Acquired resistance of pancreatic cancer cells to treatment with gemcitabine and HER-inhibitors is accompanied by increased sensitivity to STAT3 inhibition

NIKOLAOS IOANNOU[1], ALAN M. SEDDON[1], ANGUS DALGLEISH[2], DAVID MACKINTOSH[1], FLAVIO SOLCA[3] and HELMOUT MODJTAHEDI[1]

[1]School of Life Science, Pharmacy and Chemistry, Kingston University London, Kingston; [2]Department of Cellular and Molecular Medicine, St George's University of London, London, UK; [3]Boehringer Ingelheim RCV GmbH & Co KG, Vienna, Austria

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Abstract. Drug-resistance is a major contributing factor for the poor prognosis in patients with pancreatic cancer. We have shown previously that the irreversible ErbB family blocker afatinib, is more effective than the reversible EGFR tyrosine kinase inhibitor erlotinib in inhibiting the growth of human pancreatic cancer cells. The aim of this study was to develop human pancreatic cancer cell (BxPc3) variants with acquired resistance to treatment with gemcitabine, afatinib, or erlotinib, and to investigate the molecular changes that accompany the acquisition of a drug-resistant phenotype. We also investigated the therapeutic potential of various agents in the treatment of such drug-resistant variants. Three variant forms of BxPc3 cells with acquired resistance to gemcitabine (BxPc3GEM), afatinib (BxPc3AFR) or erlotinib (BxPc3OSIR) were developed following treatment with increasing doses of such drugs. The expression level, mutational and phosphorylation status of various growth factor receptors and downstream cell signaling molecules were determined by FACS, human phopsho-RTK array, and western blot analysis while the sulforhodamine B assay was used for determining the effect of various agents on the growth of such tumours. We found that all three BxPc3 variants with acquired resistance to gemcitabine (BxPc3GEM), afatinib (BxPc3AFR) or erlotinib (BxPc3OSIR) also become less sensitive to treatment with the two other agents. Acquisition of resistance to these agents was accompanied by upregulation of p-c-MET, p-STAT3, CD44, increased autocrine production of EGFR ligand amphiregulin and differential activation status of EGFR tyrosine residues as well as downregulation of total and p-SRC. Of all therapeutic interventions examined, including the addition of an anti-EGFR antibody ICR62, an anti-CD44 monoclonal antibody, and of STAT3 or c-MET inhibitors, only treatment with the STAT3 inhibitor Stattic produced a higher growth inhibitory effect in all three drug-resistant variants. In addition, treatment with a combination of afatinib with either c-MET inhibitor Crizotinib or Stattic resulted in an additive or synergistic growth inhibition in all three variants. Our results suggest that activation of STAT3 may play an important role in the acquisition of resistance to gemcitabine and HER inhibitors in pancreatic cancer and warrant further studies on the therapeutic potential of STAT3 inhibitors in such a setting.

Correspondence to: Professor Helmout Modjtahedi, School of Life Science, Pharmacy and Chemistry, Kingston University London, Kingston-upon-Thames, Surrey KT1 2EE, UK
E-mail: h.modjtahedi@kingston.ac.uk

Abbreviations: EGFR, epidermal growth factor receptor; FOLFIRINOX, fluorouracil, irinotecan and oxaliplatin; CSC, cancer stem cells; EMT, epithelial mesenchymal transition; RTK, receptor tyrosine kinases; ATCC, American Type Culture Collection; MAPK, mitogen-activated protein kinase; STAT3, signal transducer and activator of transcription 3; IGF-IR, insulin like growth factor receptor I; 5-FU, 5-fluorouracil; HB-EGF, heparin-binding EGF; PVDF, polyvinylidene difluoride; dCK, deoxycytidine kinase; MDR, multi-drug resistance; ABC transporters, ATP-binding cassette transporters; P-gp, P-glycoprotein; NSCLC, non-small lung cancer

Key words: pancreatic cancer, drug resistance, epidermal growth factor receptor, afatinib, erlotinib, gemcitabine, cancer stem cells, EMT markers

Introduction

Despite the advances in our understanding of cancer biology, diagnosis and therapy in the past decades, pancreatic cancer is still one of the deadliest types of human cancer, with a median survival rate of less than 6 months (1,2). Worldwide, there were an estimated 338,000 new cases of pancreatic cancer and 330,000 pancreatic cancer death in 2012 (3). It is the only type of cancer with an annual mortality rate which is so close to its annual incidence rate. This mainly stems from its late diagnosis, and its resistance to the current forms of therapy (4). Since its introduction in 1997, gemcitabine is the gold standard for the treatment of locally advanced and metastatic pancreatic cancer (5,6). In addition, of the numerous agents investigated, only the addition of erlotinib (OSI-744), an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, to gemcitabine therapy led to a modest, nonetheless

significant prolonged median overall survival in pancreatic cancer patients (7-9). The limited clinical benefit of erlotinib stems from the fact that the majority of pancreatic cancer patients simply do not respond to this treatment or acquire drug resistance following a short course of therapy (10). While the addition of two more effective chemotherapy combinations in the last decade, such as FOLFIRINOX (fluorouracil, irinotecan and oxaliplatin) and the combination of gemcitabine with Nab (nanoparticle albumin-bound)-paclitaxel improved the median survival rates for patients with metastatic disease, such combinational approaches are only suitable for patients with good performance status, with gemcitabine monotherapy remaining the only option for patients with poor performance status (6,11).

The extremely limited progress in improving survival outcomes in pancreatic cancer during the last decades, underlines the need not only for the development of more effective inhibitors for existing targets such as EGFR, but also it is imperative to develop new targeted agents and combination therapies for overcoming drug resistance.

We have shown previously that afatinib, an irreversible ErbB family blocker, is superior at inhibiting the growth of a panel of human pancreatic cancer cell lines compared to first generation reversible EGFR inhibitors such as erlotinib (OSI-774) or gefitinib (12,13). As drug-resistance is a major cause of treatment failure, development of drug-resistant pancreatic cancer models could help in unravelling the molecular mechanisms of acquired resistance and facilitate the discovery of novel and more effective approaches for the treatment of pancreatic cancer patients. We have shown previously that of seven pancreatic cancer cell lines investigated, BxPc3 cells exhibited the highest sensitivity to targeted agents afatinib and erlotinib (12). In this study, we developed three variants of the human pancreatic cancer cell line BxPc3, resistant to afatinib, erlotinib and gemcitabine and investigated the possible molecular alterations accompanying the acquisition of a drug-resistant phenotype. In particular, we determined the expression levels of EGFR ligands, putative cancer stem cells (CSCs) and epithelial mesenchymal transition (EMT) markers, as well as the expression levels and activation status of several receptor tyrosine kinases (RTKs) including the HER family of receptors and several downstream cell signalling molecules in the parental pancreatic cancer and its drug-resistant variants. We also investigated the therapeutic potential of several agents in the treatment of such drug-resistant variants.

Materials and methods

Cell culture and tumour cell lines. BxPc3 pancreatic cancer cell line was purchased from the American Type Culture Collection (ATCC). BxPc3 cells were cultured routinely at 37˚C in a humidified atmosphere (5% CO_2) in RPMI-1640 medium (Sigma-Aldrich, Dorset, UK) supplemented with 10% foetal bovine serum (PAA, UK), antibiotics penicillin (50 U/ml), streptomycin (0.05 mg/ml) and neomycin (0.1 mg/ml) and glutamine at a final concentration of 2 mM (Sigma-Aldrich), as previously described (14).

Antibodies and other reagents. Primary mouse antibodies HM50.67A and HM43.16B, were raised against the external domain of the HER-2 and EGFR, respectively (15). Mouse MAB3481 (anti-HER-3), MAB11311 (anti-HER-4), anti-insulin like growth factor receptor I (IGF-IR) mAbs and anti-E-cadherin were purchased from R&D Systems (Abingdon, UK). Secondary FITC-conjugated rabbit anti-mouse mAb STAR9B was obtained from AbD Serotec (UK). Gemcitabine was acquired from Healthcare at Home (UK) while The irreversible pan-HER family blocker afatinib was developed by Boehringer Ingelheim (Austria) as previously described (16,17). OSI-774 was kindly provided by OSI-Phamarceutical (USA). Doxycycline, 5-fluorouracil (5-FU), oxaliplatin, and mouse anti-EGFR antibody were purchased from Sigma-Aldrich. Mouse antibodies against β-actin and vimentin as well as rabbit antibodies against AKT, Mitogen-activated protein kinase (MAPK), phospho-MAPK (Thr202/Tyr204), phospho AKT (S473), Signal transducer and activator of transcription 3 (STAT3), p-STAT3 (Y705), Src, p-Src (Y416), c-MET (mouse), p-MET (Y1234/1235), p-EGFR (Y1086, 1068, 1143, 1173, 1045), p-HER3 (Y1289), HER3, HER2 and p-HER2 (Y1221/1222) were purchased from Cell Signalling, UK. The mouse anti-p-IGF-IR (Y1161) antibody and STAT3 inhibitor Stattic were purchased from Santa Cruz Biotechnology Inc. (Insight Biotechnology, UK).

Establishment of drug-resistant cell lines. We showed previously that of all pancreatic cancer cell lines investigated, BxPc3 cells exhibited the highest overall sensitivity to treatment with either HER-targeted or chemotherapeutic agents (12). Drug-resistant pancreatic cancer variants were developed by the treatment of BxPc3 cells with escalating doses of afatinib, erlotinib or gemcitabine. Cells were cultured routinely in small cell culture flasks (25 cm^2) in growth medium/10% FBS in the presence of increasing doses of an inhibitor for a period of over 6 months. Once tumour cells were able to maintain exponential growth at the presence of at least 3x the IC_{50} concentration of the drug, they were passed 15 times in drug-free medium and drug sensitivity was determined again to ensure that drug resistance acquisition was permanent.

Migration studies. For migration studies, 200 μl of cell suspension at a density of $2x10^5$ cells/ml were mixed with 50 μl of serum free medium alone or with the inhibitors and then seeded into Transwell inserts (pore size 8 μm) of 24-well plates (Becton Dickinson Ltd., UK). The lower chamber was filled with 750 ml of growth medium supplemented with 10% FBS (as chemoattractant) and cells were incubated at 37˚C for 6 h. Following incubation, non-migrated cells were removed from the Transwell insert (upper surface of the membrane) using a cotton swab, and cells were fixed with ice-cold methanol for 10 min at room temperature. Cells were stained with haematoxylin and were then washed. The number of cells that had migrated through the membrane was counted under a microscope at x100 magnification. Five fields were counted in total for each sample. Results are expressed as the average number of migrated cells.

Flow cytometry. The cell surface expression of putative pancreatic CSCs (CD44, CD24 and CD133), HER family members (EGFR, HER-2, HER-3 and HER-4), IGF-IR and c-MET was assessed by flow cytometry as previously

Table I. Changes in the sensitivity of drug resistant variants to various inhibitors.

	IC_{50}								
Cell line	Gemcitabine	5-FU	Doxycycline	Oxaliplatin	Afatinib	Erlotinib	Gefitinib	NVP-AEW541	Crizotinib
BxPc3 parental	7.5 nM	655 nM	11 μM	3.8 μM	17 nM	1.45 μM	2.3 μM	1.25 μM	1.71 μM
BxPc3AFR	386 nM	555 nM	10.68 μM	3.2 μM	1.32 μM	4.3 μM	8.3 μM	1.63 μM	1.46 μM
Fold change	51.46[a]	0.84	0.97	0.84[a]	77.64[a]	2.96[a]	3.55[a]	1.30	0.85
BxPc3GEMR	663 nM	613.5 nM	7.2 μM	1.34 μM	1.2 μM	6.1 μM	5.545 μM	1.34 μM	1.24 μM
Fold change	88.4[a]	0.93	0.75	0.35[a]	70.88[a]	4.20[a]	2.37[a]	1.07	0.72
BxPc3OSIR	507.5 nM	1.2 μM	7.3 μM	11.25 μM	3.1 μM	5.25 μM	6.4 μM	3.25 μM	1.61 μM
Fold change	67.66[a]	1.83[a]	0.66	2.96[a]	182.3[a]	3.62[a]	2.74[a]	2.58[a]	0.93

Drug sensitivity was determined by the SRB colorimetric assay and IC_{50} values were calculated as described in Materials and methods. ([a]$p<0.05$ compared to parental cell line).

described (12). A minimum of 10,000 events were recorded following excitation with an argon laser at 488 nm using the FL-1 detector (525 nm) of a BD FACsCalibur flow cytometer (Becton Dickinson Ltd.). Mean fluorescence intensity values were calculated using the CellQuest Pro software (Becton Dickinson Ltd.) and compared with those of negative controls (no primary antibody).

Growth inhibition studies. The effect of the various agents, on the growth of human cancer cell lines was investigated using the sulforhodamine B (SRB; Sigma-Aldrich) colorimetric assay as previously described (12). Interactions between the different agents when used in combination were assessed, using the combination index (CI) as described by Chou and Talalay (18). For each combination the two drugs were mixed at their 8X IC_{50} followed by 8 doubling dilutions. Interpretation of the results was based on the proposed descriptions for presenting the degrees of antagonism or synergism by Calcusyn software. In general, CI<0.9 indicates a synergistic effect while CI between 0.90-1.10 denotes an additive effect. CI>1.1 indicates antagonistic effects. Data analysis was performed using the Calcusyn software (Biosoft, Cambridge, UK).

Determination of autocrine ligand production by tumour cells. The level of autocrine EGFR ligands [EGF, TGF-α, beta-cellulin, amphiregulin and heparin-binding EGF (HB-EGF)] secreted by the tumour cells into the culture supernatant was determined using the R&D Duoset ELISA kit following the manufacturer's instructions (R&D Systems). Briefly, tumour cells were grown in wells in a 6-well plate with 5 ml of growth medium supplemented with 10% FBS until almost confluent. Growth medium was replaced with fresh serum-free medium and incubated overnight at 37˚C. Supernatants were collected from each well and then the number of cells in each well was determined for all samples.

A standard curve was created for each ligand investigated, using a four parameter logistic (4-PL) curve-fit. Concentration of ligands in cell supernatants was determined from each standard curve using GraphPad prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA).

Analysis of receptor tyrosine kinase (RTK) phosphorylation status by the Proteome Profiler 96 Human Phospho-RTK Array 1. The basal phosphorylation status of 16 RTKs (EGFR, HER-2, HER-3, HER-4, HGF-R (c-MET), IGF-IR, INS-R, M-CSFR, MSP-R, PDGFRa, PDGFRb, SCFR, Tie-2, VEGFR1, VEGFR2, VEGFR3) was investigated in BxPc3 parental cells and its drug-resistant variants using the Proteome Profiler 96 Human Phospho-RTK Array 1 (Catalog # ARZ001) following the manufacturer's instructions (R&D Systems). The plate was imaged using a G-box imaging system (Invitrogen, UK) and data analysis was performed using Q-view software (Quansys Biosciences, Logan, UT, USA).

Western blotting. Parental cancer cells and drug-resistant variants were grown to near confluency in 6-well culture plates containing 5 ml of 10% FBS RPMI growth medium. Cells were washed once with 5 ml of RPMI/0.5% FBS, lysed using 400 μl of lithium dodecyl sulfate (LDS) lysis buffer (Invitrogen) containing protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were heated at 90˚C for 5 min, protein samples (25 μg) were separated on 4% to 12% Bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen). The PVDF membranes were probed with antibodies against several cell signalling molecules at optimal concentrations according to the manufacturer's instructions. The specific signals were detected using the WesternBreeze chemiluminescence kit (Alkaline phosphatase conjugated secondary antibody) (Invitrogen). Results were visualized using the G-box imaging system (Syngene, Cambridge, UK).

Mutational analysis by next generation sequencing. Characterization of somatic mutations in the parental BxPc3 cell line and its drug-resistant clones was performed by using the Ion AmpliSeq™ Cancer Hotspot Panel v2 (CHPv2) (Life Technologies, Paisley, UK) following the manufacturer's instructions. Ion AmpliSeq CHPv2 is a next generation sequencing assay which can identify numerous somatic mutations [2855 hot spots/catalogue of somatic mutations in cancer (COSMIC) mutations] from 50 genes including EGFR, HER2, KRAS, p53, PIK3CA, PTEN and c-MET among others. For

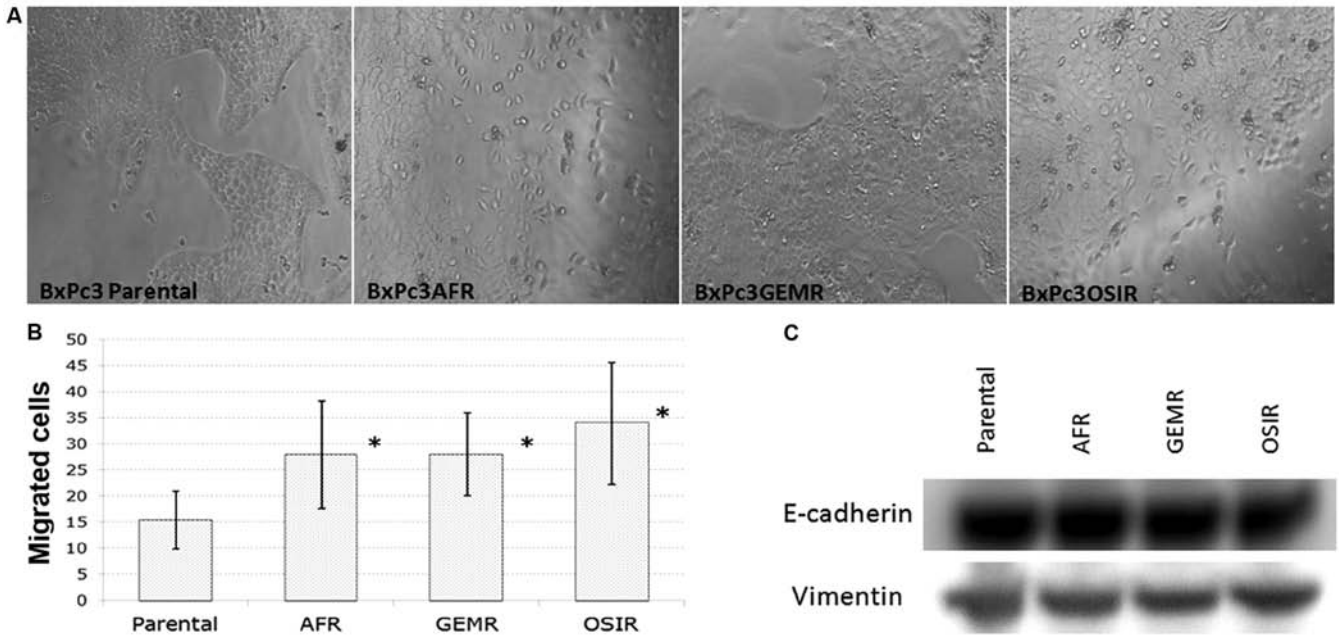

Figure 1. Morphology (A), migration ability (B), and expression of EMT markers vimentin and E-cadherin (C) in the drug-resistant pancreatic cancer cell lines and their parental counterpart (original magnification, x20). Results are expressed as the average number of migrated cells of five fields in two independent experiments (*p<0.05) [Error bars, standard deviation (SD)]. Expression of EMT markers was investigated by western blot analysis as described in Materials and methods.

a detailed list of all genes and hotspots included in the assay please see Table I). Genomic DNA was extracted from each cancer cell line using the AllPrep DNA/RNA/Protein Mini kit (Qiagen, UK) according to the manufacturer's instructions. Analysis of sequencing data was performed using the Ion Reporter software (Life Technologies) and confirmed by using NextGENe® software (Softgenetics, UK).

Statistical analysis. The unpaired two-tailed Student's t-test was used for comparing mean values between two groups. Data are presented as mean ± SD. $p<0.05$ was considered statistically significant.

Results

Establishment of drug-resistant pancreatic cancer cell lines. In this study, we established three variant forms of BxPc3 cells; with acquired resistance to gemcitabine (BxPc3GEMR), afatinib (BxPc3AFR) and erlotinib (BxPc3OSIR). IC_{50} values for each drug in the parental cells and their drug-resistant variants, following at least 15 passages in drug-free medium, are presented in Table I. The morphology of the drug-resistant variants is presented in Fig. 1A. All BxPc3 derived drug-resistant cell lines, gained increased migration ability compared to the parental cell line indicating a higher metastatic potential (Fig. 1B). However, there were no changes in the expression of EMT markers (vimentin and E-cadherin) in the drug-resistant variants compared to the parental cell line (Fig. 1C).

Growth response of parental and drug-resistant pancreatic cancer cell variants to treatment with various agents following acquisition of drug resistance. All three drug-resistant variants exhibited significant changes in their sensitivity to treatment with several agents compared to their parental counterpart (Table I). For example, BxPc3AFR variant with acquired resistance to afatinib (77.64-fold change, $p<0.05$), also developed resistance to erlotinib (2.96-fold change, $p<0.05$), gefitinib (3.55-fold change, $p<0.05$) and gemcitabine (51.46-fold change, $p<0.05$) (Table I). Similarly, BxPc3GEMR and BxPc3OSIR variants demonstrated a reduced sensitivity to treatment with gemcitabine, afatinib, erlotinib and gefitinib. For example, BxPc3OSIR variant in addition to erlotinib, became highly resistant to treatment with afatinib with 182-fold increase in its IC_{50} value ($p<0.05$) (Table I). However, the changes in sensitivity to other agents differed considerably in each of these cell lines. For example, in comparison to the parental BxPc3 cells, both BxPc3AFR and BxPc3GEMR cell lines became more sensitive to treatment with oxaliplatin, while BxPc3OSIR cells became less sensitive (Table I).

Acquisition of resistance to treatment with afatinib and gemcitabine is accompanied by upregulation of EGFR ligand amphiregulin (AR) in pancreatic cancer variants. Of all growth factors investigated, BxPc3 parental cell line was found to secrete only AR. Of note, acquired resistance to afatinib and gemcitabine in pancreatic cancer cells was accompanied by a 3-fold increase in secretion of AR. The concentration of AR secreted by BxPc3, BxPc3AFR, BxPc3GEMR and BxPc3OSIR was 120, 338.5, 367.8 and 142.9 pg/ml, respectively (Fig. 2A). Treatment of parental BxPc3 cells with 20 nM of AR for 15 min was accompanied by increased phosphorylation of all EGFR tyrosine residues and MAPK but it had no effect on the phosphorylation status of STAT3 and AKT (Fig. 2B).

Expression levels of putative pancreatic CSC markers, HER family members, IGF-IR and c-MET in drug resistance variants.

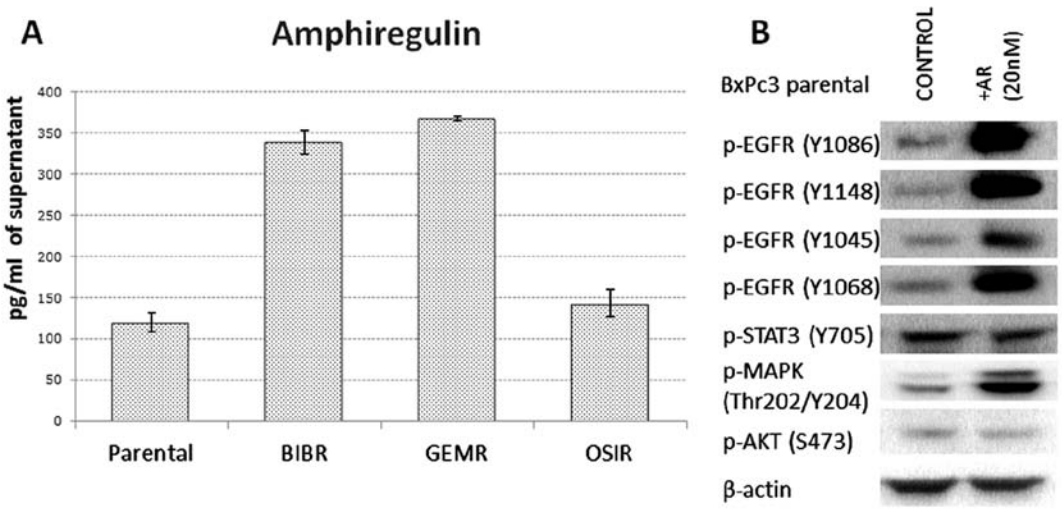

Figure 2. Amphiregulin concentration levels in culture medium as determined by ELISA (A) and effect of amphiregulin treatment on activation status of EGFR tyrosine residues, AKT, MAPK and STAT3 on BxPc3 parental cells (B). Amphiregulin was the only ligand found positive in the samples investigated (mean of two individual experiments, n=6) (error bars=SD) (A). BxPc3 cells were treated with amphiregulin (20 nM) for 15 min then lysed, proteins were separated by SDS-PAGE, transferred to PVDF membranes and probed with the antibodies of interest. Representative of two independent experiments (B).

Table II. Changes in the expression of CSC markers (CD44, CD24 and CD133), HER family members, IGF-IR and c-MET in drug resistant established cell lines relative to their parental counterparts.

Cell line	EGFR	HER-2	HER-3	HER-4	IGF-IR	c-MET	CD44	CD24	CD133
BxPc3AFR	0.97	0.59	1.07	N/A	1.04	0.96	3.14[a]	0.69	N/A
BxPc3GEMR	0.69	0.66[a]	0.70	N/A	0.76	0.84	0.86	0.60	N/A
BxPc3OSIR	1.17	0.78	1.34	N/A	1.07	0.80	2.23[a]	0.83	N/A

Cell surface expression of all markers was determined by flow cytometry as described in Materials and methods. Changes in expression are presented as expression in drug resistant cells relative to their parental cell lines (fold change) ([a]$p<0.05$). Two independent experiments were performed for each cell line including parental cells. N/A, not applicable.

The cell surface expression of putative pancreatic cancer CSC markers CD44, CD24 and CD133, as well as all members of the HER family, IGF-IR and c-MET was analysed in the parental BxPc3 cell line and its drug-resistant variants by flow cytometry. Both BxPc3AFR and BxPc3OSIR variants exhibited a statistically significant increase in CD44 expression with a 3.14- and 2.23-fold change ($p<0.05$), respectively, compared to the parental BxPc3 cells. No differences were observed in the expression of CD24 while all cell lines were found to be negative for CD133 (Table II). There was a small decrease in the expression of several markers in BxPc3GEMR, however, none of these was found to be statistically significant (Table II).

Upregulation of phosphorylated c-MET and STAT3 in drug-resistant variants. Next, we investigated the basal phosphorylation status of 16 RTKs in BxPc3 parental cells and its drug-resistant variants. Our aim was to determine whether acquisition of resistance was accompanied by any changes in the phosphorylation status of major RTKs. Noteworthy, detectable levels of phosphorylated c-MET receptor were observed only in BxPc3AFR and BxPc3GEMR variants (Fig. 3). The upregulation of p-c-MET in the BxPc3AFR and BxPc3GEMR-resistant variants was confirmed by subsequent western blot analysis, which also showed upregulation of p-c-MET in the BxPc3OSIR variant which was not detectable by the RTK assay (Fig. 4).

A detailed analysis of the phosphorylation status of several tyrosine residues of the EGFR, revealed several differences between the drug-resistant variants and the parental cell line (Fig. 4). While there were no major differences in the phosphorylation status of EGFR tyrosine residues Y1148 and Y1045 between the parental cell line and the drug-resistant variants, a significant increase in phosphorylation levels of Y1068 in all three BxPC3 variants. In addition, the phosphorylation of EGFR residues Y1173 and Y1086 was increased in some drug-resistant variants (Fig. 4). Next, we examined the activation status of several downstream signalling molecules including SRC, MAPK, AKT and STAT3 in the parental cell line and its drug-resistant variants showing downregulation of pAKT in BxPc3AFR and BxPc3OSIR but not BxPc3GEMR cells. In addition, there was an upregulation of pSTAT3 in all drug-resistant variants compared to the parental cell line. Interestingly, total and phosphorylated SRC was downregulated in all resistant clones (Fig. 4).

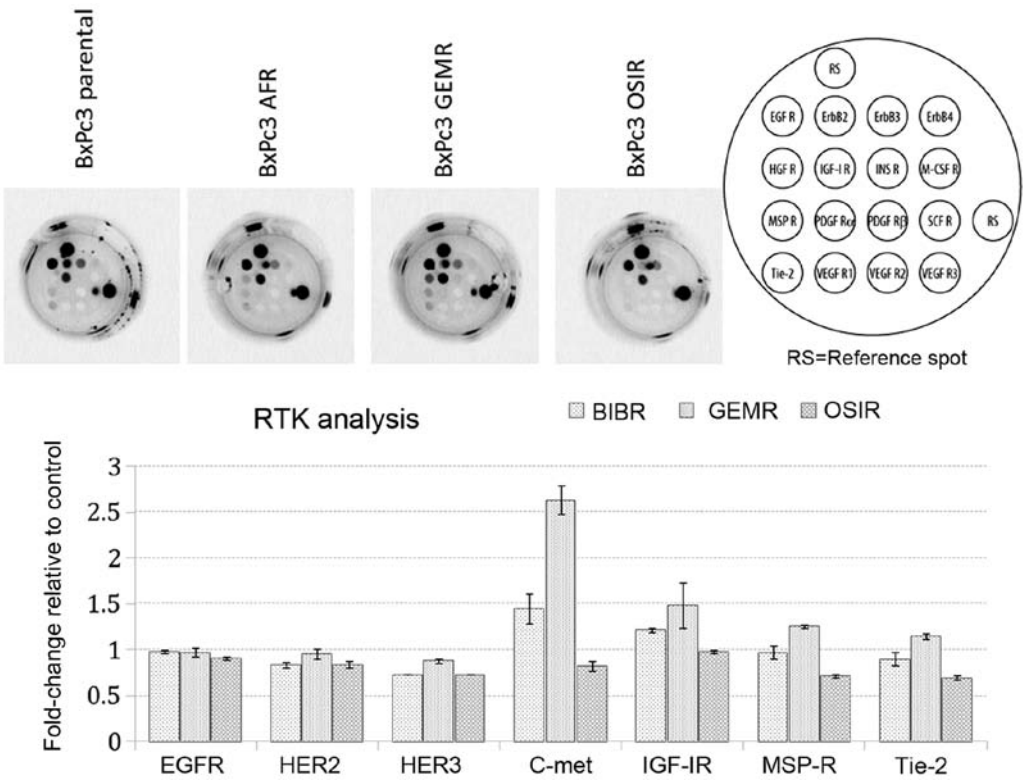

Figure 3. Basal phosphorylation status of 16 receptor tyrosine kinases (RTKs) in BxPc3 cells and its drug-resistant clones. Phosphorylation levels of all RTKs investigated are expressed relative to the levels of the parental BxPc3 cell line (control=1) (three independent experiments) (error bars=SD).

Table III. Mutations of BxPc3 cell line as determined using next generation sequencing (cancer hotspot v2 amplicons).

Locus	Genotype	Control genotype	Type	Gene	Location	AA change
chr4:1807894	A/A	G	SNV	FGFR3	Exonic	Synonymous
chr4:55141050	AGCCCGGATGGACATG/ AGCCCGGATGGACATG	AGCCCAGATGGACATG	SNV	PDGFRA	Exonic	Synonymous
chr4:55152040	C/T	C	SNV	PDGFRA	Exonic	Synonymous
chr4:55593481	A/G	A	SNV	KIT	Exonic	Synonymous
chr4:55972974	A/A	T	SNV	KDR	Exonic	p.Gln472His
chr4:55980239	T/T	C	SNV	KDR	Intronic	N/A
chr5:112175769	CAG/CAG	CGG	SNV	APC	exonic	Synonymous
chr7:55249063	G/A	G	SNV	EGFR	Exonic	Synonymous
chr11:534242	A/G	A	SNV	HRAS	Exonic	Synonymous
chr13:28610183	G/G	A	SNV	FLT3	Intronic	N/A
chr17:7578190	C/C	T	SNV	TP53	Exonic	p.Tyr181Cys
chr17:7579470	CGC/CGC	CGG	SNV	TP53	Exonic	p.Pro33Arg
chr22:24176287	A/A	G	SNV	SMARCB1	Intronic	N/A

No additional genetic variations were detected in the drug resistant cell lines when compared with the parental cell line. Control genotype refers to the reference genome. AA, amino acid; N/A, not available; SNV, single nucleotide polymorphism.

Effect of afatinib and crizotinib treatment on the phosphorylation status of EGFR, HER3, p-c-MET and downstream molecules STAT3, AKT and MAPK. Since upregulation of phorphorylated c-MET and STAT3 was found in all drug-resistant variants, we examined the effect of crizotinib, a c-MET inhibitor, when used alone or in combination with afatinib on the activation of downstream molecules STAT3, AKT and MAPK. At 400 nM, crizotinib blocked completely the activation of c-MET but had no effect on the activation status of either STAT3 or HER family members, EGFR (Y1068) and HER3 (Fig. 5). Similarly, treatment with afatinib had no effect on the phosphorylation levels of STAT3, or c-MET, however, it

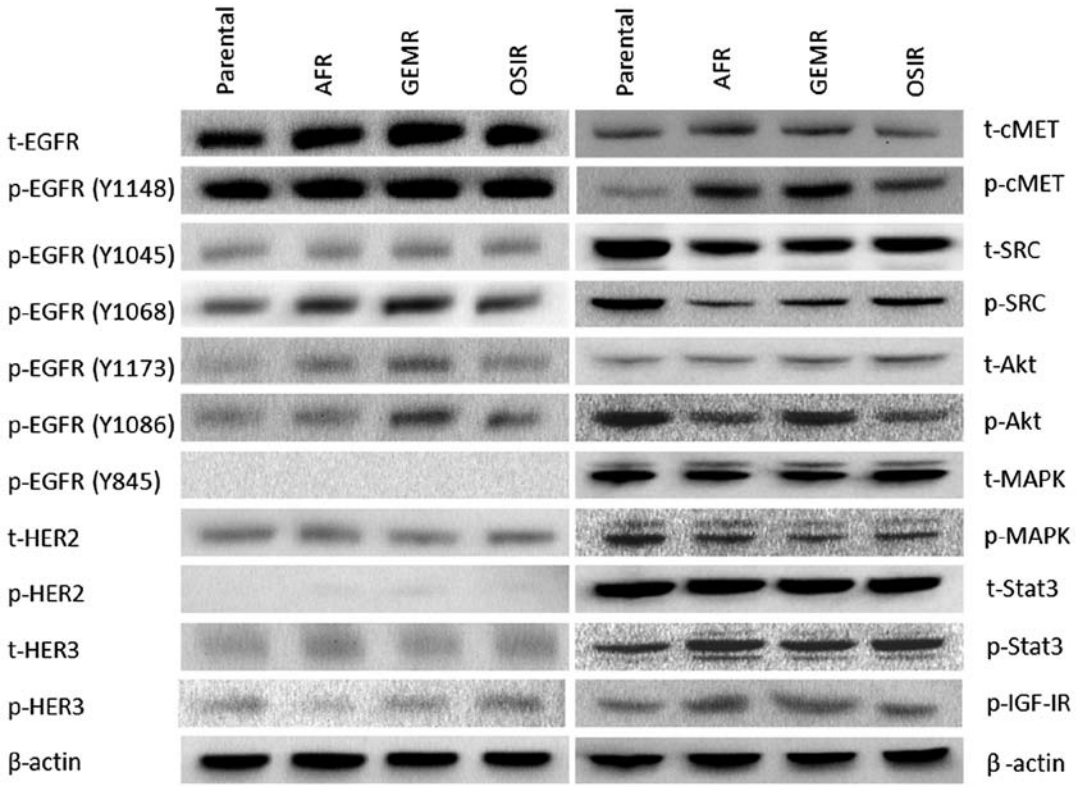

Figure 4. Basal levels of phosphorylated HER family members, c-MET and downstream molecules SRC, AKT, MAPK and STAT3 in parental BxPc3 cells and drug-resistant cell lines BxPc3AFR, BxPc3GEMR and BxPc3OSIR. Cells were grown to near confluence in 10% FBS at 37˚C, then lysed, proteins were separated by SDS-PAGE, transferred to PVDF membranes and probed with the antibodies of interest. Representative of two independent experiments.

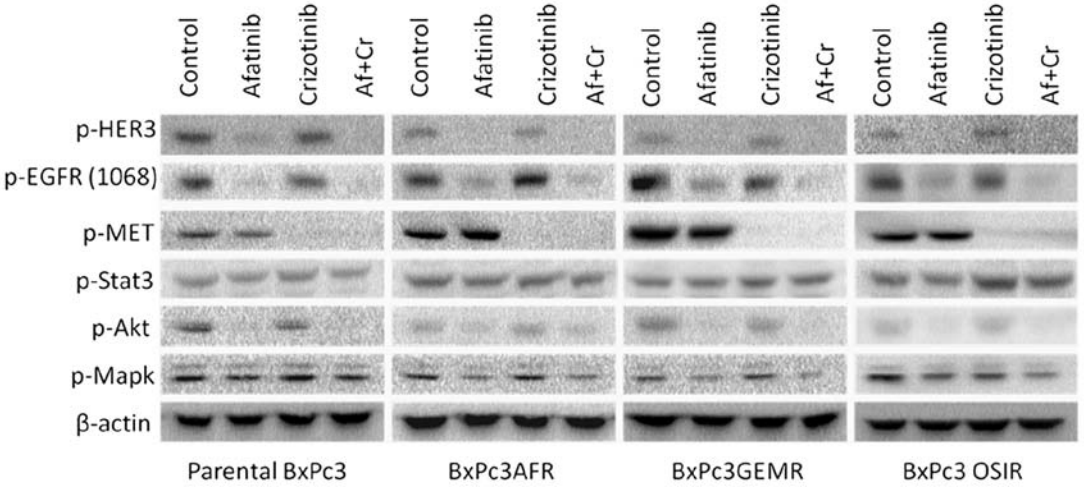

Figure 5. Basal levels of phosphorylated HER3, EGFR (Y1068) c-MET and downstream molecules AKT, MAPK and STAT3 in parental BxPc3 cells and drug-resistant cell lines BxPc3AFR, BxPc3GEMR and BxPc3OSIR either grown in 10% FBS supplemented growth medium alone or with Afatinib, Crizotinib or their combination. Cells were grown to near confluence in 10% FBS at 37˚C, and incubated overnight. Cells were then lysed, proteins were separated by SDS-PAGE, transferred to PVDF membranes and probed with the antibodies of interest. Representative of two independent experiments.

was more effective at blocking the phosphorylation of EGFR at tyrosine 1068 in the parental cell line compared to the drug-resistant variants suggesting that STAT3 activation was a result of a c-MET and HER family members-independent mechanism (Fig. 5).

Next generation sequencing using the CHPv2 revealed no differences between the parental cell line and drug-resistant clones. Next generation sequencing data analysis revealed 13 SNVs in the parental and the drug-resistant variants, 3 of which were found to be intronic. Of the 10 exonic SNVs only 3 led to amino acid substitutions, 2 of which in the TP53 gene and one in the KDR gene (Table III).

Drug-resistant variants exhibited increased sensitivity to treatment with Stat-3 inhibitor Stattic. As there were higher

Table IV. IC_{50} values for STAT3 inhibitor stattic and combination index (CI) values of afatinib plus stattic or afatinib plus crizotinib in the parental BxPc3 cell line and its drug-resistant variants (three independent experiments).

Cell line	Stattic IC_{50} range	Afatinib + Stattic CI range (effect)	Afatinib + Crizotinib CI range (effect)
BxPc3	1.36-1.9 μM	0.503-0.603 (Synergism)	0.15-0.22 (Strong synergism)
BxPc3AFR	703-774 nM[a]	0.86-0.98 (Additive)	0.73-0.87 (Slight synergism)
BxPc3GEMR	691-775 nM[a]	1.05-1.08 (Additive)	0.96-1.07 (Additive)
BxPc3OSIR	1.22-1.27 μM	0.72-0.85 (Additive/Slight synergism)	0.7-0.88 (Additive/Slight synergism)

Interpretation of the results was based on the proposed descriptions for presenting the degrees of antagonism or synergism by Calcusyn software. ([a]$p<0.05$).

levels of phosphorylation of STAT3 in all drug-resistant variants, we sought to investigate the inhibitory effect of STAT3 inhibitor Stattic, when used alone or in combination with afatinib or gemcitabine. All drug-resistant variants exhibited higher sensitivity to STAT3 inhibition with IC_{50} values of 750 nM (p=0.012), 760 nM (p=0.012) and 1.26 μM (not significant, p=0.22) in BxPc3AFR, BxPc3GEMR and BxPc3OSIR, respectively, compared to the IC_{50} value of 1.51 μM in the parental cell line. Of note, treatment of the parental BxPc3 cell line with a combination of afatinib with STAT3 led to synergism with a CI value range of 0.5-0.6 while the same combination had an additive effect in the drug-resistant variants (Table IV). Similarly, the combination of Crizotinib with afatinib led to a strong synergistic effect in the parental cell line, it had an additive or slight synergistic effect in all drug-resistant variants (Table IV).

Discussion

Drug resistance is one of the greatest challenges in clinical oncology (6). Molecular changes that lead to a decreased influx of the drug [low expression of the human equilibrative nucleoside transporter-1(hENT1)] or a low rate conversion of gemcitabine to its active metabolites, caused by a low efficacy of deoxycytidine kinase (dCK) are some of the mechanisms which have been identified so far for acquired resistance to gemcitabine (19,20). Similarly, a number of mechanisms have been identified for acquired resistance to HER inhibitors, including the HER family member modification (e.g. EGFR T790M mutation), the activation of alternative signalling pathways (e.g. c-MET, IGF-IR), production of autocrine ligands or mutations in downstream signalling molecules such as K-RAS (10,21,22).

In our previous study, we investigated and reported the growth response of a panel of seven human pancreatic tumour cells lines (BxPC-3, AsPC-1, FA6, PANC-1, CAPAN-1, MiaPaca2, PT-45) to the treatment with a wide range of agents including afatinib, and erlotinib. We have shown that of these agents, afatinib was more effective than erlotinib in inhibiting the growth of these cancer cell lines *in vitro*. More importantly, of all the cell lines examined in that study, BxPC3 cells were the most sensitive to treatment with both afatinib and erlotinib, with IC_{50} values of 11 and 1,200 nM, respectively (12). Since *KRAS* mutations have already been established as a mechanism of resistance to EGFR inhibitors, and in BxPC-3 cells it is the only one with a wild-type *KRAS* gene and consequently most sensitive to treatment with both afatinib and erlotinib, we developed variants of BxPC-3 cells with acquired resistance to these drugs.

In this study, we sought to investigate molecular changes accompanying the acquisition of drug resistance to HER-targeted therapy or gemcitabine in pancreatic cancer, and to determine therapeutic interventions that could overcome this phenomenon. We found that acquired resistance to one agent such as gemcitabine was accompanied by reduced sensitivity to afatinib and erlotinib and vice versa, indicating the acquisition of a drug cross-resistance phenotype (Table II). However, the changes in sensitivity to other chemotherapeutic agents did not follow the same pattern in the cell lines. For example, while BxPc3GEMR and BxPc3AFR cells showed an increase in sensitivity to oxaliplatin treatment, the IC_{50} value in BxPc3OSIR for oxaliplatin was increased by almost 3-fold ($p<0.05$). Similarly, while there was no significant change in the sensitivity of BxPc3AFR cells to treatment with doxycycline, both BxPc3GEMR and BxPc3OSIR cells were found to have a significantly lower IC_{50} for doxycycline compared to the parental cell line indicating that different mechanisms could be contributing to the acquisition of drug resistance in these cell lines (Table III).

Numerous studies have identified cells with stem cell characteristics, that represent a small subpopulation within haematological or solid tumours known as cancer stem cells (CSCs) which have the capacity of self-renewal, differentiation, and high tumourigenicity (23). According to the CSC model, current therapeutic strategies can eliminate the majority of tumour cells. However, due to their high intrinsic drug resistance, CSCs can escape conventional treatments and lead to tumour recurrence. The innate resistance of CSCs to treatment with conventional therapies stems from specific traits which confer high resistance to therapeutic agents, such as high detoxification capacity, increased DNA repair capability, increased drug efflux due to high expression of ABC transporters and infrequent replication (24,25). One of the most well established mechanisms involved in acquisition of multi-drug resistance (MDR) is the over-expression of drug efflux proteins, mainly the ATP-binding cassette (ABC) transporters. The ABC superfamily consists of 48 members which can use energy to facilitate the transport

of various agents and therefore, can confer a multidrug phenotype (26,27).

Therefore, we started to examine the expression levels of several CSC markers including CD133, CD24 and CD44 as well as some of the basic members of ABC transporters such as P-glycoprotein (P-gp) in the developed drug-resistant variants (28-30). Noteworthy, of all markers investigated, CD44 expression was found to be increased in BxPc3AFR and BxPc3OSIR drug-resistant variants (Table IV). However, the percentage of the population of CD44 positive cells in these drug-resistant variants was above 99%, indicating that the upregulation of CD44 was not restricted to a small subpopulation of these cells. Since CD44 can associate directly with HER family members and enhance their activation and subsequent mitogenic signals, we hypothesized that CD44 overexpression could be involved in the acquisition of resistance in these cell lines (31-33). However, addition of a blocking anti-CD44 antibody to treatment of these cell lines with HER inhibitors erlotinib and afatinib, failed to re-sensitize them to the latter (data not shown). In addition, with the exception of a small increase (not significant) in ABCG2 expression in BxPc3AFR cells, there were no major changes in the expression of ABC transporters investigated (P-gp, MRP-2 and ABCG2) in all drug-resistant variants (data not shown).

The phosphorylation status of various tyrosine residues of EGFR exhibited several differences between the parental cell line and the drug-resistant variants. For example, even though phosphorylation levels of Y1148 and Y1045 were similar between the parental cell line and its drug-resistant variants, an increase of phosphorylation of Y1068 and Y1173 was observed in the BxPc3AFR and BxPc3GEMR clones (Fig. 4). Furthermore, afatinib at 400 nM, led to an almost complete inhibition of EGFR phosphorylation at tyrosine 1068 only in the parental BxPc3 cells (Fig. 5). In addition, we found a 3-fold increase in the autocrine production of amphiregulin in the BxPc3AFR and BxPc3GMER variants but not BxPc3OSIR compared to the parental cell line, indicating that the differences in the phosphorylation of EGFR tyrosine residues or the low efficacy of afatinib could result from the presence of an amphiregulin autocrine loop. However, addition of an anti-EGFR antibody (ICR62 at 200 nM) to afatinib treatment failed to re-sensitize them to the latter, indicating that AR overexpression alone could not explain the acquisition of resistance to anti-HER treatment in these cell lines (data not shown).

Next generation sequencing using the CHPv2 pool of primers, revealed no differences between the parental cell line and its drug-resistant variants in 2855 hot spots/COSMIC mutations from 50 genes including EGFR, HER2, KRAS, p53, PIK3CA, PTEN and c-MET among others. However, the possibility of mutations in these genes leading to drug resistance cannot be excluded and could be addressed only by full gene-sequencing for these biomarkers (Table III).

Several studies have shown that c-MET signalling can be involved in the acquisition of drug resistance to either HER inhibitors or gemcitabine through overexpression of the receptor or hyperactivation of the c-MET/HGF signalling axis (34-38). Of note, despite increased phosphorylation of c-MET in all drug-resistant variants, growth inhibition analysis showed no significant change in the sensitivity to the c-MET inhibitor crizotinib. In addition, while treatment with crizotinib (400 nM) blocked the phosphorylation of c-MET completely, it had no effect on the phosphorylation status of STAT3, MAPK or AKT (Table I and Fig. 5).

Activation of STAT3 has also been implicated in resistance to HER inhibitors or cytotoxics in several malignancies such as lung cancer, non-Hodgkin's lymphoma and multiple myeloma (39-42). In non-small cell lung cancer cells, Kim *et al* found that resistance to afatinib is mediated by the activation of STAT3 via the IL-6R/JAK1 signalling axis (43). More recently, STAT3 has been found to have a critical role in conferring resistance to anoikis and promote metastasis in pancreatic cancer cells (44). STAT3 has been shown to be activated in an EGFR-dependent mechanism through association of STAT3 with the EGFR tyrosine residues 1068 and 1086 which act as docking sites, as well as EGFR-independent mechanisms, including the IL-6 receptor, SRC family kinases and JAK (45). In our study, while short term treatment with AR led to a significant increase of phosphorylation in all EGFR tyrosine residues tested in the parental cell line, it had no effect on STAT3 phosphorylation (Fig. 2B). In addition, as mentioned above, even though afatinib inhibited the phosphorylation of EGFR at Y1068 in all drug-resistant variants, it had no effect on the activation status of STAT3, indicating that upregulation of p-STAT3 was a result of an EGFR independent mechanism and warrants further investigation (Fig. 5).

Of note, we found that treatment with the STAT3 inhibitor Stattic, had a higher inhibitory effect on the drug-resistant variants compared with the parental cell line (Table IV). Other studies have also indicated that inhibition of STAT3 in some cancer types can re-sensitize cells that have acquired resistance to EGFR inhibitors or chemotherapeutics (40,46). Currently, the therapeutic potential of STAT3 inhibition is being pursued in two separate lung cancer studies: i) A phase I/II trial of Ruxolitinib (a JAK1/2 inhibitor) in combination with erlotinib in patients with lung adenocarcinoma (EGFR-mutant), with acquired resistance to erlotinib (ClinicalTrials.gov identifier: NCT02155465) and ii) a phase I trial of afatinib plus ruxolitinib in non-small lung cancer (NSCLC) patients (ClinicalTrials.gov identifier: NCT02145637) will provide more information on this new therapeutic concept. In our preclinical study, when Stattic was used in combination with afatinib, it led to an additive effect in all drug-resistant pancreatic cancer variants (Table IV). In contrast, the combination of Stattic with gemcitabine led to an antagonistic effect (data not shown). In addition, while the sensitivity of the drug-resistant variants to treatment with the c-MET inhibitor crizotinib remained unchanged, a combination of afatinib with the latter produced an additive or synergistic effect indicating the therapeutic potential of this combination in overcoming resistance of pancreatic cancer cells to HER inhibitors and warrants further investigation.

In conclusion, our results indicate that the activation of STAT3 may play an important role in the acquisition of resistance to gemcitabine and HER inhibitors in pancreatic cancer and warrant further investigations on the therapeutic potential of STAT3 inhibitors in such a setting.

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