Schedule-dependent cytotoxic synergism of pemetrexed and erlotinib in BXPC-3 and PANC-1 human pancreatic cancer cells

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Abstract. Previous studies have shown that both pemetrexed, a cytotoxic drug, and erlotinib, an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), inhibit the cell growth of pancreatic cancer cells. However, whether they exert a synergistic antitumor effect on pancreatic cancer cells remains unknown. The present study aimed to assess the synergistic effect of erlotinib in combination with pemetrexed using different sequential administration schedules on the proliferation of human pancreatic cancer BXPC-3 and PANC-1 cells and to probe its cellular mechanism. The *EGFR* and *K-ras* gene mutation status was examined by quantitative PCR high-resolution melting (qPCR-HRM) analysis. BXPC-3 and PANC-1 cells were incubated with pemetrexed and erlotinib using different administration schedules. MTT assay was used to determine cytotoxicity, and cell cycle distribution was determined by flow cytometry. The expression and phosphorylation of EGFR, HER3, AKT and MET were determined using Western blotting. Both pemetrexed and erlotinib inhibited the proliferation of BXPC-3 and PANC-1 cells in a dose- and time-dependent manner *in vitro*. Synergistic effects on cell proliferation were observed when pemetrexed was used in combination with erlotinib. The degree of the synergistic effects depended on the administration sequence, which was most obvious when erlotinib was sequentially administered at 24-h interval following pemetrexed. Cell cycle studies revealed that pemetrexed induced S arrest and erlotinib induced G_0/G_1 arrest. The sequential administration of erlotinib following pemetrexed induced S arrest. Western blot analyses showed that pemetrexed increased and erlotinib decreased the phosphorylation of EGFR, HER3 and AKT, respectively. However, both pemetrexed and erlotinib exerted no significant effects on the phosphorylation of c-MET. The phosphorylation of EGFR, HER3 and AKT was significantly suppressed by scheduled incubation with pemetrexed followed by erlotinib, but not by concomitant or sequential incubation with erlotinib followed by pemetrexed. In summary, our results demonstrated that the combined use of erlotinib and pemetrexed exhibited a strong synergism in BXPC-3 and PANC-1 cells. The inhibitory effects were strongest after sequential administration of pemetrexed followed by erlotinib. The synergistic effects may be related to activation of the EGFR/HER3/AKT pathway induced by pemetrexed.

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Introduction

Pancreatic cancer is one of the most common malignant tumors. At the time of diagnosis, less than 10% of pancreatic cancer can be resected (1). Its 5-year survival rate is the lowest one among all cancer types (2). Because of its high degree of malignancy, rapid progression, late stage diagnosis, early metastasis and poor prognosis, pancreatic cancer is known as the 'king of cancer'. According to the clinical guidelines of NCCN and ESMO, gemcitabine is the first-line chemotherapeutic drug for the treatment of metastatic pancreatic cancer. However, the major impediment in successful treatment is the limited effect of gemcitabine. The median survival time after the treatment of gemcitabine was found to be only 5.65 months, whereas the 1-year survival rate was merely 16-19% (3). Thus, the chemotherapy for pancreatic cancer has reached a plateau. In recent years, molecular-targeted therapy is regarded as an exciting research hotspot in the treatment of tumors because of its high specificity and minimal adverse effects. Epidermal growth factor receptor (EGFR), a member of the ErbB family, promotes tumor cell proliferation, inhibits apoptosis, improves migratory ability and thereby increases tumor invasion and distant metastasis. Previous studies have shown that the EGFR is overexpressed in pancreatic cancer cells. Erlotinib is one of the tyrosine kinase inhibitors of EGFR (EGFR-TKIs). A phase III clinical trial (4) showed that the combined use of erlotinib with gemcitabine prolonged median survival time (6.24 vs. 5.91 months, P=0.034) and 1-year survival rates (23 vs. 17%, P=0.023) in patients with advanced pancreatic cancer. Thus, erlotinib was approved by the US-FDA as a first-line treatment for advanced pancreatic cancer in November 2005. Although the combined use of erlotinib with gemcitabine was found to extend the survival period of cancer patients, the improvement of the median survival time and the 1-year

survival rate is limited. Therefore, it is of great importance to identify novel combinations of anticancer drugs with erlotinib for the treatment of advanced pancreatic cancer.

Pemetrexed is a novel antifolate that enters tumor cells rapidly via several membrane transporters, where it is metabolized to polyglutamate derivatives that are potent inhibitors of thymidylate synthase and, to a much lesser extent, glycinamide ribonucleotide formyltransferase (5,6). Pemetrexed arrests cells mainly in the S phase and induces apoptosis in a broad spectrum of solid tumors. Previous basic research has also shown that pemetrexed inhibits the proliferation of PANC-1 pancreatic cancer cells and exerts an additive effect when it is combined with gemcitabine (7). Phase II clinical studies have shown that pemetrexed significantly improves advanced pancreatic cancer treatment with few adverse side effects (8).

A timing effect was noted when erlotinib was combined with pemetrexed for the treatment of non-small cell lung cancer (NSCLC) (9), but reports on the alteration of cytotoxicity by a combined regimen of pemetrexed and erlotinib on pancreatic carcinoma are limited. Hence, the purpose of this study was to investigate the sequence-dependent effects of pemetrexed and erlotinib on BXPC-3 and PANC-1 cells and to probe the possible cellular mechanism. In the present study, the molecular mechanisms underlying the synergistic cytotoxicity between erlotinib and pemetrexed *in vitro* were first investigated. Furthermore, several factors, including modulation of EGFR, HER3 and Akt phosphorylation, which may contribute to this synergistic interaction when used in combination were characterized. We further hope that this study will aid in exploring combination treatment options for the cure of advanced pancreatic cancer.

Materials and methods

Chemicals and reagents. Erlotinib (Tarceva®) was obtained from Roche (Nonnenwald, Penzberg, Germany) and dissolved in DMSO as a stock solution of 10 mmol/l. Pemetrexed (Alimta®) was obtained commercially from Eli Lilly and Co. and dissolved in 0.9% NaCl to a final concentration of 40 g/l. Both compounds were stored at -20˚C in tightly sealed sterile tubes and diluted to the desired concentrations in PBS within 24 h of each experiment. Antibodies and their sources were as follows: anti-EGFR antibody (Santa Cruz) and anti-phosphorylated EGFR (Tyr1068) antibody (Cell Signaling); anti-AKT antibody (Santa Cruz) and anti-phosphorylated AKT (Ser473) antibody (Cell Signaling); anti-HER antibody (Santa Cruz) and anti-phosphorylated HER (Tyr1289) antibody (Cell Signaling); anti-MET antibody and anti-phosphorylated MET (Tyr1003) antibody (Cell Signaling).

Cell lines. BXPC-3 and PANC-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum at 37˚C in a humidified atmosphere of 95% air and 5% CO_2.

Growth cytotoxicity assay. The effect of erlotinib and pemetrexed on cell survival using different exposure schedules *in vitro* was assessed by the MTT colorimetric assay carried out in 96-well plates. Cells were then treated with increasing concentrations of erlotinib or pemetrexed for a duration of 72 h. To determine the proliferative effects of simultaneous or sequential administration of erlotinib and pemetrexed, cells were incubated for 72 h after concurrent or sequential treatment with erlotinib and pemetrexed for a 24-h interval. At the end of the 72-h incubation, viable cell numbers were measured using MTT assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was added at a final concentration of 0.5 mg/ml to each well. Cells were then incubated at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 for 4 h, the supernatant was removed, and the converted dye was solubilized with 150 μl DMSO. The absorbance was measured at 540 nm. Growth inhibition was expressed as a percentage of surviving cells in drug-treated vs. PBS-treated control cells (which was considered as 100% viability). The IC_{50} value was the concentration resulting in 50% cell growth inhibition after the 72 h exposure to the drug(s) compared to the untreated control cells and was calculated using CalcuSyn software (Biosoft, Inc.).

Mutation analysis of EGFR and K-ras genes. Genomic DNA was extracted from each cell line with the TIANamp DNA extraction kit (Tiangen Biotech). Exons 18-21 of the *EGFR* and exons 2 and 3 of the *K-ras* showed mutational changes by quantitative PCR high-resolution melting (qPCR-HRM) analysis (Suzhou Microdiag Biomedicine Tech. Co., Ltd.).

Cell cycle distribution analysis. Exponentially growing BXPC-3 cells were seeded in 6-well plates and treated with erlotinib and pemetrexed alone or in combination concurrently or sequentially for a defined time interval. The cells were trypsinized and pelleted by centrifugation. After washing the pellet with PBS, the cells were counted, and $1x10^6$ cells were fixed in 70% ethanol at -20˚C for 24 h. These fixed cells were then washed with PBS and incubated with RNase A (0.25 mg/ml) for 30 min at 37˚C; 5 μl of propidium iodide (KeyGen, China) was then added to the cell suspension. The mixture was incubated at room temperature (RT) for 30 min in the dark. The suspended cells were then analyzed for cell cycle distribution using the FACSCalibur Flow Cytometer (BD, USA).

Immunoblot analysis. Whole-cell extracts were obtained by lysis of cells in a 2X sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer [125 mmol/l Tris-HCl (pH 6.8), 6% SDS, 10% glycerol, 10 mmol/l of 2-mercaptoethanol]. Whole-cell extracts were separated by electrophoresis on 8% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes in 25 mmol/l Tris-HCl (pH 8.3) containing 90 mmol/l glycine and 20% methanol for 2 h at 100 mA. The membranes were blocked with Tween Tris-buffered saline (TBS) with 0.1% Triton X-100 and 5% nonfat milk for 2-3 h at RT. Primary antibodies were diluted in a blocking solution (1:200 for EGFR, AKT and HER3; and 1:5,000 for p-EGFR, p-HER3, p-AKT, MET and p-MET) Membranes were incubated overnight at 4˚C with the primary antibodies. After washing with TBS Tween-20 for 30 min, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at RT for 1 h. The membranes were then washed for 1 h with TBS Tween-20. Autoradiography was performed using chemiluminescent detection reagents according to the manufacturer's instructions, and signal detection and quantification were carried

Table I. Cytotoxicities of erlotinib or pemetrexed and the mutation status of the *EGFR* and *K-ras* genes in the BXPC-3 and PANC-1 cell lines.

Cell line	*EGFR* gene	*K-ras* gene	Erlotinib IC_{50} (μm)	Pemetrexed IC_{50} (μm)
BXPC-3	WT	WT	8.86±0.19	39.86±1.68
PANC-1	WT	Mut (2 exon)	>20	83.76±1.75

Characteristics of the pancreatic cancer cell lines and *in vitro* cytotoxicity of erlotinib or pemetrexed. The mutation status of the *EGFR* and *K-ras* genes was determined by qPCR-HRM analysis. Cytotoxicity of erlotinib and pemetrexed was determined by the cell count after 72 h of drug treatment, and is expressed as the IC_{50} calculated using the CalcuSyn software. Each value represents the means ± SD from at least three independent experiments. WT, wild-type; Mut, mutation.

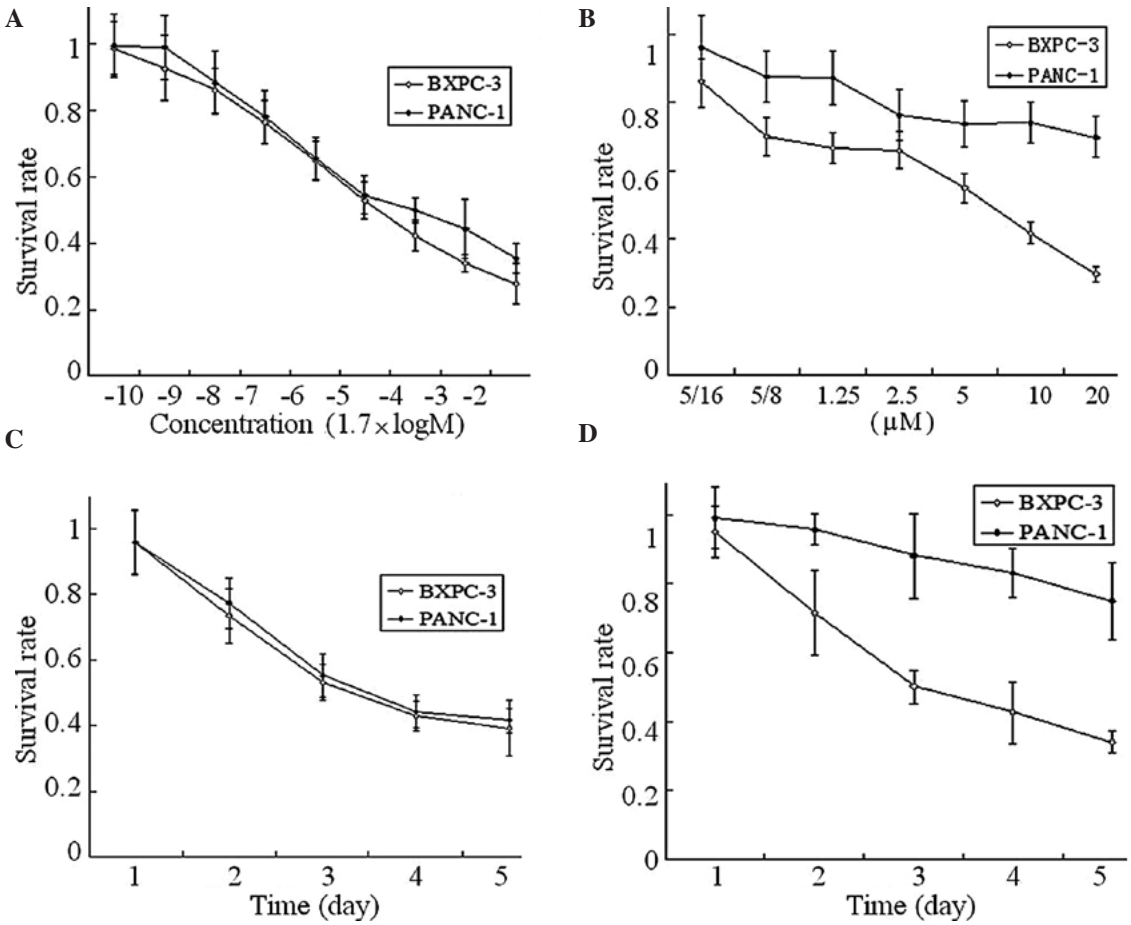

Figure 1. Effects of erlotinib and pemetrexed on the cell proliferation of BXPC-3 and PANC-1 cells. (A) Erlotinib and pemetrexed inhibited cell proliferation in a dose-dependent manner when they were added to quiescent BXPC-3 cells. (B) Erlotinib and pemetrexed inhibited cell proliferation in a dose-dependent manner when they were added to quiescent PANC-1 cells. (C) Time course of erlotinib and pemetrexed-induced cell proliferation in BXPC-3 cells. (D) Time course of erlotinib and pemetrexed-induced cell proliferation in PANC-1 cells. The curves represented cell survival rate in the presence of different concentrations of erlotinib and pemetrexed. Cells in 96-well plates were rendered quiescent in RPMI-1640 medium. Quiescent monolayers were stimulated with erlotinib or pemetrexed at the indicated concentrations and then cultured for a further 72 h. ◇ and ◆ represent the BXPC-3 and PANC-1 cells, respectively. Data are reported as the means ± SE; n=12.

out with an ECL system (Amersham Biosciences, Piscataway, NJ, USA) and Image Quant analysis software (Quantity One; Bio-Rad, Hercules, CA, USA).

Statistical analysis. All assays were performed in triplicate. Data are expressed as the means ± SD of values. Statistical analyses were performed using the Student's t-test. A value of $P<0.05$ was considered statistically significant.

Results

Cytotoxicity of pemetrexed and erlotinib and correlation with genetic background. Table I and Fig. 1 summarize the genetic background and cytotoxicity of erlotinib and pemetrexed on the human pancreatic cancer BXPC-3 and PANC-1 cells. Exons 18-21 of the *EGFR* and exons 2 and 3 of the *K-ras* gene by qPCR-HRM and mutational analysis showed

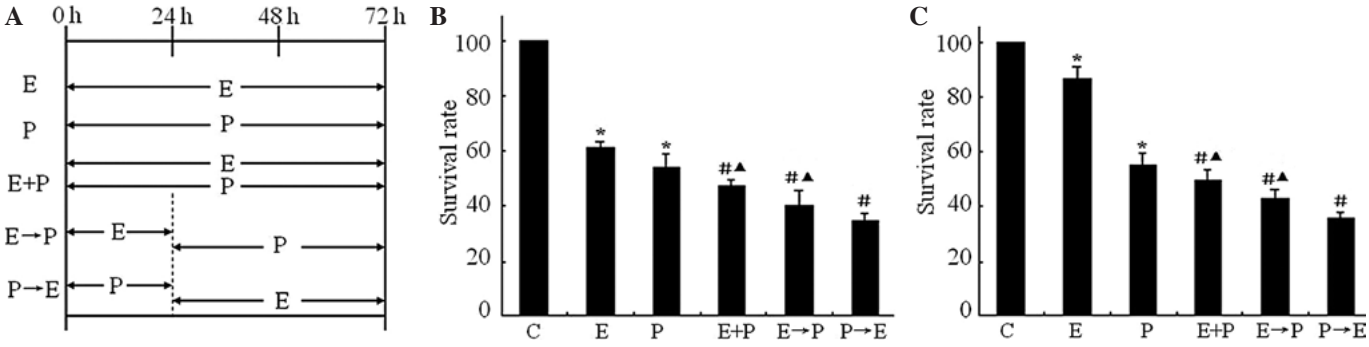

Figure 2. Effects of different exposure schedules of erlotinib and pemetrexed on cell survival *in vitro* measured by means of the MTT colorimetric assay. (A) The five exposure schedules tested: i) erlotinib treatment alone (E group); ii) pemetrexed treatment alone (P group); iii) concurrent treatment of erlotinib and pemetrexed for 72 h (E+P group); iv) 24-h exposure of erlotinib followed by pemetrexed for a total of 72 h (E→P group); v) 24-h exposure of pemetrexed followed by erlotinib for a total of 72 h (P→E group). (B) Effect of different administration schedules of erlotinib and pemetrexed on the proliferation of BXPC-3 cells. (C) Effect of different administration schedules of erlotinib and pemetrexed on the proliferation of PANC-1 cells. Data are reported as the means ± SE; n=6. *P<0.05 vs. control; #P<0.05 vs. E and P groups; ▲P<0.05 vs. P→E group.

that BXPC-1 cells were wild-type and PANC-1 cells harbored a specific mutation in *K-ras* exon 2. The cytotoxicity results showed that erlotinib (0.3125-20 μmol/l) and pemetrexed ($1.7x10^{-7}$-17 mmol/l) suppressed BXPC-3 and PANC-1 cell proliferation, and the effect was concentration-dependent (Fig. 1A and B) and time-dependent (Fig. 1C and D). The IC_{50} of pemetrexed in the BXPC-3 and PANC-1 cells was 39.86±1.68 and 83.76±0.19 μmol/l, respectively. The IC_{50} of erlotinib in the BXPC-3 and PANC-1 cells was 8.86±1.68 and >20 μmol/l, respectively. Concentrations of erlotinib >20 μmol/l could not be achieved due to its low solubility in culture medium. Accordingly, 10 μmol/l was chosen as the concentration of erlotinib used in the PANC-1 cells for subsequent studies. At the IC_{50} of pemetrexed and erlotinib, the inhibitory effects were time-dependent and reached the maximal effect at day 5.

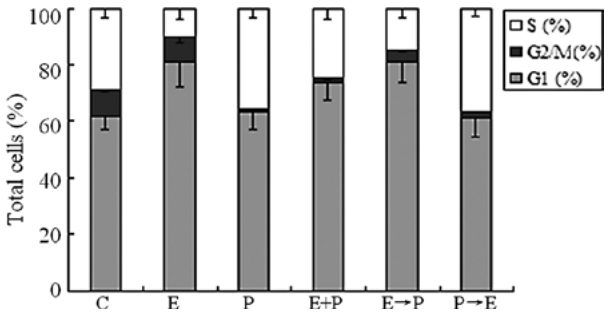

Figure 3. Alterations is the cell cycle upon different exposure schedules of pemetrexed and erlotinib in BXPC-3 cells. BXPC-3 cells were treated with different schedules of pemetrexed (39.86 μmol/l) and/or erlotinib (8.86 μmol/l) alone or in combination. The exposure schedules tested and the concentrations used in the cell cycle distribution were consistent with their effects on cell growth. Columns, the mean of three independent experiments; bars, SD.

Effects of different schedules of erlotinib and pemetrexed on cell proliferation. We observed the growth inhibitory effects of erlotinib in combination with pemetrexed concurrently or sequentially at a 24-h interval. Fig. 2A illustrates the five exposure schedules tested that mimic possible clinical scenarios: i) erlotinib or pemetrexed alone for 72 h (E and P), ii) erlotinib and pemetrexed concurrently (E+P) for 72 h, iii) erlotinib for 24 h followed by pemetrexed for 48 h (E→P), and iv) pemetrexed for 24 h followed by erlotinib for 48 h (P→E). At IC_{50}, the effects of pemetrexed in combination with erlotinib on cell proliferation depended on the sequence. The results showed that in the BXPC-3 cells (erlotinib-sensitive cells), the cell survival rate was 60.83% for erlotinib alone and 54.01% for pemetrexed alone, 47.31% for erlotinib and pemetrexed concurrently (E+P), 40.15% for erlotinib followed by pemetrexed (E→P) and 34.56% for pemetrexed followed by erlotinib (P→E) (Fig. 2B). The results showed that in the PANC-1 cells (erlotinib-resistant cells), the cell survival rate was 86.46% for erlotinib alone and 55.29% for pemetrexed alone, 49.38% for erlotinib and pemetrexed concurrently (E+P), 42.75% for erlotinib followed by pemetrexed (E→P) and 35.84% for pemetrexed followed by erlotinib (P→E) (Fig. 2C). Compared to cells treated with erlotinib or pemetrexed alone, significant additive effects on cell proliferation were found when erlotinib was added concurrently or sequentially with pemetrexed (P<0.05). Furthermore, sequential administration of erlotinib following pemetrexed significantly enhanced the cell growth inhibition in BXPC-3 and PANC-1 cells, compared to pemetrexed administered concurrently or prior to erlotinib (P+E or P→E) (P<0.05).

Cell cycle effects of pemetrexed and erlotinib. To probe the possible mechanism of growth inhibition of erlotinib in combination with pemetrexed in BXPC-3 cells, cell cycle analysis was performed. The BXPC-3 cells were treated for 24 h with 8.86 μmol/l erlotinib, 39.86 μmol/l pemetrexed alone and in combination concurrently or sequentially at a 24-h interval. The concentrations of erlotinib or pemetrexed used were the IC_{50} concentrations, respectively. As shown in Fig. 3, 48 h of exposure to erlotinib alone induced G_0/G_1 phase arrest (P<0.05) and decreased the number of cells in the S phase (P<0.05), resulting in a 20% cell increase in the G_0/G_1 phase compared to the untreated control cells. However, pemetrexed alone induced S arrest (P<0.05) and decreased the number of cells in the G_2/M phase (P<0.05). Compared to pemetrexed alone, both concurrent cotreatment of erlotinib with pemetrexed and sequential treatment of erlotinib followed by pemetrexed resulted in an accumulation of cells in the G_0/G_1 phase (P<0.05) and a decrease in the G_2/M phase (P<0.05).

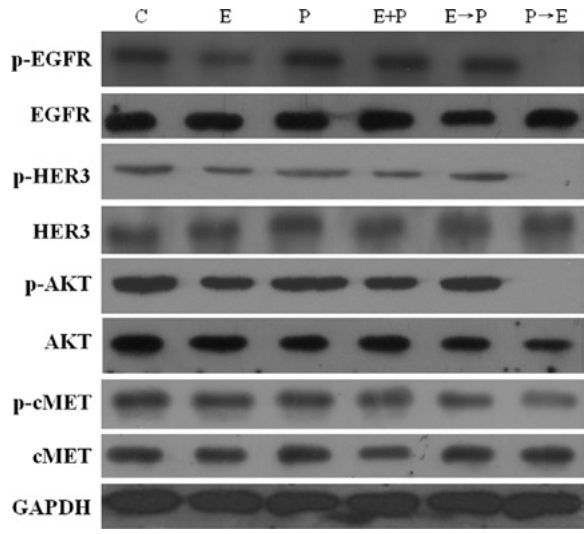

Figure 4. Effect of different exposure schedules of erlotinib and pemetrexed on EGFR/HER3/AKT signaling pathways and cMET in BXPC-3 cells. Western blotting indicates the expression and phosphorylation of EGFR, HER3, AKT and MET in BXPC-3 cells. Cells were treated as described in Fig. 2A.

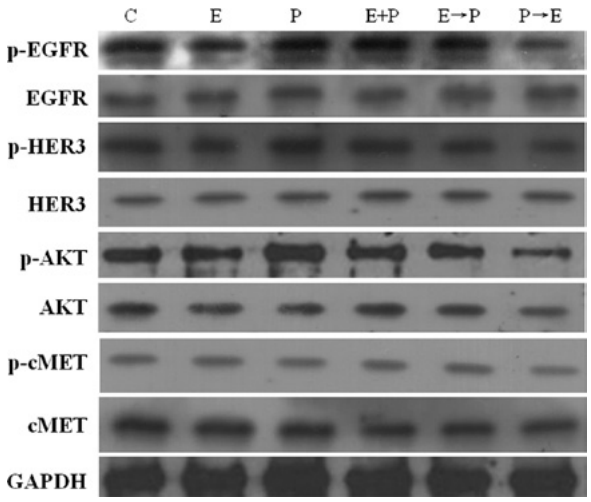

Figure 5. Effect of different exposure schedules of erlotinib and pemetrexed on EGFR/HER3/AKT signaling pathways and cMET in PANC-1 cells. Western blotting indicates the expression and phosphorylation of EGFR, HER3, AKT and MET in PANC-1 cells. Cells were treated as described in Fig. 2A.

By contrast, sequential administration of pemetrexed followed by erlotinib resulted in S arrest ($P<0.05$) and a decrease in cells in the G_2/M phase ($P<0.05$) compared to erlotinib alone.

Pemetrexed activates the EGFR, HER3 and AKT signaling pathway in human pancreatic BXPC-3 and PANC-1 cells. To gain insight into the mechanism(s) underlying the cytotoxic synergism between pemetrexed and erlotinib, the effect of pemetrexed on the EGFR pathway in the BXPC-3 and PANC-1 cells was further examined by Western blot analysis (Figs. 4 and 5). As expected, erlotinib induced a significant suppression of EGF-induced phosphorylation of EGFR in the BXPC-3 and PANC-1 cells; the percent reductions in EGFR-phosphorylated protein were 47.4 and 30% in BXPC-3 and PANC-1 cells, respectively. Conversely, pemetrexed significantly enhanced EGFR phosphorylated levels, and the protein levels were 26.3 and 13.6% higher compared to the control cells, respectively. Moreover the administration schedule (P→E) significantly reduced the phosphorylation status of EGFR when compared to treatment with erlotinib alone.

Since EGFR signaling is transduced mainly through the HER3/AKT pathways, we investigated the phosphorylation status of HER3 and AKT to determine their activity after drug treatment. Erlotinib resulted in the inhibition of p-HER3 and p-AKT in the BXPC-3 and PANC-1 cell lines. The p-HER3 levels were potently (~17 and 14%) down-regulated by erlotinib in the BXPC-3 and PANC-1 cells, respectively. p-AKT levels were potently (~45%) down-regulated by erlotinib in the BXPC-3 cells. Conversely, pemetrexed significantly enhanced EGFR, HER3 and AKT phosphorylation levels. p-EGFR levels were up-regulated (~26 and 14%) by pemetrexed in the BXPC-3 and PANC-1 cells, respectively. p-HER3 levels were up-regulated (~58 and 26%) by pemetrexed in the BXPC-3 and PANC-1 cells, respectively. p-AKT levels were up-regulated (~15 and 26%) by pemetrexed in the BXPC-3 and PANC-1 cells. As a mechanism of escape of cancer from anti-EGFR therapy, the phosphorylation level of MET was determined by immunoblotting. The results conclusively showed that no significant change in the phosphorylation level of MET was noted for the different schedules of erlotinib and pemetrexed exposure (Figs. 4 and 5).

Discussion

The aim of the present study was to investigate the cytotoxic activity of erlotinib and pemetrexed in combination and to define the optimal schedule and the cellular mechanism involved in drug interaction against human pancreatic cancer cells. Two significant findings of this study are as follows: i) Synergistic effects on cell proliferation were found when pemetrexed was used in combination with erlotinib. The degree of the synergistic effects depended on the treatment sequence, which was most significant when erlotinib was sequentially administrated at a 24-h interval following pemetrexed. ii) These synergistic effects may be related to the activation of the EGFR/HER3/AKT pathway induced by pemetrexed.

In the present study, a set of experiments was designed to elucidate the combination effects and possible cellular mechanism underlying the interaction between pemetrexed and erlotinib in PANC-1 and BXPC-3 cells. PANC-1 and BXPC-3 cells were exposed to pemetrexed and erlotinib using five treatment schedules. In agreement with previous studies (10,11), the results of the present study showed that pemetrexed and erlotinib alone significantly inhibited cell proliferation. We found that the synergistic effects of pemetrexed and erlotinib on cell proliferation were sequence-dependent. Furthermore, the cytotoxic synergism was observed in both erlotinib-sensitive and erlotinib-resistant human pancreatic cell lines. This was independent of the mutation status of the *EGFR* or *K-ras* gene. These results were in accord with previous pre-clinical findings that the existence of a synergistic interaction between EGFR-TKIs and chemotherapeutic agents in NSCLC cell lines was schedule-dependent (9,12).

Previous reports have illustrated the importance of modulating the cell cycle to exploit the optimal effect of drug combinations. Furthermore, there is growing laboratory

evidence of a possible sequence-dependent antagonism between EGFR-TKIs and cytotoxic agents as a result of the well-known G_1-phase arrest of tumor cells by EGFR-TKIs, which protect tumor cells from cell cycle-specific cytotoxic agents (13,14). In the present study, flow cytometry demonstrated that both pemetrexed and erlotinib caused an accumulation of cells in the S and G_1 phases, respectively. Compared to pemetrexed alone, concurrent treatment of both agents as well as erlotinib followed by pemetrexed resulted in an accumulation of cells in the G_1 phase. Compared to erlotinib alone, concurrent treatment resulted in an accumulation of cells in the S phase. Our result also showed that sequential administration of pemetrexed followed by erlotinib resulted in S phase arrest when compared to erlotinib treatment alone. These results may explain why the concomitant or sequential treatment of pemetrexed followed by erlotinib exerted significant additive effects on cell proliferation. By contrast, sequential administration of erlotinib following pemetrexed induced G_1 arrest. The increase in cells in the G_1 phase could not provide a plausible explanation for discordant results of sequence-dependent effects on cell proliferation when erlotinib followed pemetrexed. It has been reported that modulation of EGFR activity following chemotherapeutic agents determines the response to pemetrexed in NSCLC cells (9), and erlotinib strengthens the cytotoxic effect of pemetrexed in NSCLC cells by harboring specific molecular characteristics (15). Further studies are required to explore the possible cellular mechanism.

Furthermore, the possible molecular mechanism of the cell signaling pathways involved was determined. The dependency of NSCLC and pancreatic cancer cells on the EGFR pathway for growth and survival was found to be an important determinant of sensitivity towards EGFR-TKI monotherapy (16,17). Previous reports have shown that the mammalian target of rapamycin inhibitor rapamycin activates the phosphatidylinositol 3-kinase (PI3K)/AKT pathway and enhances cytotoxicity of the PI3K/AKT inhibitor, LY294002 (18). In pre-clinical studies, the combination of pemetrexed and erlotinib yielded conflicting results in various NSCLC tumor cell lines. Li *et al* discovered that schedule-dependent synergism of pemetrexed and erlotinib was associated with pemetrexed-induced EGFR phosphorylation and AKT phosphorylation in NSCLC tumors (9). On the contrary, a previous study demonstrated that the effect of pemetrexed reduced Akt phosphorylation in NSCLC tumors (15). However, the contradictory findings may be related to the discrepancy between drug exposure conditions and sensitivity of the experimental methods. In agreement with these findings, *in vitro* experimental data obtained in this study indicate enhanced phosphorylation of EGFR/Akt after exposure to pemetrexed. Our results suggest that pemetrexed enhances the antitumor activity of erlotinib by activation of the EGFR-AKT pathway.

The EGFR family comprises four distinct receptors: EGFR/ErbB1, HER-2/ErbB2, HER-3/ErbB3 and HER-4/ErbB4. Pancreatic cancer cells frequently overexpress EGFR, HER-2, HER-3 and, less frequently, HER-4, as well as six ligands that bind directly to EGFR (19). Overexpression of EGFR, HER-2 and HER-3 has also been implicated in the development and progression of pancreatic cancer (20,21). Giovannetti *et al* found that 60-70% of pancreatic cancer cases have an amplification of HER-3 (22). Previous reports have shown that phosphorylated HER-3 acts as a scaffold to recruit signaling proteins, including PI3K (23,24). Phosphorylated HER3 binds the regulatory subunit of PI3K (p85) leading to activation of PI3K (p110α) and its downstream kinase AKT, essential for cell growth and survival (25,26). The EGFR kinase inhibitor gefitinib prevents phosphorylation of both EGFR and HER3, as a result, the PI3K-AKT pathway is uncoupled from HER3 and inactivated. A recent study suggests that HER-3 may be a useful biomarker for the selection of patients who are most likely to respond to the EGFR inhibitor erlotinib (27). The phosphorylation status of HER3 and AKT was studied here to determine their activity after the scheduled drug treatment. The data showed that pemetrexed significantly enhanced HER3 and AKT phosphorylation levels. The p-HER3 levels were potently (~17 and 14%) down-regulated by erlotinib in the BXPC-3 and PANC-1 cells, respectively. Our findings in this study indicate that pemetrexed enhances the antitumor activity of erlotinib by activating the EGFR/HER3/AKT pathway, thus rendering the cells more dependent on the EGFR pathway and more susceptible to the inhibition by erlotinib.

Although the above-mentioned results justify the synergistic effects of sequential administration of pemetrexed followed by erlotinib, it cannot explain the synergistic effects noted upon the concomitant exposure of both agents or erlotinib followed by pemetrexed. The expression of MET phosphorylation was also observed in this study. In gefitinib-resistant cells, Engelman *et al* (28) found that the oncogenic receptor tyrosine kinase MET could also phosphorylate HER3, leading to activation of the PI3K/AKT pathway, and amplification of MET caused gefitinib resistance by driving ERBB3 (HER3)-dependent activation of PI3K, a pathway thought to be specific to EGFR/ERBB family receptors (29). Pharmacological inhibition of either EGFR or MET alone was insufficient to inactivate the HER3-PI3K-AKT axis or stall resistant cell growth (30,31). However, our data showed that there was no significant change in MET phosphorylation.

In conclusion, the present study characterized several molecular mechanisms and determinants involved in the synergistic effect between erlotinib and pemetrexed against pancreatic cancer BXPC-3 and PANC-1 cells, regardless of their genetic signature. Our results suggest that pemetrexed enhances the antitumor activity of erlotinib by activating the EGFR/HER3/AKT pathway, thus rendering the cells more dependent on the EGFR pathway and more susceptible to the inhibition by erlotinib.

Although the extrapolation of *in vitro* data to the clinical setting should be considered with caution, these results may have implications for the rational development of chemotherapeutic regimens, including erlotinib and pemetrexed, for the treatment of pancreatic cancer. Although our results explain the synergistic effects of a sequential administration of pemetrexed followed by erlotinib, we are unable to explain the synergistic effects of a concomitant exposure of both agents and erlotinib treatment followed by pemetrexed. This raises the possibility of the involvement of another cellular signaling mechanism, which will be the focus of our next stage of research.

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