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Title: Lack of growth inhibitory synergism with combined MAPK/PI3K inhibition in preclinical models of pancreatic cancer.

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Despite substantial advances in chemotherapy and biology understanding, pancreatic ductal adenocarcinoma (PDAC) remains one of the deadliest solid tumors. Attempts at exploiting PDAC biology for therapeutic purposes have failed and the likelihood of approval for new agents that enter phase I testing in this disease is down to a dismal 2.3%[1]. Recently, a combination of MEK (selumetinib) and AKT (MK-2206) inhibitors failed to demonstrate clinical benefit in unselected PDAC patients[2], adding to a long list of targeted agents that have failed clinical testing (EGFR/VEGFR, SMO, and Notch inhibitors, to name a few). We thus asked ourselves whether such failure could have been predicted preclinically.

We explored pharmacologic interactions between MEK inhibitors (trametinib) and PI3K pathway inhibitors [gedatolisib (PI3K/mTOR inhibitor) andMK-2206 (AKT inhibitor)] *in vitro*, using 6 human PDAC cell lines and the "normal pancreatic epithelium" cell line HPDE. Single-agent inhibition of MEK, PI3K/mTOR, or AKT inhibited cell growth to a variable extent in all cell lines examined. However, combined inhibition of MEK and PI3K/mTOR (trametinib/gedatolisib) afforded frankly antagonistic effects in Panc1, MiaPaCa2, T3M4, PaCa44, and HPDE, slightly additive effects in HPAFII, and synergistic effects only in L3.6pl cells (Fig. 1A); similarly, combined MEK/AKT inhibition (trametinib/MK-2206) was antagonistic in all cell lines tested (Fig. 1A-C). Overall, no growth inhibitory synergism *in vitro* was observed in any of the cell lines tested, with the exception of L3.6pl cells in response to trametinib/gedatolisib combination.

Our group has recently shown that combined inhibition of the MAPK and PI3K pathways affords synergistic anti-tumor effects almost exclusively in cancer cells without a functional PTEN gene/protein (PTEN-loss)[3]. We thus examined PTEN expression in the panel of PDAC cell lines examined: no PTEN mutations or bi-allelic loss have been reported for these cells and all displayed detectable levels of PTEN protein (Fig 1A, D), thus falling in the PTEN-competent category

according to the definition recently proposed by our group[3]. Consistent results (lack of growth inhibitory synergism) had, indeed, already been obtained in the PTEN-competent cell lines HPAFII and MiaPaCa2, using another combination of MEK and mTOR inhibitors (trametinib and everolimus)[3].

Inactivating PTEN point mutations or LOH rarely occur in human PDAC[4]; thus, based on the preclinical data presented here, the failure of selumetinib/MK-2206 to achieve clinical benefit in unselected PDAC patients would have been largely anticipated. Extensive preclinical modeling and early selection biomarker development are, in our opinion, crucial to successful drug development, in addition to uniform trial eligibility criteria, stringent statistical methods, and detection of robust activity signals in early phase trials. Unfortunately, such rules have been often overlooked in advanced PDAC, resulting in the identification of only 3 clinically meaningful agents or combinations out of 32 phase III trials enrolling >13,000 patients[5]. This line of reasoning especially applies to combined pathway inhibition, which implies increased monetary and toxicity costs, representing a high risk for all stakeholders, should it fail to demonstrate more than additive benefits, and to advanced PDAC, a disease setting in which novel effective therapeutic approaches are urgently needed.

Disclosures

The authors have declared no conflicts of interest.

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	IC_{50} nM			CI		
Cell Line	trametinib	gedatolisib	MK-2206	T+G	T+MK	PTEN
HPAFII	7.40	9.80	6.40 x 10^3	0.80	1.00	+
L3.6	0.15	0.01	1.40 x 10^5	0.40	1.46	+
PANC1	8.00	376.70	6.40 x 10^3	12.30	2.84	+++
MiaPaca2	1.77	8.60	n.a.	24.00	n.a.	++
T3M4	36.90	40.60	n.a.	1.60	n.a.	+++
PACA44	0.01	18.50	n.a.	61.60	n.a.	+++
HPDE	15.60	n.a.	1.60 x 10^4	8.10	33.80	+

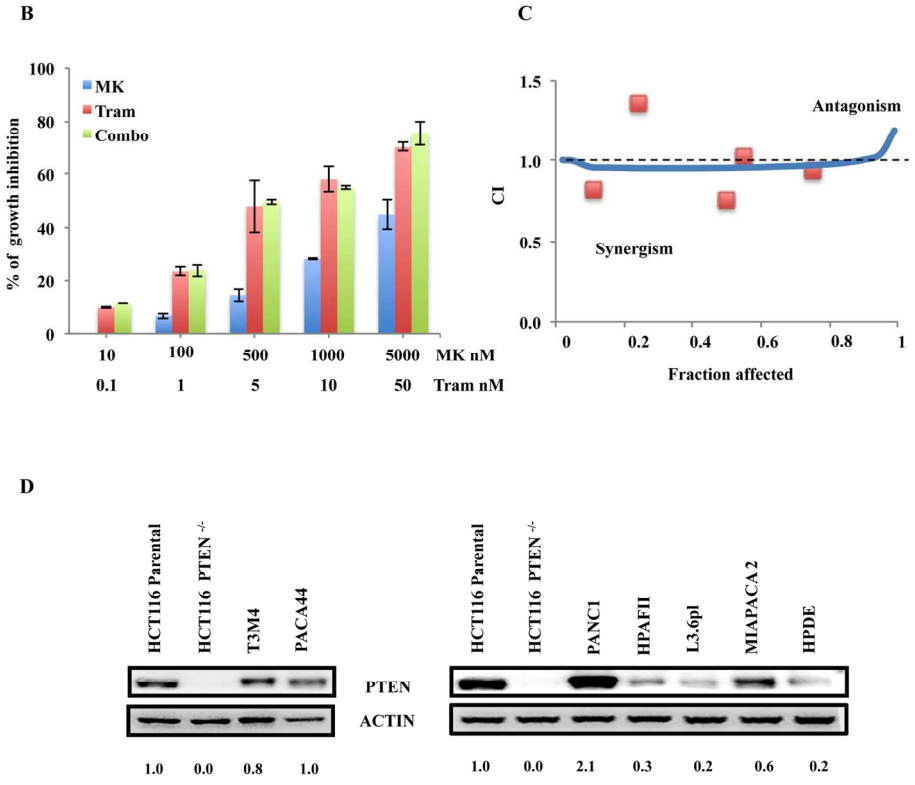

Figure 1. A. Growth inhibitory interactions (synergism, antagonism, additivity) between trametinib and gedatolisib or MK-2206 were assessed in a panel of 6 pancreatic adenocarcinoma cell lines and 1 "normal pancreatic epithelium" cell line (HPDE), using a fixed-ratio (10:1 or 1:100, for the combination of trametinib with either gedatolisib or MK-2206, respectively). Experiments were designed using a wide range of concentrations (0.1-1000 nM for trametinib; 0.01-100 nM for gedatolisib; 10-5000 nM for MK-2206). Viability was then assessed after 72 h and pharmacologic interactions were evaluated using the Chou-Talalay method and the Calcusyn software. With this method, an average combination index (CI) at the ED50, ED75, and ED90 <1 indicates synergism, =1 indicates additivity, >1 indicates antagonism. IC50 and CI values were derived from the average of at least three independent experiments. PTEN expression was summarized based on PTEN/b-actin ratio (as assessed by WB, see also panel D), as follows: +, ratio 0.1-0.3; ++, ratio 0.3-0.6; +++, ratio 0.6-1. B-C. HPAF II cells were exposed to increasing concentrations of trametinib (0.1-1000 nM) and the allosteric AKT inhibitor MK-2206 (10-5000 nM), alone or in combination at a 1:100 ratio. Results are expressed as percentage of growth inhibition relative to untreated control and

represent the average±SEM of three independent experiments (B). CI were calculated by conservative isobologram analysis and plotted against the fraction affected; actual experimental data are shown as red squares (C). D. Pancreatic cell lines were lysed and analyzed by Western Blotting using rabbit polyclonal antibodies specific for PTEN (Cell Signaling Technologies, Danvers, MA, USA). Western blot with antibodies specific for β-actin are shown as protein loading and blotting control. X-ManTM HCT116 Parental and HCT116 PTEN-/- isogenic cell lines (Horizon Discovery; www.horizondiscovery.com) were used as a positive and negative control for PTEN expression.

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