcitabine and their combination presented the typical apoptotic morphology with cell shrinkage, nuclear condensation and fragmentation, and rupture of cells into debris, after 72-hour exposure (data not shown). In all cell lines, 8-10% of apoptotic cells were observed after gemcitabine treatment, whereas crizotinib exposure was associated with a higher percentage (6-15%) of apoptotic cells; in all the PDAC cells the drug combination significantly increased the apoptotic index with respect to both control cells and gemcitabine-treated cells.

**Crizotinib and its combination with gemcitabine significantly inhibits cell migration and vimentin expression**

The effect of crizotinib, gemcitabine and their combination on migratory behavior was investigated in Capan-1 and Capan-1-R using a scratch motility assay. Capan-1-R cells were characterized to have a higher migratory ability (approximately +80% at 24 hours), compared to Capan-1 cells. As shown in Fig. 5A-B, crizotinib significantly reduced cell-migration in both Capan-1 and Capan-1-R. This effect was already detectable after 8 hours of exposure, with percentage of wound healing area of 8% in Capan-1-R compared to 58% in untreated cells (Fig. 5A).

Figure 5. Effects of crizotinib, gemcitabine and their combination on Capan-1 and Capan-1-resistant cells migration. (A-B) Representative results of wound-healing assay in Capan-1 and Capan-1-R after exposure to 10 µM crizotinib, gemcitabine at IC₅₀, or their combination. Images were taken at the beginning of the exposure (time 0), and after 4, 8, 12, 16, 20 and 24 hours and the ability of the cells to migrate into wound area was evaluated by the LeicaDMI300B migration station integrated with the Scratch Assay 6.1 software. (C) Modulation of *vimentin* mRNA expression was measured after 24 hours exposure to 10 µM crizotinib by quantititative RT-PCR. Columns, mean values obtained from three independent experiments; bars, SEM. *Significantly different from controls (P<0.05).
Furthermore, the percentages of migrated cells after 8-hour exposure in Capan-1 were approximately 46%, 43%, 14% and 13% in untreated, gemcitabine, crizotinib and crizotinib/gemcitabine combination treated cells, respectively. These data demonstrate that crizotinib was able to significantly inhibit the migration of Capan-1 cells compared to both untreated and gemcitabine-treated cells (Fig. 5B).

The PCR analyses of the EMT markers, E-cadherin and vimentin, showed that the increased migration in Capan-1-R, compared to Capan-1, was associated with decreased E-cadherin (-36%) and increased vimentin mRNA-expression (+27%). However, the mRNA expression of vimentin in Capan-1-R cells was significantly reduced after 24-hour treatment with crizotinib (Fig. 5C), whereas we observed only a slight modulation of E-cadherin expression (data not shown).

**Crizotinib and its combination with gemcitabine modulates the expression of CSC markers and gemcitabine determinants**

Previous studies have suggested that gemcitabine resistance is associated with a stem-cell like phenotype, characterized by altered cell surface marker expression, and gemcitabine resistance in PDAC cells can be reverted by RNA interference of the cancer stem cell marker CD44 [5]. We therefore evaluated whether crizotinib was able to modulate the mRNA expression of CSC markers, focusing on the Capan-1-R cells. As shown in Fig. 6A, crizotinib significantly reduced the expression of CD44, CD326 and CD133 (P<0.05). In particular, a 24-hour crizotinib exposure at 10 μM resulted in a 2-fold reduction in CD44 expression compared to untreated cells. Similar results were observed for the expression of CD326, which was reduced by crizotinib up to -38%, while there was -72% reduction of CD133 expression.

Since c-Met has recently been recognized as a new marker for pancreatic CSCs, as well as a valuable therapeutic target [8], we evaluated whether crizotinib affected its activation. As shown in the representative blot in Fig. 6B, Capan-1-R cells treated with crizotinib were marked by a significant downregulation of phospho-c-Met.

Moreover, in order to unravel possible molecular mechanisms affecting gemcitabine mechanism of action and underlying the crizotinib/gemcitabine synergistic interaction, we evaluated whether crizotinib affected the expression of the main determinants of gemcitabine activity. Crizotinib significantly increased hCNT1 and decreased CDA, while minimally affecting the expression of hENT1, RRM1 and RRM2 (Fig. 6C). In particular, hCNT1 expression was 2.2-fold increased, whereas CDA expression was reduced of about 50%.

**Discussion**

The present study demonstrated that the combination of the novel c-Met/ALK inhibitor crizotinib and gemcitabine was synergistic in several PDAC cells, including a cell line resistant to gemcitabine and a primary pancreatic cancer cell culture. This synergistic interaction was associated with induction of apoptosis, as well as with a significant reduction of cell proliferation and migration of the PDAC cells (Fig. 7).

Gemcitabine is the most commonly used chemotherapeutic agent in the treatment of pancreatic cancer, however, this therapeutic regimen results in less than 12% response rate in PDAC patients [34]. Thus, there is an urgent need to develop novel anticancer agents in
order to improve the efficacy of gemcitabine, as well as inhibit or prevent PDAC invasiveness and chemoresistance.

Recently, pancreatic CSC markers have been associated with PDAC aggressiveness, metastatic behavior and intrinsic resistance to chemotherapy [3, 8]. The existence of CSC in PDAC was initially observed by Li and colleagues, who identified a subpopulation of highly tumorigenic cancer cells expressing the cell surface markers CD44, CD24, and CD326 [7]. Of note, Shah and colleagues reported that PDAC cells treated with therapeutic doses of gemcitabine underwent EMT and gemcitabine-resistant cells also had increased expression of the CSC markers CD44, CD24 and CD326 [35]. Nevertheless, c-Met emerged as a marker of pancreatic CSCs and a novel therapeutic target in treatment of PDAC [8]. C-Met is overexpressed in advanced and metastatic disease in many solid tumors and is associated with poor prognosis/chemoresistance of PDAC [8, 14, 17, 19].

Consistent with these data, our current results showed that the expression of CD44, CD24 and CD326 were increased at the mRNA and protein levels in Capan-1-R compared to Capan-1 cells. However, we also evaluated the expression of c-Met and phospho-c-Met, and our results showed significantly higher expression of these proteins in Capan-1-R, compared to Capan-1 cells. These findings suggest that the c-Met signaling pathway could be a valuable target to overcome chemoresistance in PDAC.

Figure 7. Molecular mechanisms involved in the synergistic interaction of crizotinib with gemcitabine. Crizotinib enhances the cytotoxicity effect of gemcitabine through its pronounced pro-apoptotic effect, as well as by inhibiting cell migration through the c-Met signaling pathway. Furthermore, crizotinib promotes gemcitabine uptake and reduces gemcitabine catabolism.
Crizotinib is an ATP competitive inhibitor of ALK and a c-Met receptor tyrosine kinase and has recently been recognized as an effective targeting agent in non-small cell lung cancer (NSCLC) patients with a chromosomal rearrangement that generates a fusion gene between echinoderm microtubule-associated protein-like 4 (EML4) and ALK, which results in constitutive kinase activity that drives the malignant phenotype [36-37]. Previous studies in gastrointestinal tumors, including PDAC, did not find cancers positive for expression of EML4–ALK [38]. However, crizotinib suppresses auto-phosphorylation of both c-Met and ALK receptor tyrosine kinases and competes with ATP binding in both kinases through its binding at the hinge region of c-Met [39]. Indeed Ou and colleagues showed that NSCLC patients with amplification of MET, but without ALK rearrangement, experienced a rapid and durable response to crizotinib, demonstrating its therapeutic role also as a bona fide c-Met inhibitor [40].

To the best of our knowledge, this is the first study evaluating the pharmacological interaction of crizotinib with gemcitabine in PDAC cells. This combination was synergistic in PANC-1 and Capan-1 cells, and synergistic/additive in the primary cell culture LPC028. This synergistic effect was associated with increased apoptosis. In addition, crizotinib was active against Capan-1-R cells, where it down-regulated the expression of CSC markers (CD44+/CD133+/CD326+), as well as that of c-Met, compared to Capan-1-R-untreated cells. Our findings suggest that the positive interaction of crizotinib with gemcitabine was also mediated by other mechanisms, which decreased the aggressiveness of PDAC and enhanced sensitivity to gemcitabine. In this respect, we evaluated whether crizotinib could affect PDAC cell migration. These results showed that crizotinib and its combination with gemcitabine significantly reduced the migratory ability of Capan-1 and Capan-1-R cells after 8-hour exposure with inhibition of ≈30% and >50%, respectively, compared to the untreated and gemcitabine-treated cells. Several studies demonstrated that the c-Met signaling pathway is responsible for stimulation of cell motility and invasion, and c-Met inhibitors significantly impaired the metastatic phenotype in different preclinical models [41]. Therefore, the c-Met inhibition by crizotinib in PDAC cells suggests that this agent may have therapeutic utility in targeting PDAC invasive behavior and metastasis.

Since EMT is thought to play a fundamental role during the early steps of invasion and metastasis of PDAC [42], we also evaluated the expression of EMT markers by quantitative PCR. The levels of E-cadherin were not affected, whereas vimentin expression was increased in Capan-1-R compared to Capan-1 cells. These results are in agreement with a previous study, showing that gemcitabine-resistant cells were more invasive and migratory and had increased vimentin expression [43]. Vimentin is expressed in several epithelial carcinomas and its expression has been related to invasiveness and poor prognosis, especially in PDAC [44]. A recent study suggested that suppression of vimentin expression inhibited migration and invasion of colon and breast cancer cell lines [45]. Consistent with these observations, our data showed that crizotinib caused a significant reduction of vimentin expression in Capan-1-R cells, compared to the untreated cells, resulting in an impaired migration, which might be attributed, at least in part, to a reversal of their EMT phenotype.

Gemcitabine is a nucleoside analogue which requires active cellular uptake by members of the Solute Carriers SLC28 (e.g. SLC28A, hCNT1) and SLC29 (e.g. SLC29A, hENT1) families, followed by intracellular activation to its mono-, di- and triphosphorylated
(dFdCMP, dFdCDP and dFdCTP, respectively) metabolites in a rate limiting step catalyzed by the deoxycytidine kinase (dCK) [46]. Active phosphorylated forms of gemcitabine are finally incorporated into DNA/RNA and induce apoptosis [33]. Nevertheless, gemcitabine can also be inactivated in the blood, liver and kidneys, by CDA [47]. Several in vitro studies have shown that overexpression of CDA is associated with gemcitabine resistance [48-49]. Other studies showed that resistance to gemcitabine could be reversed by addition of the CDA inhibitor tetrahydrouridine [32, 50]. Furthermore, studies in NSCLC patients showed that high expression of CDA is associated with shorter survival after gemcitabine-based therapy [23, 51]. Conversely, deficiency in CDA has been associated with an increased risk of experiencing severe or even lethal adverse effects in patients [52-53].

In order to investigate whether molecular mechanisms affecting gemcitabine mechanism of action might underlie the synergistic effect of crizotinib and gemcitabine, we evaluated the expression of the main determinants of gemcitabine activity in Capan-1-R and Capan-1 cells, as well as the effect of crizotinib on the modulation of these genes. The mRNA expression of the \textit{hCNT1}, \textit{dCK}, \textit{RRM1} and \textit{RRM2} was significantly reduced in Capan-1-R (gemcitabine-resistant) cells compared to Capan-1, while the mRNA expression and enzyme activity of CDA in Capan-1-R cells was significantly higher than in Capan-1 cells. This can potentially explain the significantly lower levels of gemcitabine metabolites in Capan-1-R compared to Capan-1. However, crizotinib significantly reduced the expression of \textit{CDA} and increased \textit{hCNT1} expression, potentially reducing gemcitabine catabolism, while increasing gemcitabine uptake.

In aggregate, our data provide novel insights into the antitumor activity of crizotinib in PDAC cells, unraveling its ability to specifically target CSC-like-subpopulations, interfere with cell-proliferation, induce apoptosis, reduce migration and synergistically interact with gemcitabine, supporting further studies on this novel therapeutic approach for PDAC.

\textbf{Grant Support}

This work was supported by grants from the Netherlands Organization for Scientific Research (NWO, Veni grant, Elisa Giovannetti), AIRC-Marie Curie (International Fellowship, Elisa Giovannetti), and CCA-VICI foundation (grant#2012-5-07, Amir Avan, Godefridus J. Peters, Elisa Giovannetti).
Modulation of c-Met signalling pathway in pancreatic cancer

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