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REPORT

Anti-tumoral activity of the Pan-HER (Sym013) antibody mixture in gemcitabine-resistant pancreatic cancer models

Emilia Rabia[a], Véronique Garambois[a], Julie Hubert[a], Marine Bruciamacchie[a], Nelly Pirot[a,b], Hélène Delpech[a], Morgane Broyon[a,b], Charles Theillet[a], Pierre-Emmanuel Colombo[c], Nadia Vie[a], Diego Tosi[a,c], Celine Gongora[a], Lakhdar Khellaf[c], Marta Jarlier[c], Nina Radosevic-Robin[d], Thierry Chardès[a], André Pèlegrin[a], and Christel Larbouret[a]

[a]Institut De Recherche En Cancérologie De Montpellier (IRCM), INSERM U1194, Université De Montpellier, Institut Régional Du Cancer De Montpellier (ICM), Montpellier, France; [b]BioCampus Montpellier, Université Montpellier, CNRS UAR3426, INSERM US09, Université De Montpellier, Montpellier, France; [c]Institut Régional Du Cancer De Montpellier (ICM), Montpellier, France; [d]Centre Jean Perrin, Université Clermont Auvergne, INSERM U1240, Clermont-Ferrand, France

ABSTRACT

Chemoresistance, particularly to gemcitabine, is a major challenge in pancreatic cancer. The epidermal growth factor receptor (EGFR) and human epidermal growth factor receptors 2 and 3 (HER2, HER3) are expressed in many tumors, and they are relevant therapeutic targets due to their synergistic interaction to promote tumor aggressiveness and therapeutic resistance. Cocktails of antibodies directed against different targets are a promising strategy to overcome these processes. Here, we found by immunohistochemistry that these three receptors were co-expressed in 11% of patients with pancreatic adenocarcinoma. We then developed gemcitabine-resistant pancreatic cancer cell models (SW-1990-GR and BxPC3-GR) and one patient-derived xenograft (PDX2846-GR) by successive exposure to increasing doses of gemcitabine. We showed that expression of EGFR, HER2 and HER3 was increased in these gemcitabine-resistant pancreatic cancer models, and that an antibody mixture against all three receptors inhibited tumor growth in mice and downregulated HER receptors. Finally, we demonstrated that the Pan-HER and gemcitabine combination has an additive effect *in vitro* and in mice xenografted with the gemcitabine-sensitive or resistant pancreatic models. The mixture of anti-EGFR, HER2 and HER3 antibodies is a good candidate therapeutic approach for gemcitabine-sensitive and -resistant pancreatic cancer.

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EGFR; HER2; HER3; pancreatic cancer; gemcitabine; Pan-Her; chemoresistance

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive tumors, and patients with this disease have an extremely poor prognosis. PDAC is projected to become the second leading cause of cancer-related death by 2030.[1] At the time of diagnosis, 85% of patients already have advanced and/or metastatic disease, and very limited treatment options.[2] Only patients with resectable tumor (10% of all cases) might hope for an efficient treatment by surgery, but in most cases the tumor is too advanced and already metastatic.[3] To date, three drugs or drug combinations have been approved for the treatment of advanced and metastatic PDAC: gemcitabine, gemcitabine-Nab-paclitaxel, and FOLFIRINOX (oxaliplatin, irinotecan, fluorouracil and leucovorin). Gemcitabine (approved in 1997) is the oldest and the most prescribed drug, but shows limited efficacy. Interesting results have been obtained with FOLFIRINOX in patients with metastatic PDAC. Specifically, the overall survival was significantly increased in the FOLFIRINOX group compared with the gemcitabine group (11.1 months *versus* 6.8 months, respectively).[4] However, its high toxicity limits the number of patients who can benefit of this combination.[5] The gemcitabine and nab-paclitaxel combination increases the intratumoral concentration of gemcitabine and slightly improves survival compared with gemcitabine alone, but this benefit is not sufficient for a wide use in Europe.[6] Until now, no clear data are available about second-line therapies for patients with metastatic or advanced PDAC that progresses after chemotherapy, particularly with gemcitabine.

Receptor tyrosine kinases (RTKs), such as the human epidermal growth factor receptor (HER) family, MNNG HOS transforming (MET)/hepatocyte growth factor receptor, and insulin–like growth factor 1 (IGF1) receptor. are expressed at the cell surface of most pancreatic cancer cells, and are involved in signaling pathways leading to tumor progression, migration and angiogenesis.[7,8] In PDAC, the expression of EGFR, HER2 and HER3 has been correlated with advanced disease and poor prognosis.[9–11] In the past 15 years, many RTK-targeted therapies (e.g., tyrosine kinase inhibitors, monoclonal antibodies) have been developed, and some of them are currently used in the clinic for patients with colorectal or breast cancer. A Phase 3 clinical trial to test the combination of gemcitabine and erlotinib (EGFR inhibitor) in PDAC showed a modest survival benefit, but this was better than the result obtained with the cetuximab and gemcitabine combination.[12] In addition, the discovery of resistance mechanisms to chemotherapy or to anti-EGFR agents prompted researchers to

CONTACT Christel Larbouret christel.larbouret@inserm.fr; Institut De Recherche En Cancérologie De Montpellier (IRCM), INSERM U1194, Université De Montpellier, Institut Régional Du Cancer De Montpellier (ICM), Montpellier, France
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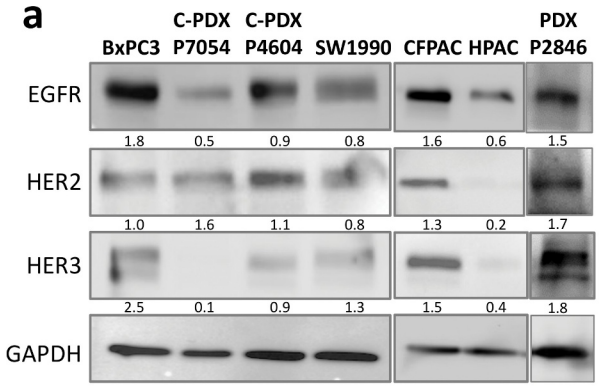
propose use of new combinatorial strategies, such as cetuximab and trastuzumab,[13] an anti- HER3/IGF1 receptor istiratumab (MM141),[14] anti-AXL and anti-HER3 antibodies,[15] anti-MET with anti-EGFR tyrosine kinase inhibitors,[16] and the combination of two anti-EGFR, two anti-HER2, and two anti-HER3 antibodies (Sym013 or Pan-HER mixture).[17] As an example of preclinical study results, Jacobsen et al. showed the efficacy of the Pan-HER mixture in a broad panel of cancer cell lines with different genetic mutations, including patient-derived xenografts (PDXs) of pancreatic cancer harboring *KRAS* mutations. The Pan-HER mixture induced receptor cross-linking at the cell surface, leading to the internalization and degradation of the targeted receptors.[18,19] This indicated the importance of inhibiting more than one HER family member to maximally block the HER signaling network and also to increase the antitumor response. In addition, acquired resistance to anti-HER therapies and chemotherapy has been correlated with the modulation of HER expression.[20] Most of these combinations effectively decrease tumor growth in animal models, but their clinical efficacy still must be demonstrated. For this reason, a response biomarker, such as receptor or ligand expression, is necessary to assess and optimize the clinical response to these combinations.

In this context, the development of resistant pancreatic cancer cell models could help to understand the underlying mechanisms and to find new approaches to treat patients. Therefore, in this study, we developed and characterized *in vitro* and *in vivo* gemcitabine-resistant (GR) models derived from pancreatic cancer cell lines and PDXs. Resistance to gemcitabine was mainly associated with HER2 and HER3 overexpression and ligand modulation. Acquired gemcitabine resistance was efficiently overcome by the Pan-HER (Sym013) antibody mixture. Finally, the gemcitabine and Pan-HER combination demonstrated an additive effect in limiting pancreatic tumor growth in gemcitabine-sensitive PDAC models.

Results

EGFR/HER2/HER3 expression in human PDAC cell lines, PDX and formalin-fixed paraffin-embedded PDAC tissue sections

First, the expression of EGFR, HER2 and HER3 was analyzed by western blotting in four PDAC cell lines (BxPC3, SW1990, CFPAC and HPAC), two PDX-derived cell (C-PDX) lines (P7054 and P4604), and one PDX (P2846) (Figure 1a). The three PDXs were generated from resected hepatic metastasis samples from three patients with PDAC treated with gemcitabine (P7054 and P4604) or untreated at the time of surgery (P2846) (PDX Platform, Institut de Recherche en Cancérologie de Montpellier). The C-PDX P7054 and P4604 were derived from their respective PDX after *in vitro* culture. The four PDAC cell lines, the two C-PDX lines, and the PDX co-



b

Receptor	No. Pos	% Pos
EGFR	33/45	73
+++	15/45	36
HER2	11/45	24
+++	2/45	4
HER3	12/44	27
EGFR+HER2+HER3	**5/44**	**11**

c

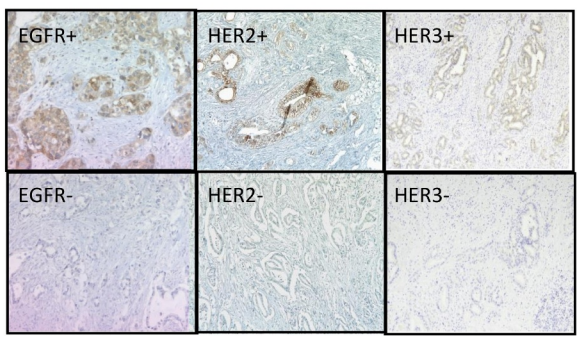


Figure 1. Co-expression of EGFR, HER2 and HER3 in pancreatic cancer models. a. Western blot analysis of EGFR, HER2 and HER3 expression in the BxPC-3, SW1990, CFPAC and HPAC pancreatic cancer cell lines, two cell lines derived from the PDX P7054, P4604 (C-PDX), and the PDX P2846, using the relevant antibodies. GAPDH served as loading control. Quantification of each protein band was normalized to GAPDH. b. EGFR, HER2 and HER3 expression was assessed by IHC in 45 FFPE tumor samples from patients with pancreatic cancer. The number and the percentage of positive (Pos) tumor samples are shown. c. Examples of EGFR, HER2 and HER3 positive and negative cases.

expressed EGFR, HER2 and HER3, but at different levels (Figure 1a). Specifically, the expression of HER2 and HER3 was low in HPAC cells and PDX P2846. Moreover, HER3 was not detected in the C-PDX P7054 line.

Then, the co-expression of EGFR/HER2/HER3 was investigated by immunohistochemistry (IHC) in formalin-fixed paraffin-embedded (FFPE) tumor tissue sections from 45 PDAC samples (Figure 1b, 1c). The patients in this cohort had no chemotherapy before the resection. EGFR was detected in 33/45 PDAC samples (73%), with strong (3+) expression in 15 of them (36%). HER2 was detected in 11/45 PDAC specimens (24%), but expression was strong (3+) only in 2 samples (4%). HER3 (moderate staining) was detected in 12/44 PDAC samples (27%). EGFR/HER2/HER3 co-expression was observed in 5/44 PDAC samples (11%).

In vitro generation of the gemcitabine-resistant cell lines SW1990-GR, BxPC3-GR, HPAC-GR and CFPAC-GR

To develop *in vitro* gemcitabine-resistant cell lines, HPAC, CFPAC, BxPC3, and SW1990 cells were exposed to increasing concentrations of gemcitabine with repeated subculture until the cells became resistant. The maximal gemcitabine dose to which cells could be exposed without affecting their viability was 40 nM for gemcitabine-resistant CFPAC cells (named CFPAC-GR), 130 nM for BxPC3-GR, and 200 nM for HPAC-GR and SW1990-GR cells. The comparison of gemcitabine dose-dependent effect on cell viability in the sensitive parental (wt) and resistant (GR) cell lines showed that the IC_{50} of each resistant cell line was higher than that of the parental cell line (Figure 2a and Table 1). Specifically, the gemcitabine IC_{50} value was increased by 12-fold and by 7-fold in HPAC-GR and CFPAC-GR cells, respectively, compared with the parental cell line (Table 1). Resistance to gemcitabine was higher in BxPC3-GR (IC_{50} increased by 65-fold) and in SW1990-GR cells (nearly fully resistant to gemcitabine) (Figure 2a; lower panels and Table 1). The gemcitabine resistance of SW-1990-GR and BxPC3-GR cells was confirmed *in vivo* (Figure 2b), as indicated by the similar survival curves obtained for mice xenografted with BxPC3-GR and SW1990-GR cells treated with vehicle or gemcitabine.

In vivo generation of the gemcitabine-resistant PDX model P2846-GR

To develop a gemcitabine-resistant PDX, the PDX P2846, which co-expresses EGFR, HER2 and HER3 and was derived from a PDAC of a patient never treated with gemcitabine, was xenografted in two nude mice (8363 and 8364). Alpha Smooth Actin (αSMA) and Hematein-

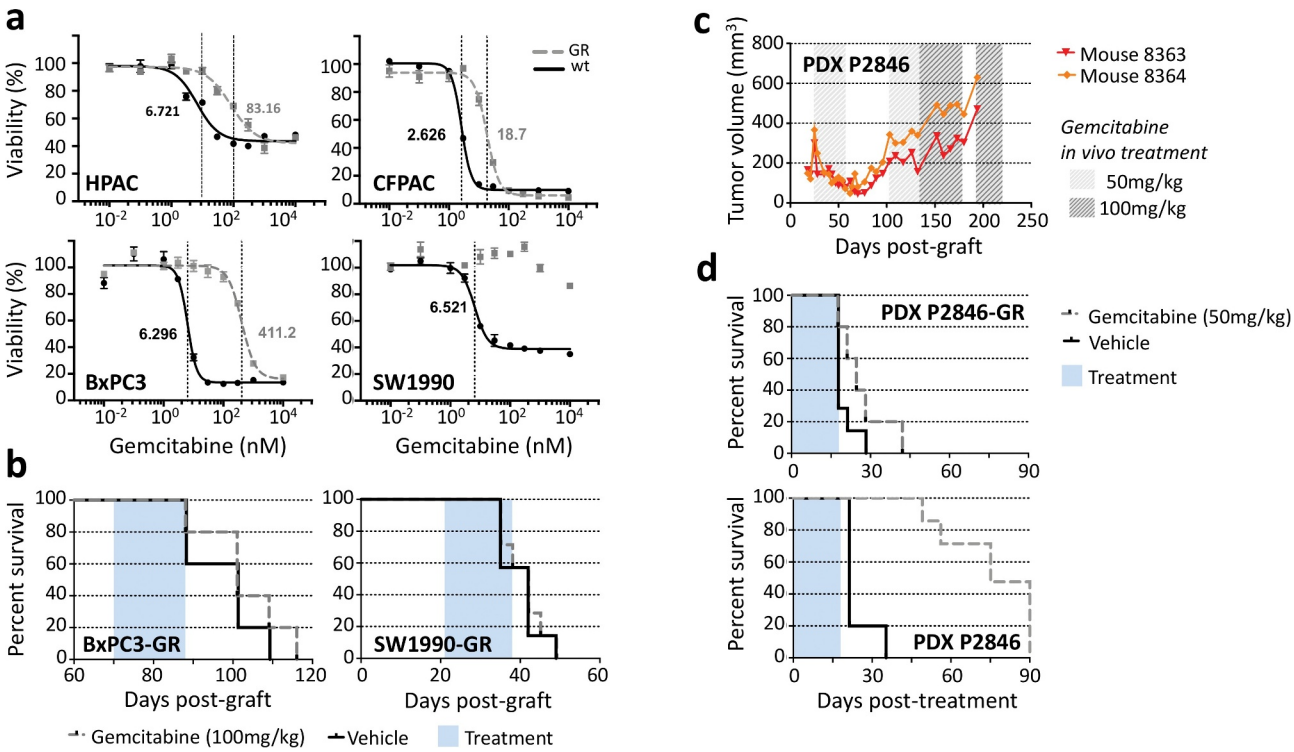


Figure 2. Development of gemcitabine-resistant pancreatic cancer models. a. Gemcitabine sensitivity of the gemcitabine-resistant (GR; gray) and parental (sensitive; wt; black) pancreatic cancer cell lines HPAC, CFPAC, BxPC3 and SW1990 was assessed *in vitro* using the MTS assay. Cells were plated in 96-well plates in complete medium and incubated with increasing doses of gemcitabine for 5 days (n = 3). The IC_{50} of each cell line is indicated in the graphs. b. Gemcitabine sensitivity of BxPC3-GR and SW1990-GR cells were tested *in vivo* in xenografted mice. Mice harboring 200 mm^3 BxPC3-GR and SW1990-GR cell xenografts were treated with 100 mg/kg gemcitabine, or vehicle, twice per week for 3 weeks. Mice were euthanized when tumors reached 1500 mm^3 and Kaplan-Meier curves were computed. The treatment period is highlighted in blue. c. *In vivo* generation of the P2846-GR. Two mice (8363 and 8364) were xenografted with the PDX P2846 and treated with two cycles of 50 mg/kg gemcitabine followed by two cycles of 100 mg/kg gemcitabine until tumors continued to grow during treatment with 100 mg/kg gemcitabine. Tumor volume was checked throughout the experiment. d. Gemcitabine sensitivity of PDX P2846-GR and parental PDX 2846 was tested *in vivo* in xenografted mice. Mice were treated with 100 mg/kg of gemcitabine, or vehicle, twice per week for 3 weeks. Mice were euthanized when tumors reached 1500 mm^3 and Kaplan-Meier curves were computed. The treatment period is highlighted in blue.

Table 1. Gemcitabine IC50 values and Fold increase of WT and GR cell lines.

		HPAC	CFPAC	BxPC3	SW1990
IC50 (nM)	WT	6.7 ± 1.7	2.6 ± 0.1	6.3 ± 1.9	6.2 ± 0.1
	GR	83.2 ± 3.06	18.7 ± 1.4	411.2 ± 3.6	>100
Fold increase		12.3	7.1	65	>100

Eosin-Saffron (HES) labeling of the P2846 patient's tumor revealed a dense micro-environment composed of cancer-associated fibroblasts (CAF) and collagen (Figure S1a). Furthermore, the structure of the patient's tumor and the corresponding PDX (Supplementary Figure 2a) was very similar. These mice were then treated with 50 mg/kg gemcitabine every 7 days for 2 weeks (Q7D-2 W) (two cycles) followed by 100 mg/kg gemcitabine Q7D-2 W (two cycles), and the tumor growth was monitored over time (Figure 2). PDX tumors (P2846-GR) in these mice continued to grow despite treatment with 100 mg/kg gemcitabine. Gemcitabine resistance of the PDX P2846-GR was confirmed *in vivo* by the comparable Kaplan-Meier survival curves of nude mice xenografted with P2846-GR treated with vehicle or 50 mg/kg gemcitabine (Figure 2d; upper panel). Conversely, the parental PDX P2846 was still sensitive to gemcitabine (Figure 2d; lower panel).

Expression of HER2 and HER3 increases in SW1990-GR, BxPC3-GR cells and in the PDX P2846-GR

To evaluate the impact of induced gemcitabine resistance on the HER family, the receptor expression was compared by western blotting and quantitative PCR analysis of parental (sensitive) and gemcitabine-resistant models (Figure 3). EGFR protein expression was comparable in parental and gemcitabine-resistant SW1990 and BxPC3 cells (Figure 3a). Conversely, HER2 and HER3 protein levels were strongly increased in BxPC3-GR and SW1990-GR cells compared with the parental cell lines (Figure 3a). This was confirmed in gemcitabine-resistant PDX where the expression level of the three receptors (EGFR, HER2 and HER3) was also increased in the PDX P2846-GR, compared with the parental PDX (Figure 3b). By immunofluorescence, the increased expression of HER2 and HER3 was confirmed on P2846GR compared to parental tumor (Figure 3c). A slight EGFR, HER2 and HER3 mRNA increase was observed in SW1990-GR cells (Figure 3d, upper panel), but not in BxPC3-GR and P2846-GR xenograft (Figure 3d, middle and lower panels). mRNA expression of the EGFR ligand EGF was increased in all gemcitabine-resistant models, whereas HB-EGF and TGFα mRNA levels was increased only in SW1990-GR cells (Figure 3d, lower panel). Neuregulin1 (NRG1) level was increased only in P2846-GR

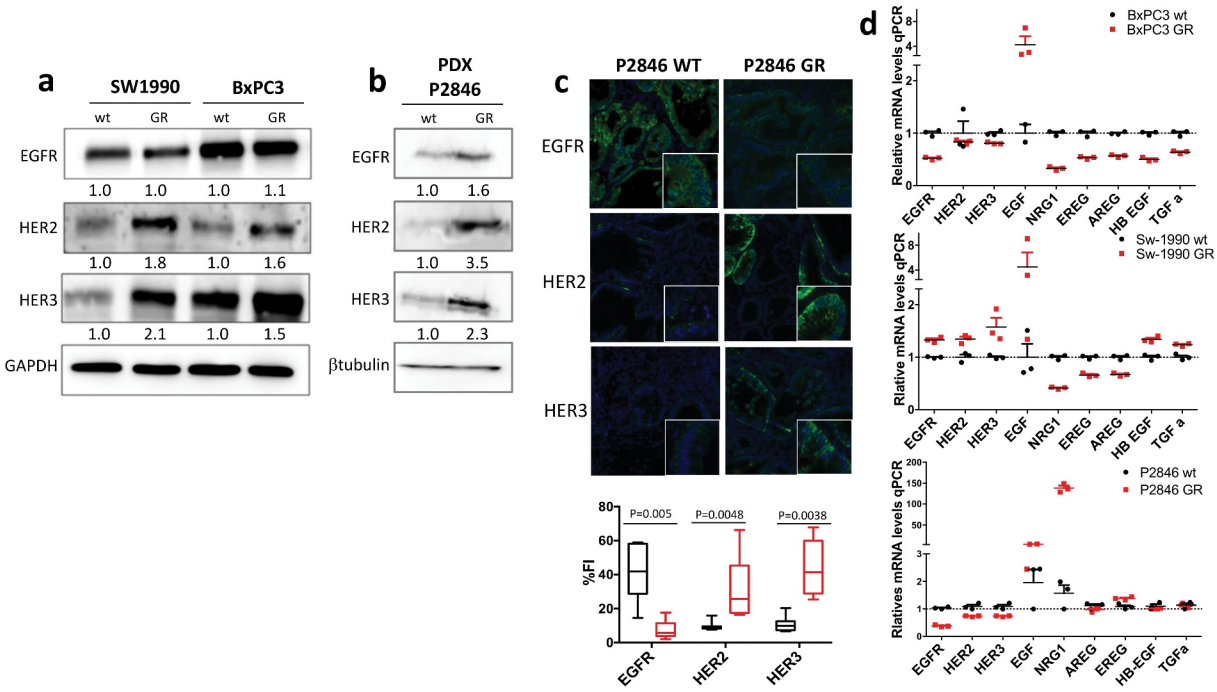


Figure 3. Expression of EGFR, HER2, HER3 and their ligands in gemcitabine-resistant pancreatic cancer models. a. Western blot analysis of EGFR, HER2 and HER3 expression in the gemcitabine-resistant BxPC3-GR and SW1990-GR and parental (wt) cancer cell lines, using the relevant antibodies. b. Western blot analysis of EGFR, HER2 and HER3 expression in the gemcitabine-resistant P2846-GR and parental P2846 (wt) PDX, using the relevant antibodies. GAPDH served as the loading control. Quantification of each protein band was normalized to GAPDH or β tubulin and then represented as fold relative to WT cells band. c. Immuno fluorescence analysis of EGFR, HER2 and HER3 expression in PDX P2846 WT and GR. The pictures are representative image (X40; X80). Quantification of fluorescence (for HER receptors) was done by ImageJ (4 randomly images/tumors) on four different tumors of each condition and normalized with the fluorescence of DAPI (nucleus). FI (fluorescence intensity) represents a mean average of fluorescence of 4 randomly images per tumors. d. Q-PCR analysis of EGFR, HER2, HER3 and EGF and NRG1 expression in SW1990 wt and SW1990-GR cells (upper panel), BxPC3 wt and BxPC3-GR cells (lower panel) and PDX P2846 WT and GR (upper panel). Concentrations were then normalized to the concentration of the housekeeping gene 18S rRNA, and expressed relatively to untreated samples.

models and reduced in the two other resistant cells (Figure 3d, lower and middle panels). Thus, gemcitabine resistance could affect expression (mRNA and protein) of receptors and ligands of the HER family.

The Pan-HER mixture Sym013 inhibits proliferation of parental and gemcitabine-resistant PDAC cells

Increasing doses of the Pan-HER mixture inhibited both the parental and gemcitabine-resistant SW1990 and BxPC3 cell lines in a dose-dependent manner (Figure 4a-b). At 100 µg/ml, the effect of the Pan-HER mixture was comparable for SW1990 and SW1990-GR cells (Figure 4a). However, at lower concentrations, the inhibitory effect of Pan-HER was higher on SW1990 cell viability. This observation can be explained by the increased mRNA expression of *EGF* ligands in SW1990-GR cells (Figure 3d) that probably leads to higher stimulation of HER receptors. This might counterbalance the overexpression of HER2 and HER3 that are targeted by Pan-HER, and thus reduce the antibody mixture efficiency. On the other hand, BxPC3-GR cells were more sensitive to the Pan-HER mixture than the parental BxPC3 cells (IC_{50}: 0.17 µg/ml and 2.18 µg/ml, respectively) (Figure 4b). In this case, HER2 and HER3 overexpression in BxPC-3-GR cells, as actionable targets for Pan-HER might act synergistically with HER ligands mRNA down-regulation to enhance Pan-HER efficiency. Furthermore, the mutational status of KRAS, wild type for BxPC-3 and mutated for SW1990 can also explain this difference of efficacy. The C-PDX P7054 and P4604 were less sensitive to the Pan-HER mixture. Indeed, cell viability was only reduced by 20% after exposure to 100 µg/ml of this antibody mixture (Figure 4c). In C-PDX P7054, this might be explained by the absence of HER3 expression.

In vivo, the Pan-HER mixture delays tumor growth in mice xenografted with SW1990-GR, BxPC3-GR and PDX P2846-GR

In nude mice xenografted with SW1990-GR, BxPC3-GR cells or the PDX P2846-GR, the Pan-HER mixture strongly inhibited tumor growth, and increased survival compared with mice treated with vehicle (control) (Figure 5a-c). At the end of the treatment (day 38 post-graft for SW1990-GR, day 88 post-graft for BxPC3-GR, and day 18 post-treatment for P2846-GR), complete tumor stasis (mean tumor size <100 mm^3) was observed in the Pan-HER-treated groups, whereas the mean tumor size was approximately 1000 mm^3 in controls (Figure 5a-c, upper panels). After the end of the treatment, tumors started to grow again until the experiment end (day 78 for SW1990-GR, day 118 for BxPC3-GR, and day 60 for P2846-GR). In Pan-HER-treated mice, the median survival (Kaplan-Meier curves) was increased by 43 days in SW1990-GR-, 15 days in BxPC-3-GR-, and 45 days in PDX P2846-GR-xenografted mice compared with vehicle-treated mice (Figure 5a-c; lower panels). The parental PDX P2846 also was very sensitive to Pan-HER (Figure 5d). At day 18 (end of treatment), complete tumor stasis (mean tumor size <100 mm^3) was observed in Pan-HER-treated mice xenografted with PDX P2846, whereas the mean tumor size was approximately 1000 mm^3 in the control group (vehicle) (Figure 5d, upper panel). Moreover, tumor re-growth in the Pan-HER-treated group was limited at 60 days post-treatment and also later. The median survival of vehicle-treated mice xenografted with PDX P2846 was 21 days, whereas no Pan-HER-treated mouse reached the tumor size of 1000 mm^3 before 60 days after treatment (Figure 5d; lower panel).

The effect of Pan-HER on HER expression in P2846-GR and WT tumors was analyzed by immunofluorescence. After three weeks of treatment, 4 mice treated with the vehicle or Pan-HER were sacrificed, tumors harvested and the expression of EGFR, HER2 and HER3 analyzed (Figure 6a). In PDX-P2846, a significant downregulation of EGFR after Pan-HER treatment was observed with no clear effect on HER2 and HER3 expression (Figure 6b, upper panel). Interestingly, up-regulation of HER2 and HER3 receptors, initially observed in non-treated PDX P2846-GR (Figure 3b) was confirmed *in vivo* from extracted tumors of mice xenografted with PDX P2846-GR (Figure 6b, lower panel) and further treated. In this case, Pan-HER treatment significantly reduced up-regulated receptor expression, particularly for HER2 and HER3, compared to vehicle treatment.

The Pan-HER and gemcitabine combination has an additive effect on tumor growth inhibition in mice xenografted with PDAC cell lines or PDAC PDX

As gemcitabine is one of the standard treatments for pancreatic cancer, we asked whether the gemcitabine and Pan-HER combination had a synergistic or an additive effect on tumor growth inhibition. A SRB colorimetric assay showed that *in vitro*, proliferation of parental BxPC3, C-PDX P4604, and SW1990 cells was inhibited in a dose-dependent manner after incubation with the Pan-HER mixture and gemcitabine for 5 days (Figure 7a; left panels). This effect was additive (Figure 7a; right panels). In SW1990 and C-PDX P4604 cells, the most efficient drug concentrations for the additive effect ranged between 0.1 and 0.3 mM for gemcitabine, and 11 and 100 µg/ml for the Pan-HER mixture (Figure 7a; right panels). This additive effect was confirmed *in vivo* in mice xenografted with C-PDX P4604 or SW1990 cells (Figure 7b). Specifically, tumor growth inhibition was higher in mice treated with the gemcitabine plus Pan-HER combination compared with gemcitabine (for C-PDX P4604 $p = .068$; for SW1990 $p < .001$) or Pan-HER (for C-PDX P4604 $p < .001$; for SW1990 $p = .029$) alone (Figure 7b; upper panels). Survival (Kaplan-Meier curves) was significantly increased by 29 and 28 days in mice xenografted with C-PDX P4604 and SW1990 cells, respectively, treated with the gemcitabine and Pan-HER combination, compared with gemcitabine-treated mice, and by 33 and 34 days compared with the vehicle-treated group (Figure 7b; middle and lower panels).

Discussion

Antibody mixtures of antibodies that bind multiple targets or several epitopes of one target are widely studied and have impressive therapeutic potential to inhibit tumor growth and bypass or delay therapeutic resistance. As drug resistance is a major challenge in pancreatic cancer, the development of

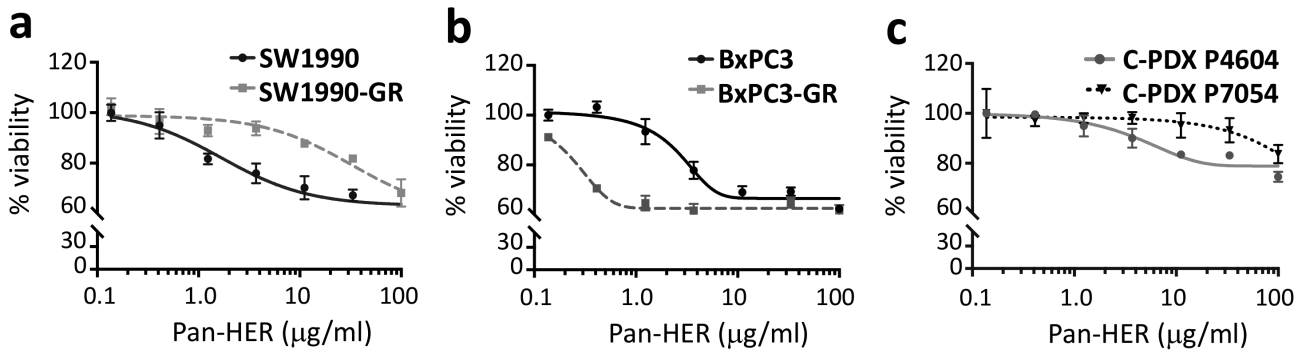


Figure 4. *In vitro* effect of Pan-HER on cell proliferation of gemcitabine-resistant pancreatic cancer cell lines. a. Gemcitabine-resistant SW1990-GR and parental SW1990 cells, b. Gemcitabine-resistant BxPC3-GR and parental BxPC3 cells, and c the C-PDX P4604 and P7054 were plated in 96-well plates in complete medium and incubated with increasing doses of Pan-HER (0.1 to 100 µg/ml) (n = 3) for 5 days. Cell proliferation was analyzed using the MTS assay. Results are indicated as the percentage of cell relative to untreated cells.

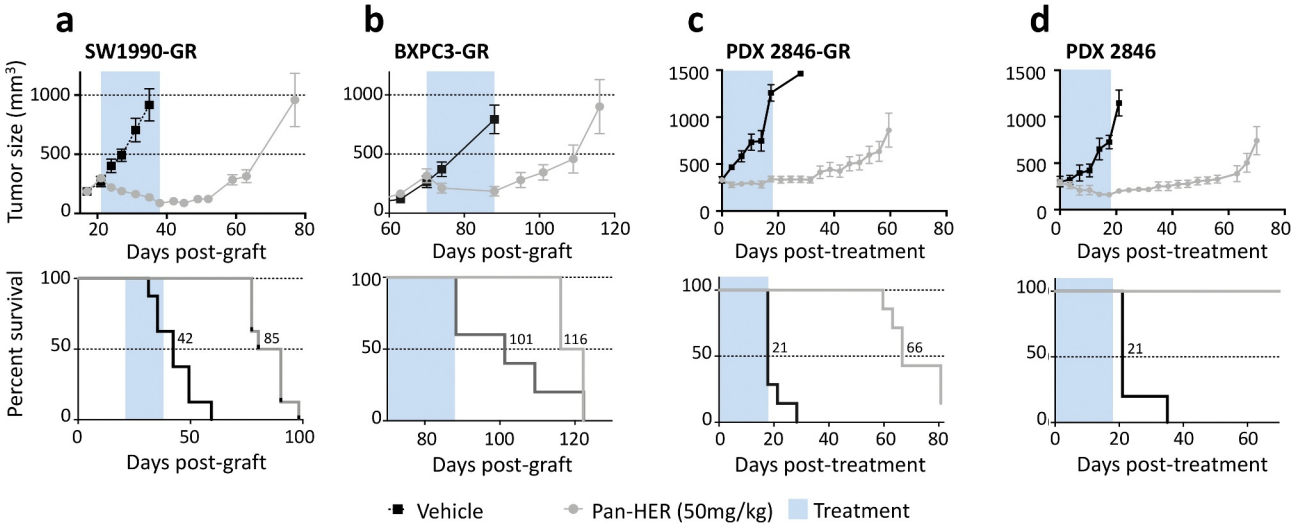


Figure 5. *In vivo* effect of Pan-HER on tumor growth of gemcitabine-resistant pancreatic cancer cell xenografts and PDX. Mice were xenografted with gemcitabine-resistant SW1990-GR (a), BxPC3-GR (b) and PDX 2846-GR (c). d The parental gemcitabine-sensitive PDX P2846 was used as a control. When tumors reached 150 mm^3, xenografted mice (n = 10/group) were treated with 50 mg/kg Pan-HER (gray curve) or vehicle (black curve) twice per week for 3 weeks. Tumor size was measured throughout the experiment (upper panel) and Kaplan Meyer curves were computed (lower panels). The treatment period is highlighted in blue.

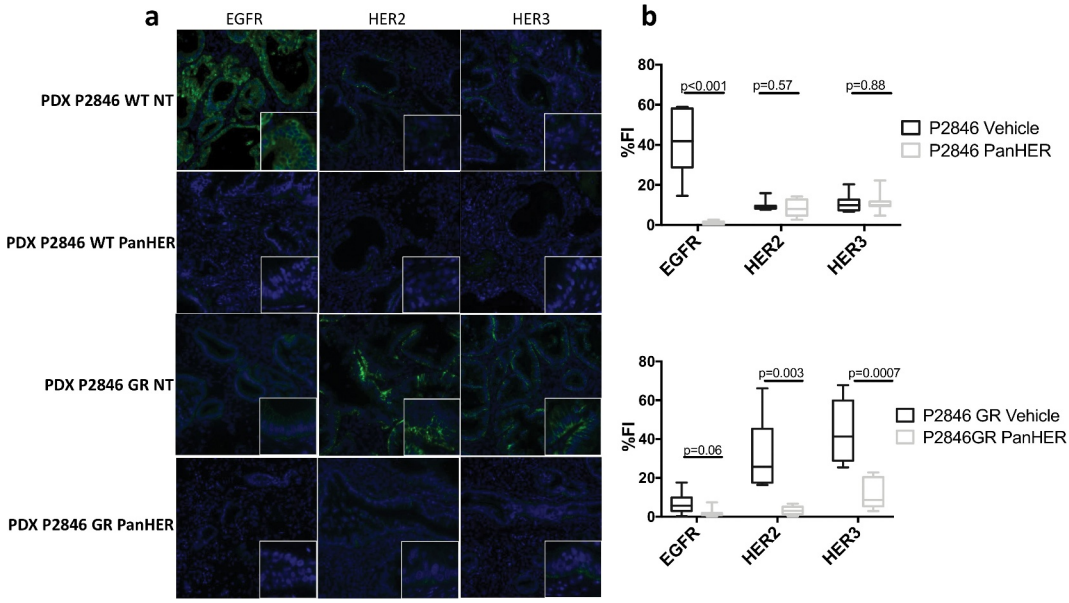
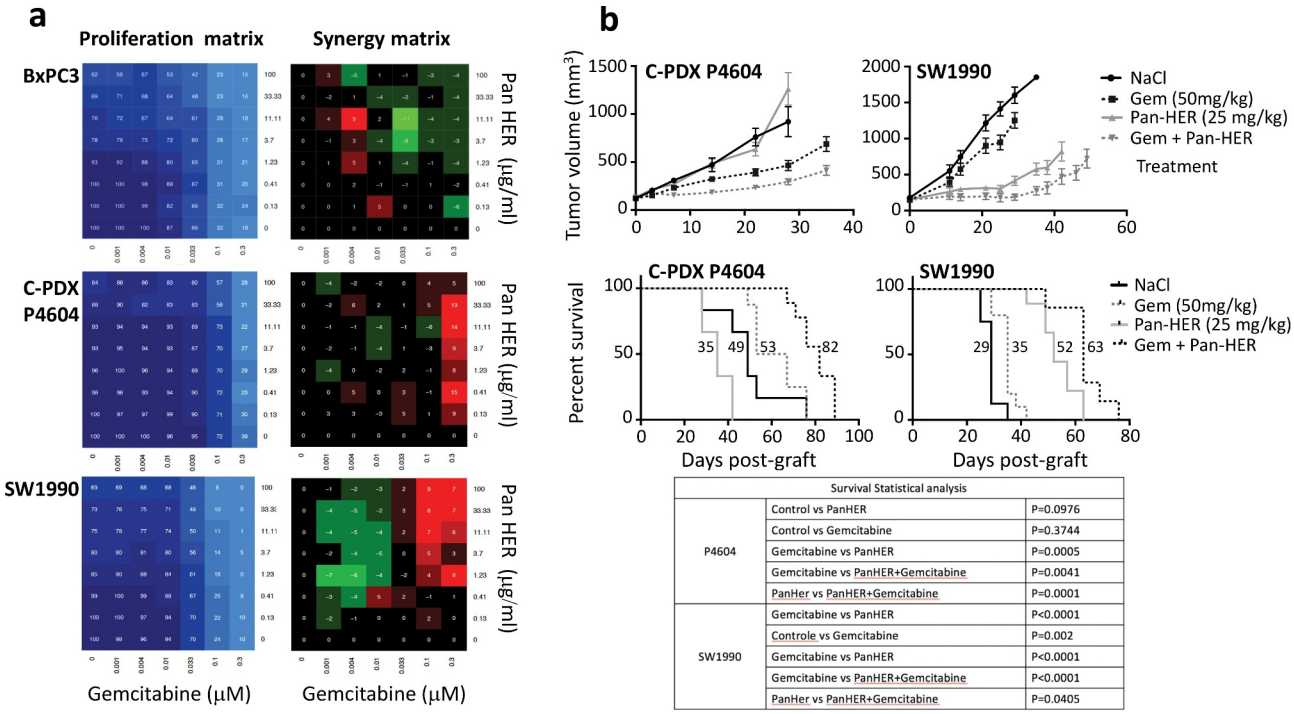


Figure 6. Effect of Pan-HER on EGFR, HER2 and HER3 expression in PDX P2846 WT and GR. a. HER receptors expression was analyzed by immunofluorescence on tumors treated with Pan-HER or the vehicle. The photos are representative image (X40; X80). b. Expression quantification was done by ImageJ on four tumors for each condition of treatments.

resistant pancreatic cancer cell models could help to understand the underlying mechanisms and to find new approaches to treat patients. In this study, we developed pancreatic cancer cell and PDX models to assess acquired gemcitabine resistance in pancreatic cancer. We demonstrated that resistance to gemcitabine was mainly associated with HER2 and HER3 overexpression, and in some cells with HER ligand expression. This was particularly true for the gemcitabine-resistant PDX P2846. In these models, acquired gemcitabine resistance was efficiently overcome by the Pan-HER (Sym013) antibody mixture, a cocktail of anti-EGFR, anti-HER2 and anti-HER3 antibodies. Finally, the combination of gemcitabine plus Pan-HER had an additive effect on pancreatic tumor cell xenograft growth inhibition. Although we detected co-expression of EGFR, HER2 and HER3 only in 11% of the tested PDAC samples, the number of patients with HER2- and HER3-positive PDAC is probably underestimated because of the limit of the IHC detection method. This technique needs to be optimized in PDAC, as already done for HER2 expression in breast and gastric cancers. Previous studies reported HER2 overexpression in 17% to 33% of PDAC, EGFR overexpression in 40% to 70% of the samples, and low HER3 expression levels in almost all PDAC specimens.[10,11,21]

In this study, we used a dose escalation strategy to develop gemcitabine resistance. This method leads to stable resistant models and was previously used for other cancer types and is



Survival Statistical analysis		
P4604	Control vs PanHER	P=0.0976
	Control vs Gemcitabine	P=0.3744
	Gemcitabine vs PanHER	P=0.0005
	Gemcitabine vs PanHER+Gemcitabine	P=0.0041
	PanHer vs PanHER+Gemcitabine	P=0.0001
SW1990	Gemcitabine vs PanHER	P<0.0001
	Controle vs Gemcitabine	P=0.002
	Gemcitabine vs PanHER	P<0.0001
	Gemcitabine vs PanHER+Gemcitabine	P<0.0001
	PanHer vs PanHER+Gemcitabine	P=0.0405

Figure 7. Effect of the Pan-HER plus gemcitabine combination on pancreatic cancer models. a *In vitro* interaction of gemcitabine and Pan-HER. BxPC3, C-PDX P4604 and SW1990 cells were exposed to the Pan-HER + gemcitabine combination at the indicated concentrations for five days. Cell proliferation was measured using the SRB colorimetric assay (n = 3). Results are presented as the percentage of proliferating cells relative to untreated cells (proliferation matrices; left panel). The interaction (synergy *vs* additivity) between the tested drugs was investigated using dose-response matrices (right panel). In the blue matrices, values indicate the percentage of surviving cells. In the synergism matrice, the green color indicates antagonist combinations, the red the synergistic combination and the black cells show additive combinations. b *In vivo* effect of the gemcitabine and Pan-HER combination on tumor growth in mice xenografted with C-PDX P4604 (left panels) and SW1990 cells. Mice (n = 10/group) were treated with Pan-HER (25 mg/kg) or/and gemcitabine (50 mg/kg) twice per week for 3 weeks. Tumor growth (upper panels) was monitored and Kaplan Meyer curves (middle panels) were computed at the end of experiment. Survival statistics are in the table (lower panel).

suitable for studying drug resistance.[22,23] To date, the mechanisms underlying gemcitabine resistance remain unclear. Many signaling pathways, including those implicated in the regulation of drug transporters and metabolism, apoptosis and pro–survival mechanisms, DNA damage, and epithelial mesenchymal transition, have been implicated in gemcitabine resistance in pancreatic cancer.[24–26] In the gemcitabine-resistant PDAC models we developed, we observed a tendency to mesenchymal transformation together with increased growth compared with the parental cell lines. These morphological and growth changes, as well as cell cycle arrest in G1 phase, have been already related to drug resistance by Zhou et al.,[23] with different features according to the cell type. Moreover, two studies indicated that c-MET signaling and STAT3 activation play an important role in acquired resistance to gemcitabine.[27,28] Cancer stem cells are also implicated in PDAC chemoresistance due to their ability to escape conventional therapies.[29] Finally, the tumor micro-environment, through its different cell types (e.g., cancer-associated fibroblasts, immune cells), secreted factors (i.g., growth factors, cytokines) and extracellular matrix composition (i.g., fibronectin, hyaluronan), plays a key role in chemoresistance to pancreatic cancer.[30] In pancreatic cancer cells, resistance to one drug leads often to cross-resistance to multiple drugs.[23] This phenomenon has been confirmed in clinical settings where the limited efficacy of combination chemotherapies has been documented. In our study, the gemcitabine-resistant SW1990-GR and BxPC3-GR cell lines were also moderately-resistant to FOLFIRINOX, one of the standard chemotherapies used in pancreatic cancer (Figure S2: FOLFIRINOX sensitivity of BxPC3 GR and SW1990 GR models).

In our study, both *in vitro*- and *in vivo*-induced resistance to gemcitabine were accompanied by an increase in the expression of EGFR, HER2 and HER3. This was never reported before in gemcitabine-resistant pancreatic cancer, but it confirms previous preclinical results obtained in trastuzumab-resistant gastric and breast cancer,[31,32] and in cetuximab-resistant non-small cell lung carcinoma (NSCLC) and head and neck squamous cell carcinoma models.[33] In these cases, upregulation of HER family member was sometimes associated with increased expression of EGFR ligands. The overexpression of RTK ligands has been also reported in innate and acquired resistance to antibodies, such as trastuzumab,[34] and tyrosine kinase inhibitors.[35] In our models, EGF and NRG1 expression level varied according to the cell line. Indeed, EGF expression was always increased in both BxPC-3 and SW1990-GR cells, and GR-PBX P2846. In contrast, augmented expression of NRG1 was only observed in the gemcitabine-resistant PDX P2846-GR.

To strengthen our hypothesis, the number of HER receptors expressed at the cell surface and their mutation status, which could be changed by gemcitabine pressure, should be precisely analyzed. Other parameters, such as EGF and NRG1 expression, could also affect Pan-HER sensitivity in gemcitabine-

sensitive vs gemcitabine-resistant models, and we showed that the EGF/NRG1 balance (and other ligands) changes during gemcitabine resistance development. In addition, compensatory crosstalk between receptors from the HER family and other RTKs (i.e., cMET, AXL or IGFR) has been also described, particularly after gemcitabine pressure and induced resistance. All these compensatory mechanisms could explained why Pan-HER treatment did not exactly translate into a lower tumor growth inhibition in the gemcitabine-sensitive pancreatic cancer model.

RTK plasticity and compensatory signaling pathways limit the efficacy of drugs that target a single receptor in several cancer types. Simultaneous inhibition of different HER family members is an attractive alternative to prevent the resistance to monotherapy. Here, we showed that Pan-HER, a cocktail of anti-EGFR, anti-HER2 and anti-HER3 antibodies, inhibited cell viability of our gemcitabine-resistant and -sensitive PDAC cell lines and PDX.

Interestingly, this effect was observed in *KRAS*-wild type BxPC3 cells, but also in *KRAS*-mutated SW1990 cells. Pan-HER was an effective treatment for mice xenografted with gemcitabine-resistant pancreatic cancer cells (*ex vivo* acquired resistance) and with the PDX P2846-GR (*in vivo* acquired resistance).

Few studies have suggested targeting several HER receptors together rather than one to maximize the biological effects and overcome potential resistance. The four-in-one antibody FL518, which concomitantly-inhibits VEGFR, EGFR, HER2 and HER3, demonstrated an anti-tumoral effect in colorectal, breast and gastric cancers.[36] In trastuzumab-resistant HER2-overexpressing breast cancer, the combination of lapatinib (an EGFR/HER2-specific tyrosine kinase inhibitor), trastuzumab (anti-HER2 antibody) and U3-1287 (anti-HER3 antibody) abrogated trastuzumab resistance and showed superior activity than single treatment.[37] We previously demonstrated that simultaneous targeting of HER2 and HER3 with antibodies is more efficient in HER2-low breast cancer.[38] The triple combination of anti-EGFR, anti-HER2 and anti-HER3 antibodies[39] by-passed resistance to erlotinib in NSCLC cancer to induce greater tumor growth inhibition by degradation of HER receptors and cellular senescence. In pancreatic cancer, we showed that simultaneous targeting of EGFR and HER2 with cetuximab and trastuzumab induce a synergistic therapeutic affect.[40] We confirmed and extended this strategy by combining pertuzumab (anti-HER2 antibody) with 9F7-F11 (anti-HER3 antibody) in pancreatic cancer.[41] In these preclinical settings, the antibody combinations decreased cell proliferation, blocked the MAPK and AKT pathways by disrupting hetero- and homo-dimers, and inducing receptor down-regulation. We also demonstrated that these combinations are more efficient than monotherapy to favor antibody-dependent cell-mediated cytotoxicity (ADCC) of pancreatic tumor cells.

In the study reported here, the Pan-HER mixture probably enhanced ADCC of tumor cells, but could also positively affect immune system responses that are essential for cancer therapy. This is particularly true *in vivo*. Pan-HER-induced activation of immune cells, such as natural killer cells and macrophages, could explain the better efficacy we observed *in vivo* in mice xenografted with parental and gemcitabine-resistant PDAC models compared with the *in vitro* results where immune cells are absent. Together with immune activation, Pan-HER treatment might activate other mechanisms, such as degradation of HER receptors, impairment of ligand binding, and inhibition of downstream signaling pathways, as previously described.[34,35,42] In our study, a clear up-regulation of all HER receptors is observed *in vivo* in the PDX models, notably in gemcitabine-resistant tumors; such down-regulation being efficiently targeted by Pan-HER. It is worth noting that other receptors from the RTK family, through their crosstalk with HER receptors, may be involved in the mechanisms of gemcitabine resistance in pancreatic cancer and more studies are necessary.[23] However, a mixture of non-binding IgG control will be interesting to show the specificity of Pan-HER on tumor, but the production of such an irrelevant mixture would be very complex. To generate the antibodies that compose PanHER, mice were immunized with soluble antigens or human cells that express the target.[17] The resulting antibodies were then cloned from single splenic B cells using the Symplex technology. They are expressed as chimeric or humanized IgG1 in mammalian cells. An irrelevant mixture would need to be produced using the same conditions, by maintaining the stoichiometry and concentration of each antibody.

Considering the treatments for metastatic pancreatic cancer approved to date, studying the effects of combining Pan-HER with standard-of-care agents, such as gemcitabine or FOLFIRINOX, is essential. In this work, we demonstrated an additive effect on tumor growth of Pan-HER plus gemcitabine treatment *in vitro* and in mice xenografted with PDAC cancer cell lines or PDX. Interestingly, the therapeutic benefit of the combination was observed in intrinsic gemcitabine-resistant and -sensitive pancreatic cancer cells, which is a very promising development that should be considered for clinical trials in pancreatic cancer in the future.

Materials and methods

Reagents and antibodies

Gemcitabine was purchased from ICM hospital. Pan-HER and vehicle (control) were provided by Symphogen A/S. Antibodies against EGFR, HER2 and HER3 were from Cell Signaling Technology (Cell Signaling Technology, 2085, 2242, 4754).

Cell lines and culture conditions for inducing gemcitabine resistance

The BxPC3, SW1990, CFPAC and HPAC pancreatic cell lines were obtained from ATCC (Rockville) and cultured following the ATCC recommendations. Routine characterization was done by morphological observation, and mycoplasma testing was performed using the MycoAlert™ Mycoplasma Detection Kit (LT07-318, Lonza). The P4604 and P7054 cell lines were derived from PDX of peritoneum and liver metastases of human pancreatic tumor specimens, respectively (PDX Platform, Institut de Recherche en Cancérologie de Montpellier). Gemcitabine-resistant cell lines were generated by exposure to progressively increasing doses of gemcitabine. Cells were first exposed to 20 nM of gemcitabine and doses

were increased after each passage. Finally, gemcitabine-resistant BxPC-3 (BxPC3-GR) and SW1990 (SW1990-GR) cells were maintained in medium with 130 and 200 nM gemcitabine, respectively.

Immunohistochemistry

Surgically excised, human FFPE PDAC specimens were cut into 4-µm sections that were deparaffinized in xylene and hydrated in graded alcohols. For EGFR and HER2 detection, the CONFIRM anti-EGFR (clone 3C6) and PATHWAY anti-HER2 (clone 4B5) assays were used, according to the manufacturer's instruction, in a Benchmark XT automate (Ventana/Roche Diagnostics). For HER3 detection, antigen retrieval was performed with EnVision® Target Retrieval Solution High pH (GV804, Dako) at 97°C for 20 min, followed by incubation of tissue sections at 37°C with the mouse monoclonal anti-HER3 antibody (clone DAK-H3-IC, Dako; 1:50) for 2 h. The antigen-antibody reaction was revealed using EnVision® Flex DAB System in a Dako Autostainer Plus automate. IHC staining was interpreted by an expert pathologist who was blind to patient information.

Cell viability assay and treatment interaction

The effect of gemcitabine and Pan-HER on cell viability was evaluated using MTS and sulforhodamine B (SRB) colorimetric assays, as already described.[42] After 24 h, cells were incubated with increasing drug concentrations at 37°C for 5 days. The percentage of viable cells (i.e., proliferating cells) was calculated relatively to the number in untreated cultures. All experiments were performed three times. The effect of the gemcitabine and Pan-HER combination was evaluated using the SRB assay. Treatment interactions were analyzed using dose-response matrices, according to the Bliss equation.[43]

$$fu_c = fu_A\, fu_B$$

where fu_c is the expected fraction of cells unaffected by the drug combination in the case of effect independence, and fu_A and fu_B are the fractions of cells unaffected by treatment *A* and *B*, respectively. The difference between the fu_c value and the fraction of living cells in the cytotoxicity test was considered as an estimation of the interaction effect, with positive values indicating synergism and negative values antagonism.

Western blot analysis

As previously described,[43] cells were lysed (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1% Triton, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 100 mM sodium fluoride, 1 mM sodium orthovanadate, and one tablet of complete protease inhibitor mixture (11697498001, Merck)) for 30 min. After electrophoresis on 7% SDS-PAGE in reducing conditions, proteins were transferred to polyvinylidene difluoride membranes (IPVH00010, Millipore) and incubated with the appropriate dilutions of polyclonal rabbit anti-human EGFR, HER2 and HER3 antibodies (4267, 2242, 4754 respectively, Cell Signaling Technology). Protein loading was assessed using an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AB2302, Millipore). Blots were visualized using a chemiluminescent substrate (NEL105001EA, Western Lightning Plus-ECL, Perkin Elmer).

Quantitative PCR analysis

RNA was isolated using the Quick-RNA MiniPrep Kit (R1054, Zymo Research Corp.) according to the manufacturer's protocol. RNA samples were first reverse-transcribed using the SuperScript III reverse transcriptase (18080093, Thermo Fisher Scientific) according to the manufacturer's protocol. Gene expression was then assessed by quantitative PCR using the TB Green® Premix Ex Taq™ (Tli RNase H Plus) (Takara). The primer sequences are shown in supplementary Table 1. Concentrations were then normalized to the concentration of the housekeeping gene *18S rRNA*, and expressed relatively to untreated samples.

Immunofluorescence analysis of tumors

Tumor xenografts were collected after treatment and fixed in 10% neutral buffered formalin for 24 h, dehydrated, and embedded in paraffin. Paraffin-embedded tissue was cut into 3-µm-thick sections, mounted on slides, and then dried at 37°C overnight. For EGFR and HER2 detection, the anti-EGFR (5B7, Roche) and anti-HER2 (4B5, Roche) assays were used, according to the manufacturer's instruction, in a VENTANA Discovery Ultra automated staining instrument (Ventana/Roche Diagnostics). Amplification signal were performed using the DISCOVERY OmniMap anti-rabbit HRP detection Kit (05269679001) and a Tyramid- Cy5 solution (0755121500). For HER3 detection, antigen retrieval was performed with EnVision® Target Retrieval Solution High pH (Dako) at 97°C for 20 min, followed by incubation of tissue sections at 37°C with the mouse monoclonal anti-HER3 antibody (clone DAK-H3-IC, Dako; 1:50) for 1 h at 37°C. The antigen-antibody reaction was revealed using Rabbit anti-mouse IgG (Abcam #133469) then using the DISCOVERY HQ conjugated antibody anti-rabbit IgG (07017812001) and Tyramid– Cy5 solution (0755121500). Slides were counterstained with QD Dapi (760-4196) and coverslipped with Mowiol mounting medium. Fluorescent slides were observed on Thunder microscope (Leica). Quantification of the fluorescence intensity was done with Image Scope software.

Mouse xenograft models and patient-derived xenograft

All *in vivo* experiments were performed in compliance with the French regulations and ethical guidelines for experimental animal studies in an accredited establishment (Agreement No. C34-172-27). The PDX were obtained as previously described.[44] BxPC3-GR ($4*10^6$ cells per mice), SW1990-GR ($2*10^6$ cells per mice), PDX P2846, PDX P2846-GR, SW1990 ($4*10^6$ cells per mice) or C-PDX P4604 ($5*10^6$ cells per mice) cells were injected subcutaneously in the right flank of 6 week/old female athymic mice, purchased from Harlan (Le Malcourlet, France). Tumor-bearing mice were randomized in the different treatment groups (10 animals/group) when tumors reached a minimum volume of 150 mm^3. Tumor

Abbreviations	
ADCC	antibody-dependent cell-mediated cytotoxicity
CAF	cancer-associated fibroblast; EGF: epidermal growth factor
FFPE	formalin-fixed paraffin-embedded
GR	gemcitabine resistant
HER	human epidermal growth factor receptors 1, 2 and 3
NRG1	neuregulin1
PDAC	pancreatic ductal adenocarcinoma
PDX	patient-derived xenograft
RTK	receptor tyrosine kinases

volumes were calculated with the formula: D_1 x D_2 x D_3/2. For survival comparison, mice were sacrificed when tumor reached a volume of 1500 mm^3. Mice were treated with the Pan-HER mixture (50 or 25 mg/kg), vehicle, or gemcitabine (50 or 100 mg/kg) by intraperitoneal (ip) injection twice per week for 3 weeks. The PDX P2846 model was chosen to generated a gemcitabine-resistant PDX due to his high initial sensitivity compared to PDX P4604 and PDX P7054 (Fig. S1b: Gemcitabine sensitivity of PDX P2846, P7064 and P4604). The PDX models were established as previously described.[44] Animals underwent several cycles of treatment with 50 and 100 mg/kg of gemcitabine until tumor growth continued despite the injection of 100 mg/kg gemcitabine (Figure 2c).

Statistical analysis

The relationship between tumor growth and treatment was analyzed using a linear mixed regression model. The fixed part of the model included the variables number of days post-graft and treatment group; interaction terms were also evaluated. Random intercepts and random slopes were included to take into account the time effect. The model coefficients were estimated by maximum likelihood. A survival analysis was performed and the event considered was a tumor volume of 1500 mm^3. Survival rates were estimated using the Kaplan-Meier method and survival curves were compared with the log rank test. Statistical significance was set at $p < .05$. Statistical analysis was done with the STATA 16 software (Stata Corporation, College Station, TX, USA).

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ORCID

Christel Larbouret http://orcid.org/0000-0001-8531-9552

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