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Original Research

A novel regimen for pancreatic ductal adenocarcinoma targeting MEK, BCL-xL, and EGFR

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ABSTRACT

Oncogenic KRAS signaling plays a critical role in pancreatic ductal adenocarcinoma (PDAC) biology. Recent studies indicate that the combination of MEK and BCL-xL inhibition is synthetically lethal and holds promise for some types of solid cancers, however, patient response was poorly observed in PDAC predominantly due to amplified EGFR signaling. Here, we leverage the advantage of the combinational treatment strategy and designed a triplet regimen targeting the comprehensive RAS activation networks through simultaneously blocking MEK/BCL-xL/EGFR. The cytotoxicity of trametinib (MEK inhibitor), DT2216 (BCL-xL degrader) and afatinib (pan-EGFR inhibitor) and their combination was tested in patient-derived, primary PDAC cells using a live cell imaging system. Patient-derived xenograft (PDX) model was employed for the evaluation of the therapeutic efficacy and safety of the combinational regimen. Targeted pathway cascades activities were analyzed using multiplex phosphor-immune assays. *In vitro* comparisons showed the addition of afatinib as a third agent was statistically superior compared to a doublet of trametinib+DT2216 in suppressing cell growth and inducing cell death in all cell lines tested. This triplet similarly demonstrated significant superiority over the doublet of MEK/BCL-xL inhibition in the *in vivo* murine model. The triplet regimen was well tolerated *in vivo*. Overall tumor growth rates were significantly reduced in doublet treatment compared to controls, and further reduced in the triplet treatment group. Pathway analysis revealed the addition of afatinib in triplet regimen further inhibited PI3K/AKT effectors of p90RSK, p70S6K, and GSK3α/β along with a secondary pathway of P38 MAPK. Our study identifies an important contribution of EGFR inhibition to elevate the response of PDAC, supporting a clinical assessment of this triplet combination in patients.

Introduction

Approximately sixty-two thousand new cases account for approximately 50,000 deaths each year from pancreatic cancer, making it the third most common cause of cancer deaths in the US [1]. The majority of new cases present with advanced and incurable disease. These patients are managed primarily with palliative, systemic regimens of chemotherapy. The best available regimens are associated with overall survival in the range of just under 1 year [2]. Precision approaches are rarely indicated and essentially untested and thus unproven. Hence there is a great need for new therapeutic options for patients with pancreatic cancer.

More than 90% of pancreatic cancers and a large proportion of lung, colorectal, and other cancers are associated with RAS mutations. The recent development of KRAS-targeting drugs, while more promising in other cancers, their clinical efficacy in pancreatic cancer is still under

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evaluation [3]. Using a synthetic lethal shRNA screen in RAS mutant cell lines, Corcoran and colleagues identified BCL-xL as a critical target that cooperates with MEK inhibitors to enhance their activity in RAS mutant cell lines [4]. This observation was translated into a clinical trial combining a MEK inhibitor with a BCL-xL inhibitor in patients with several types of RAS mutant cancers and identified a striking response rate in refractory gynecologic cancers (across subtypes of cervical, endometrial and ovarian) but, no response in other solid tumor types with RAS mutations, such as colorectal, non-small cell lung cancer, and pancreatic cancer [5]. Previous investigations showed that inhibition of the MAP kinase pathway in RAS mutant PDAC cell lines results in upregulation of EGFR/HER-family pathway activity, with consequent signaling down the PI3K/AKT pathway negating the potential activity of MEK inhibition [6]. It was further shown that the combination of anti-EGFR therapy with MEK inhibition abolished MEK induced signaling through the PI3K/AKT pathway, resulting in significant anti-cancer activity [7,8]. The concept of combining a MEK inhibitor with anti-EGFR therapy has emerged in gastrointestinal oncology that EGFR inhibition is necessary for MAPK-targeted therapy [9]. This concept was tested in colorectal cancer patients whose tumor contained a BRAF mutation [10]. This combination was shown to be associated with significant activity when compared with standard second line chemotherapy in this selected patient population, alas it has only resulted in modest antitumor activity in pancreatic cancer in a phase II clinical trial [11].

Taken together, the above preclinical and clinical investigations suggest that optimal inhibition of RAS-mutant cancers may require the combination of RAS/MEK inhibition with a BCL-xL inhibitor/degrader plus continued inhibition of surface receptors, in particular EGFR to prevent downstream of MEK signaling via the PI3K/AKT pathway [12]. We tested this hypothesis in RAS-mutant, early passage pancreatic cancer cell lines derived from either the primary or a metastatic site of patients with pancreatic cancers, given that these cancers are often also EGFR driven [13]. Small molecule inhibitors of BCL-xL have been associated with on-target and dose-limiting platelet toxicity. Thus, we also investigate two novel BCL-xL proteolysis targeting chimeras (PROTACs) degraders of BCL-xL in combination with MEK inhibition. Furthermore, as one mechanism of resistance to anti-EGFR therapy includes upregulation of alternative members of the HER family of growth receptors, we focus on a pan-HER-receptor kinase inhibitor.

Materials and methods

Cells and culture

Patient-derived PDAC cell lines, PDAC1, PDAC2, PDAC-lm1, and PDAC-lm3 were generated from patients who had operations performed at the University of Florida, Department of Surgery with informed consent under University of Florida IRB protocol 201600873. All cell lines were authenticated by short tandem repeat profiling [14]. Cells were grown in Advanced DMEM-F12 medium supplemented with 10 % FBS and 1 % anti–anti. Cell viability was tested using trypan blue staining on TC20 automated cell counter and only batches with cell viability over 98 % were used for experiments. For monoculture, cells were plated in at 2000 cells per well in 96-well flat-bottom and allowed overnight attachment before treatment. All experiments were performed in technical quintuplicate and repeated three times.

Drug response assay

All drugs including trametinib (MEK1 and MEK2 kinases inhibitor, GSK1120212), afatinib (an irreversible pan-EGFR inhibitor), and Navitoclax (BCL-2 and BCL-xL dual inhibitor, ABT-263) were purchased from AdooQ bioscience (Irvine, CA) and dissolved in DMSO to make a concentration of 100 μM stock. PROTACs DT2216 and 753b are synthesized as previously described [15,16]. Doublet treatment consisted of the combination of trametinib plus navitoclax or DT2216 or 753B; afatinib was added in addition for triplet treatment. Drugs were diluted to a final concentration of 0.5 μM. The Incucyte S3 (Satorius) live-cell imaging system was used for the measurements of drug response. Cell growth was assessed by confluence and cell death was labelled with Cytotox NIR Dye (for counting dead cell integrated intensity over total cell area).

In vivo model

The study was performed with IACUC approval. Eight weeks-old male NSG mice were subcutaneously inoculated with 2.5×10^5 of PDAC-lm1 tumor cells to establish tumor xenografts. When the tumor volume exceeded 100 mm^3, the animals were randomized into three groups of 15 mice to receive treatments with vehicle, doublet, and triplet treatments, respectively. All drugs were administered bi-weekly by the intraperitoneal (i.p.) route at the concentrations of trametinib: 2 mg kg^{-1}, DT2216: 15 mg kg^{-1}, and afatinib: 80 mg kg^{-1}. For i.p. administration of trametinib and afatinib, the selection of the doses for these drugs were calculated according to others' work ([17-20], and the dose of DT2216 is based on our previous experience (recommended by D. Zhou). Animal weights and xenograft tumor size were monitored and recorded twice a week. Tumor volume was calculated based on the formula of ½ (length x $width^2$). All animals were euthanized when the tumor reached humane endpoints or at the end of the experiment, except for animals euthanized for tumor harvest for mechanistic studies of pathway inhibition 4 hours after the 3rd dose (n = 3 per group).

Multiplex immunoassays

Three multiplexes were employed to assess the targeting molecules. These included: Bio-Plex Pro RBM apoptosis panel 2 multiplex assay detecting BAD, BAX/BCL-2 dimer, BCL-xL, Bim, and MCL-1; Bio-plex Pro cell signaling AKT 8-plex panel detecting AKT(Ser^{473}), BAD(Ser^{136}), GSK3α/β(Ser^{21}/Ser^{9}), IRS-1(Ser^{636}/Ser^{639}), mTOR(Ser^{2248}), p70S6K (Thr^{389}), (19,20)PTEN(Ser^{380}), and S6 ribosomal protein(Ser^{235}/Ser^{236}); and MAPK 9-plex panel detecting ATF-2(Thr^{71}), ERK1/2($Thr^{202/185}$/$Tyr^{204/187}$), HSP27(Ser^{78}), JNK(Thr^{183}/Tyr^{185}), MEK1(Ser^{217}/Ser^{221}), p38 MAPK (Thr^{180}/Tyr^{182}), p53 (Ser^{15}), p90RSK(Ser^{380}), and STAT3 (Ser^{727}).

Statistical analysis

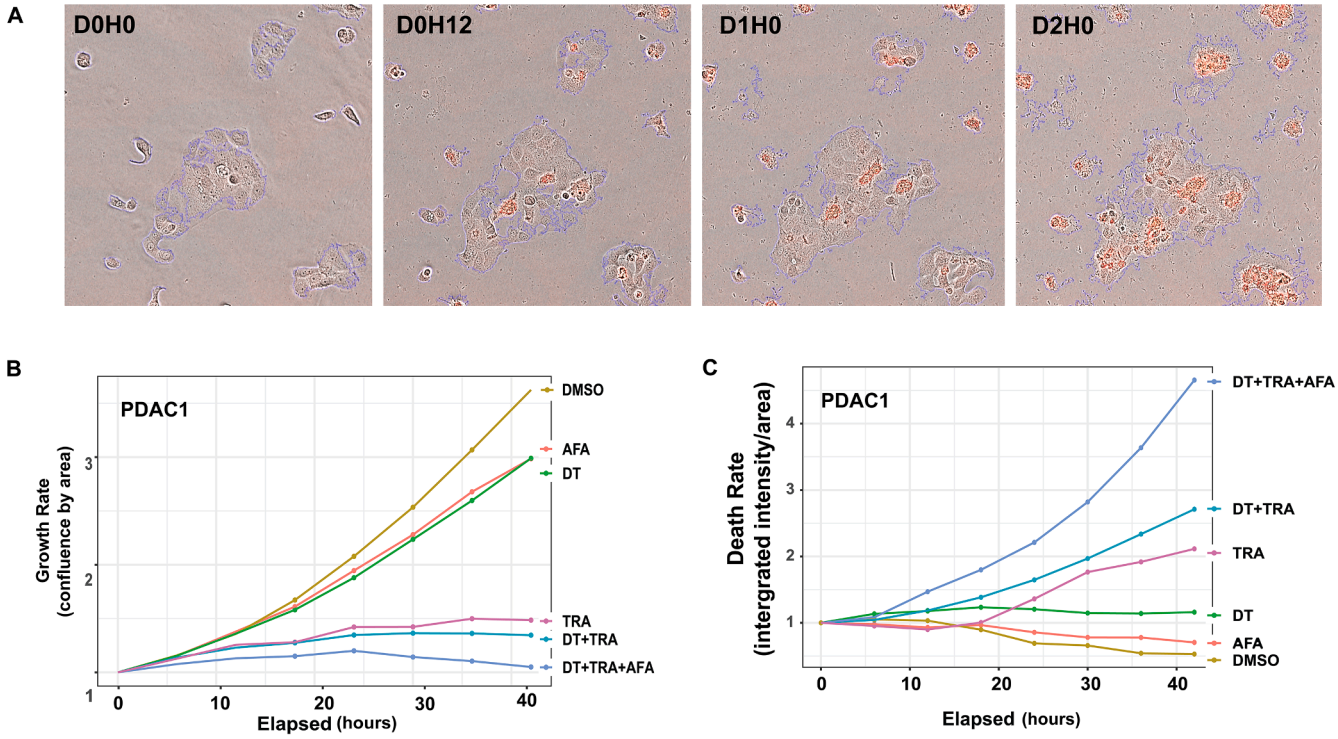
To assess the differential growth rates between treatment groups, we utilized linear mixed models by R package lme4. The longitudinal measurements of tumor sizes were set as the dependent variable. The predictors included the treatment group, time, and the interaction between time and treatment. To ensure parity, we adjusted for the initial size of the tumor as a covariate. The interaction term represents the difference in growth rate (denoted as change in tumor size per hour) between the two treatment groups. This method of analysis was conducted individually for each pair of treatment groups, as well as for each cell line. Group comparisons were performed using GraphPad Prism v10 software. Unpaired Welch's test was used to compare two groups and one-way or two-way analysis of variance (ANOVA) was used for multiple groups. False discovery rate adjusted p-value (q-value) [21] was set at 0.05 as statistical significance cut off.

Results

Inhibition of MEK, BCL-xL, and EGFR (triplet) treatment reduced PDAC cell growth and enhanced apoptosis *in vitro*. KRAS via downstream MEK drives the MAPK signaling pathways to promote cell proliferation and prevent apoptosis. To test whether the addition of EGFR-family inhibitors can further increase the efficacy of blocking this aberrant signaling in PDAC cells, we targeted EGFR-family receptors with afatinib in combination with baseline doublet treatment of

trametinib and one-of-three BCL-xL targeting strategies: the small molecule inhibitor navitoclax or the PROTACs DT2216 or 753B [11,12]. A live-cell imaging platform was employed to monitor cell growth as well as cell death during treatment (Fig. 1). Complete data for individual drug efficacy assessments and various three drug-combinations is presented in Suppl. Figure S1 for cell growth and Suppl. Figure S2 for cell death measurements. Moderate cell growth suppression was observed for mono-drug treatment, and among the various agents, trametinib monotherapy appears most effective. Navitoclax and afatinib are less potent as single agents (Fig. 2A). The PROTACs DT2216 and 753b appear more effective in inducing apoptosis than navitoclax. In line with the findings from other laboratories [22], trametinib profoundly inhibited cell proliferation as shown in all four cell lines. The degraders of DT2216 and 753B induced cell death in three and two of the four cell lines, respectively, showing potency over the inhibitor navitoclax (Fig. 2A). When comparing doublet against triplet treatments and importantly, supporting our hypothesis, we found that blocking EGFR-family signaling with afatinib in addition to MEKi/BCL-xLi resulted in greater growth inhibition and increased cytotoxicity. The synergy was superior with DT2216 compared to navitoclax or 753B; the DT2216-combined triplet regimen effectively suppressed cell growth and induced cell death in all four primary human pancreatic cancer cell lines tested (Fig. 2B).

It is not surprising that each cell lines responded differently to the treatment, and this phenomenon was first suspected due to their KRAS mutation status. The cell lines tested in this study either carry a KRAS G12D or G12V mutation, however, there is no clear connection with the response to the doublet or triplet treatments. For a better understanding of the genomic makeup that might be associated with the differential response, we looked at the data in DepMap (https://depmap.org/portal) where 32 PDAC cell lines were reported with single treatments of tramatinib and afatinib respectively. Among these cell lines, 14 cell lines appear responsive (-log2 fold change, LFC < 0) to both drugs, 14 cell lines responsive to trametinib but resistant (-log2 fold change, LFC > 0) to afatinib, and the remaining 4 cell lines are resistant to both drugs (Suppl. Table S1 and Fig. 6a). Although it is noticeable that KRAS G12V mutation appears to correlate with the cell lines that respond to both trametinib and afatinib, and KRAS G12D appears more trametinib responsive and afatinib resistant, there is no definite correlation in our data as demonstrated in Fig. 6a. This observation is in line with previous findings [11]. With further investigation of full mutation status from COSMIC data, 121, 143 and 48 unique genes are clustered in the groups of responsive to both drugs, responsive to trametinib but resistant to afatinib, and resistant to both drugs respectively. KEGG pathway



Treat_A	Treat_B	rate_diff	q value_diff
DMSO	AFA	-0.014	0.028
DMSO	DT	-0.015	0.026
DMSO	TRA	-0.052	0.000
DT	DT+TRA	-0.040	0.000
TRA	DT+TRA	-0.004	0.115
AFA	DT+TRA+AFA	-0.048	0.000
DT+TRA	DT+TRA+AFA	-0.007	0.018

Treat_A	Treat_B	rate_diff	q value_diff
DMSO	AFA	0.007	0.006
DMSO	DT	0.016	0.000
DMSO	TRA	0.045	0.000
DT	DT+TRA	0.040	0.000
TRA	DT+TRA	0.011	0.114
AFA	DT+TRA+AFA	0.092	0.000
DT+TRA	DT+TRA+AFA	0.043	0.001

Fig. 1. Live cell imaging assessment of treatment responses. (A) Phase contrast images of live and dead cells. A set of four representative images of PDAC1 cells were taken at 20x magnification from time points of day 0, 12 hours, day 1, and day 2 as indicated. Cytotox NIR Dye (red) staining indicates dead cells. (B) Quantification of cell proliferation in cultures was assessed by confluence (area covered by cells), and (C) Quantification of dead cells was measured by Cytotox NIR Dye staining (ratio of integrated intensity of red staining over total cell area). Growth rate and death rates were plotted with y-axis reporting rate change against x-axis in elapsed hours. Experiments were performed in quintuplicates and four images were taken from each well. A q-value < 0.05 was considered statistically significant.

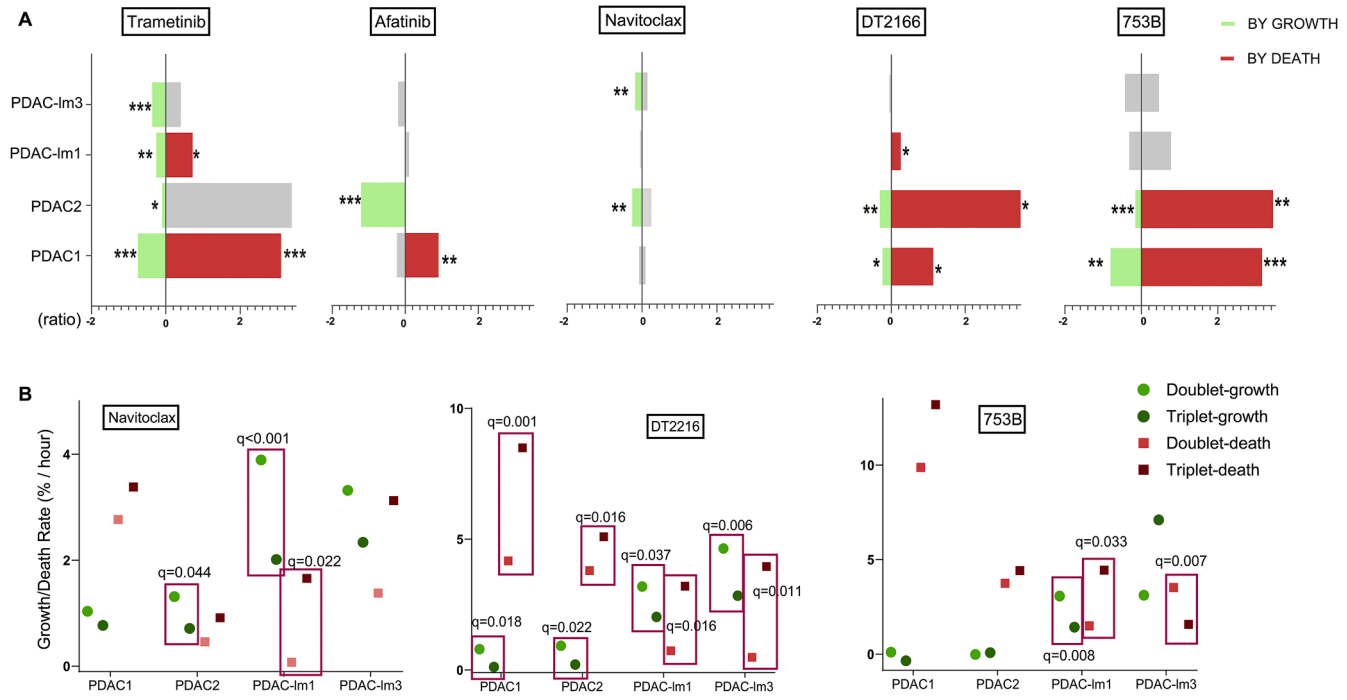


Fig. 2. Treatment effectiveness comparison of various combinations. (A) Mono-treatment of the five compounds on cell growth and cell death assessed on four PDAC patient-derived cell lines. *x*-axis is scaled by ratio of rate change between the individual compounds and the control treatment of DMSO. Ratio of changes comparing to DMSO control for decreased cell growth was scaled towards the left and increased cell death was scaled towards the right. Green and red bars indicate significant changes and gray bars indicate changes without statistical significance. * $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$; and (B) Comparisons between triplet treatment of MEK, BCL-xL, and EGFR inhibition vs doublet inhibition of MEK and BCL-xL. Growth rates are indicated by green dots (light green for doublet and dark green for triplet treatments), and cell death rates are indicated by red dots (light red for doublet and dark red for triplet treatment). Difference between rates that met statistical significance ($q < 0.05$) were boxed and the associated q value is indicated.

analysis shows the cell lines that are sensitive to both drugs carry mutant genes enriched in Pancreatic Cancer and EGFR Tyrosine Kinase Inhibitor Resistant pathways (Fig. 6b).

Triplet targeting EGFR with MEK and BCL-xL inhibition results in *in vivo* synergism. To further preclinical evaluation of the triplet regimen, we evaluated the *in vivo* efficacy in the PDAC-lm1 cell inoculated in NOD-SCID mice. Tumor-bearing mice were treated with doublet or triplet regimens and compared between one another and to vehicle controls (DT2216 was selected for BCL-xL degradation based upon *in vitro* data). Overall tumor growth rates were significantly lower in both doublet (10.7 mm^3/day, $q = 0.0017$) and triplet treatment groups (1.37 mm^3/day, $q < 0.0001$) compared to control (27.9 mm^3/day) (Fig. 3A.) Importantly and in support of our hypothesis, the triplet regimen outperformed the doublet regimen and significantly reduced tumor growth rates ($q < 0.001$). The statistically significant reduction in tumor progression between the two regimens was observed as early as at dose 3 ($q = 0.0024$). At the end of the experiment, approximately 50 % of tumors had actually shrunk in the triplet treatment group, while 25 % and 60 % tumors continued to grow over 3 times of size in doublet and vehicle treatment groups, respectively (Fig. 3B). No significant body weight loss was detected in the mice during treatment period for all groups (Fig. 3C), except measurement at dose 2 (3 days post-dose 1) where the average body weight of mice in triplet treatment group was 3 % less than controls ($q = 0.046$).

Altered EGFR/RAS regulated survival signaling pathway cascades in treated xenograft tumors. In order to gain insights on the survival signaling pathways impacted during the treatments that suppressed tumor growth, phosphorylation status of key members in the EGFR/RAS signaling pathways were probed using multiplex immune assays (key pathway cascades tested are shown in Fig. 4). Based on the previously established fitness-for-purpose modeling in xenograft models ([23], a subset of xenograft tumors was collected 4 hours after administration of the 3rd dose. In line with the *in vivo* data of suppressed tumor growth in both doublet and triplet groups, induced apoptosis was confirmed by decreased BCL-xL protein levels and increased levels of BAD in both groups in comparison to controls (Fig. 5A). A direct effect from trametinib that led to decreased p-MEK1(Ser$^{217/221}$) and p-ERK1/2 (Thy$^{202/185,}$ Tyr^{204}/^{187}) in both doublet and triplet treatment groups compared to vehicle alone was clearly observed ($q < 0.0001$, Fig. 5B), along with the decreased phosphorylation of p-STAT3 on residue Ser^{727}. Phosphorylation at Ser^{727} residue is reported to mediate STAT3 translocating into mitochondria in favor of metabolic synthesis for cell proliferation and survival, and the cooperative role of Ser^{727} is reported needed for the full activation of STAT3 in pancreatic cancer [24,25]. In addition, phosphorylation of p-IRS1(Ser$^{636/639}$), p-AKT(Ser^{473}), and p-p70S6K(Thr^{389}) was reduced in these doublet- and triplet-treated tumors (Fig. 5C). IRS1 is suggested for MAPK and PI3K cross-signaling in regulating AKT-involved cell proliferation in pancreatic cancer cells [26, 27]. These data suggest decreased activities in signaling cascades of MEK-ERK1/2 and possibly a collateral activity that simultaneously targeted the pathway of IRS-PI3K-AKT in response to treatments. A trend in decreased p-mTOR(Ser^{2248}) did not reach statistical significance and phosphorylation of p-JNK(Thr^{183}/Tyr^{185}), but p-PTEN(Ser^{380}) remained unchanged.

To explore the mechanism driving the further benefit observed in the *in vivo* triplet treatment, decreased p-AKT phosphorylation was determined in the triplet vs doublet treatment, together with decreases in phosphorylation levels of two AKT direct effectors, p-GSK3α/β(Ser$^{21/9}$) and p-p70S6K(Thr^{389}) (Fig. 5D) In addition, phosphorylation of p90RSK (Ser^{380}), a direct downstream substrate of ERK1/2, was also diminished ($q < 0.05$). Investigating two other EFGR/RAS regulated MAPK pathways, decreased phosphorylation of p38 MAPK(Thr^{180}/Tyr^{182}) but not JNK(Thr^{183}/Tyr^{185}) was detected (Figs. 4 and 5D). These data indicate a possible alternative pathway of suppression of survival signals

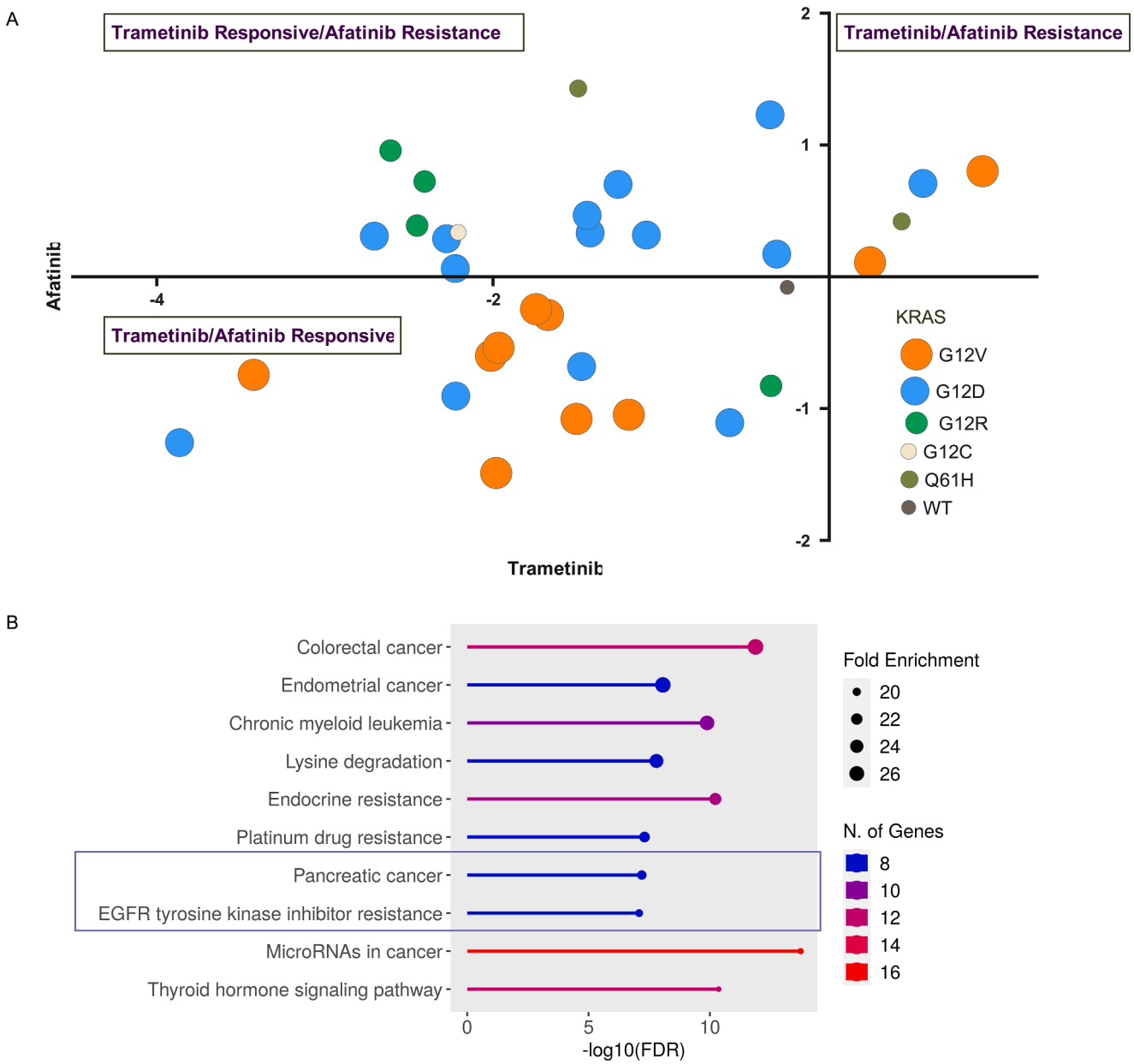


Fig. 3. In silico analysis of molecular basis of PDAC cell lines in response to trametinib and afatinib. (A) DepMap data of cell lines with KRAS mutation in response to trametinib and afatinib. Both x- and y-axis are log2 fold changes. Each dot represents one cell lines and KRAS mutation statues are colored as illustrated; (B) KEGG enriched pathways analysis. Genes with mutations in 14 PDAC cell lines that are sensitive to both trametinib and afatinib were subjected to ShinyGO 0.80 [ShinyGO 0.80 (sdstate.edu)]. FDR cutoff was set at <0.0001 with minimum 10 genes per pathway. Lollipop dots indicate fold enrichment.

attributed only to afatinib administration. As a result, BCL-xL was more effectively downregulated with the triplet treatment as shown in Fig. 5A ($q < 0.05$).

Discussion

Combinations of targeted agents are more likely to be synergistic than combinations involving chemotherapy or other targeted therapy such as EMK and BRAF inhibition [28,29]. Yet, a recent clinical study has shown PDAC patients did not respond to the combined BCL-xL and MEK inhibition strategy. As proof of concept, in this study we demonstrate the combination response of a triplet treatment regimen that simultaneously targeted EGFR/MEK/BCL-xL. This effective drug combination was demonstrated in pancreatic cancer patient-derived cell cultures as well as in an *in vivo* model. It is important to note that the designed triplet treatment regimen was also well-tolerated *in vivo*. Our study indicates that this combination regimen could be potentially effective for certain patients with KRAS mutant tumors even beyond pancreatic cancer, especially where the EGFR pathway is aberrantly activated by a variety of potential mechanisms [30].

Our study also expands upon investigations exploring the survival signaling pathways impacted by such treatments. Our data reveals potential new mechanisms of signaling with MEK and/or EGFR inhibition in pancreatic cancer cells. In line with the experimental observation of restricted tumor growth in mice in both doublet and triplet treatment groups, we observed the downregulated BCL-xL and upregulated BAD expression levels in treated xenograft tumors may be attributed to the double effects of suppressed ERK and AKT signaling. One of the novel findings is the STAT3 phosphorylation at Ser^{727} that was decreased in MEK inhibited tumors compared to controls. p-STAT3(Ser^{727}) is mainly triggered by ERK1/2, p38 MAPK, JNK, and H-7-kinase [31]. It is reported that STA3 and AKT signaling cooperate in the prostate cancer

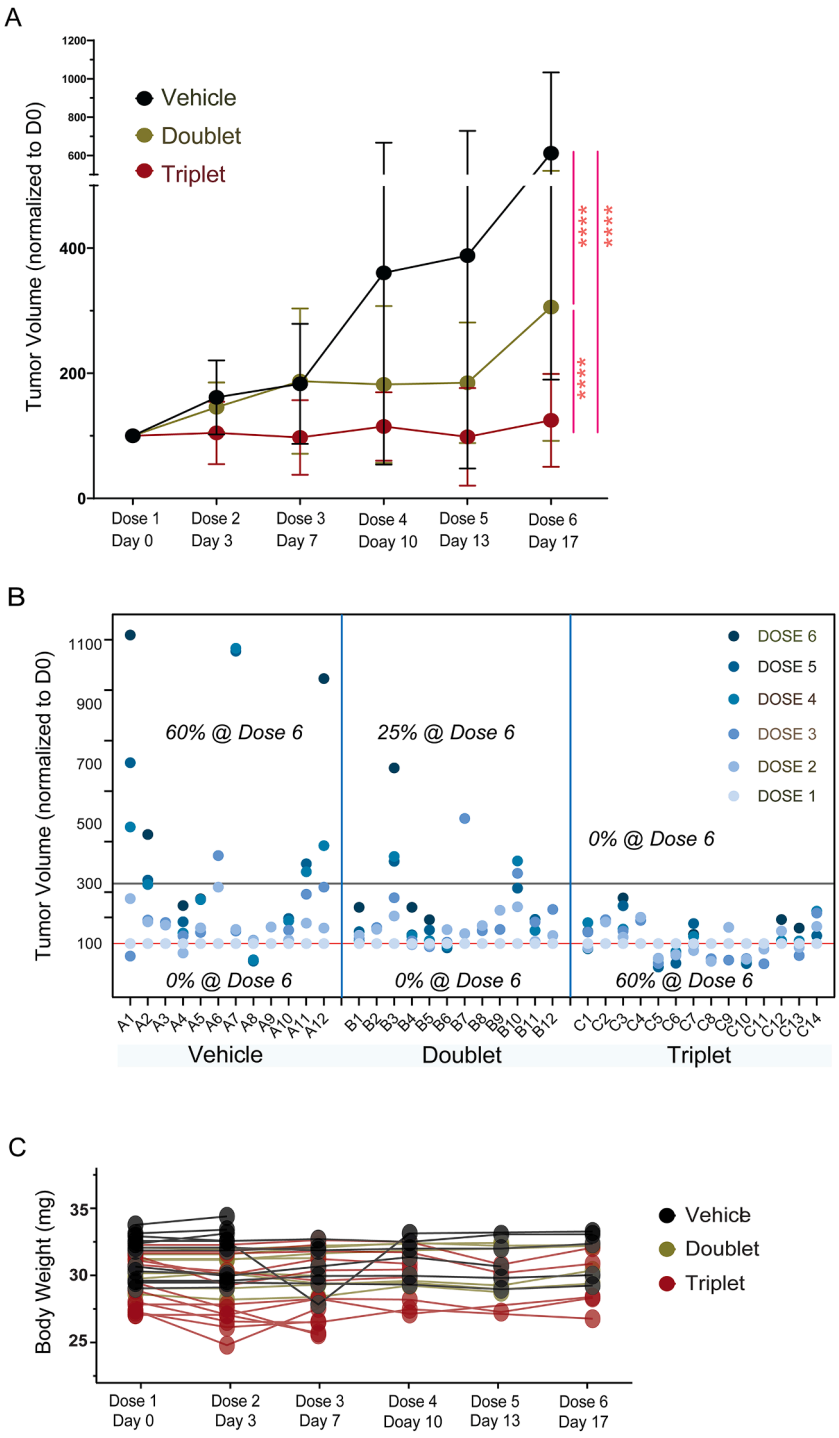


Fig. 4. Impact of the triplet regimen on tumor growth *in vivo* as compared to controls. Mice bearing PDAC-lm1 subcutaneous xenograft tumors were randomly divided into three groups and treated with vehicle (n = 12), doublet of trametinib and DT2216 (n = 12), and triplet of trametinib, DT2216, and afatinib (n = 14). Tumor volumes were normalized to their volume on dose 1 (day 0) for comparisons. Tumor growth kinetics were plotted as indicated with mean ±/ SEM (A) and individually (B), and mice body weight (mg) was plotted in (C) against treatment periods. **** $q < 0.0001$.

development and progression [32]. STAT3 activity in pancreatic cancer is well-established [25,31,33] and dual inhibition of STAT3 phosphorylation at Tyr^{705} and Ser^{727} has recently been suggested for optional pancreatic cancer treatment [34]. Decreased levels of p-STAT3(Ser^{727}) inhibits proliferation and migration of pancreatic cancer cells ([35], in line with our observation of significantly decreased p-STAT3(Ser^{727}) occurring even in doublet treatment without the help of afatinib. These data provide insight for trametinib effects upon noncanonical STAT3 pathway activation in reducing pancreatic cancer cell proliferation.

Another intriguing finding is the unexpected IRS1 phosphorylation decrease in response to MEK inhibition. Ser^{636} is located in a MAPK-consensus phosphorylation site [36]. IRS1 is phosphorylated at residue Ser^{636} in pancreatic cancer cells and suggesting a role in regulating proliferation, invasion and metastasis of pancreatic cancer through MAPK and PI3K pathways [26]. In our study, decreased $Ser^{636/639}$ phosphorylation of IRS1 was found in treated tumors, in line with previous report of MAPK inhibition with PD98059 dramatically reducing the phosphorylation of IRS1 on Ser^{636} in skeletal muscle cells from

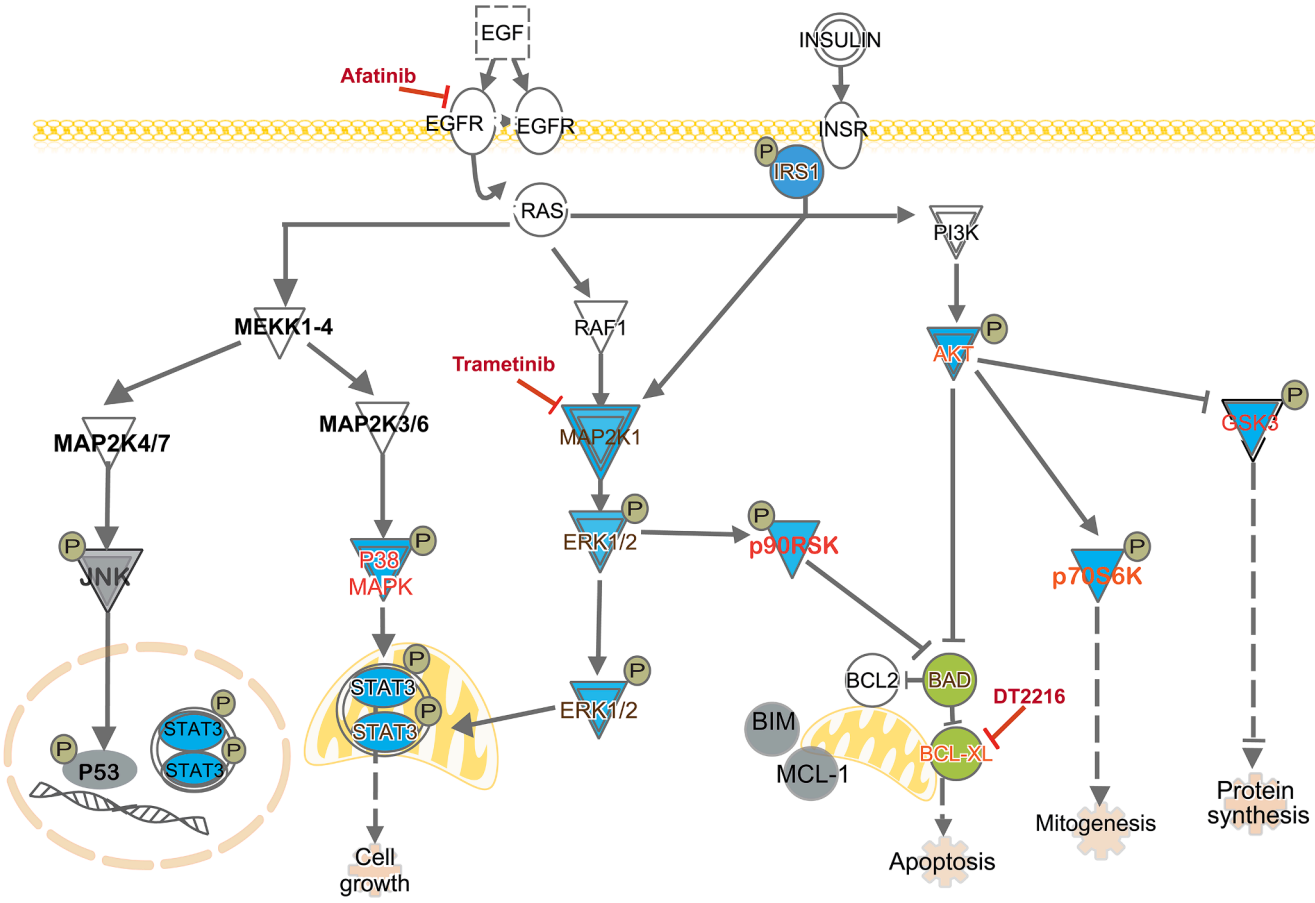


Fig. 5. Key signaling molecules in the targeted pathways. Cascades in EGFR/RAS regulated pathways are arranged and connected based upon IPA canonical/established signaling networks, including EGF, Insulin Receptor, STAT3 Pathway, PI3K/AKT Signaling, and Pancreatic adenocarcinoma Signaling. Molecules tested in the immune-plexes are colored nodes and uncolored nodes represent untested molecules. Phosphorylation of tested molecules are illustrated with a "p" in a circle.

patients with type 2 diabetes [36].

Lastly, further EGFR blockade in triplet treatment enhanced the anti-apoptotic signals through various survival pathways cascades via dephosphorylation/inactivation that were additive compared to doublet treatment, i.e., p-p70S6K, p-GSK3α/β, p-p90RSK, and p-P38 MAPK. We revealed novel pathways solely related to EGFR inhibition in pancreatic cancer cells, firstly through simultaneously enhanced inhibition of two of the three major MAPK cascades of ERK and p38 but not JNK. Afatinib alone can inhibit p38 MAPK phosphorylation in lung cancer cells [37]. p90RSK as a direct substrate of ERK1/2 has not been previously reported targeted by EGFR inhibitors. We also discovered an added supportive effort of inhibition of p38 MAPK by afatinib. Secondly and equally important, triplet treatment enhanced inhibition of the PI3K/AKT pathway. Both RAS/MEK/ERK and RAS/PI3K/AKT are the two fundamental and dysregulated EGFR downstream signaling pathways contributing to pancreatic cancer [38]. Recent studies also show promising results and invoking treatment strategies associating PI3K/AKT inhibitors with MAPK inhibitors [39].

BCL-xL as a well-validated cancer target, its use as cancer therapy however is limited due to its on-target and dose-limiting normal tissue toxicity of its inhibitors (e.g., navitoclax) [40]. We initially included a triplet treatment group using the combination of tramatinibi, afatinib and navitoclax. Alas, we encountered significant toxicity problem in this triplet combination from the 1st dose that led to an early termination (in a group of 5 mice, none surpassed the 3rd dose). On the contrary and as one of the strengths in this study, we incorporate the BCL-xL PROTAC, DT2216, a safer and more potent senolytic agents than Bcl-xl inhibitors as demonstrated in our previous studies [41,42].

Body weight loss as toxicity was not observed in our study during treatment. The safety of double inhibition of MEK and EGFR was also observed by others when an anti-EGFR antibody is used in combination with trametinib in a murine colorectal cancer model [43]. However, we are aware that this is one of the limitations of our study to measure body weight alone as a measurement for toxicity. Despite that weight loss measurement as a common and straightforward evaluation for dose-limiting toxicity in *in vivo* therapeutic testing ([44,45], body weight loss is not a sufficient readout of toxicity (safety) in clinical practice, especially in combination therapies. Each single drug has its own dose-limiting toxicity, when combined, amplified risks of toxicity have been observed, and thus it is important to access the safety in depth. For example, the common side effects of both trametinib and afatinib are skin irritation, diarrhea, and nausea. Fortunately, studies have shown that with appropriate treatment, these side effects can be managed, minimizing patient discomfort and reducing the need for therapy interruption or discontinuation in clinical trial [46]. While combinational target regimens show promise in cancer treatments, challenges remain in determining the optimal doses for safety. Another limitation of this study is the dose selections were not fully optimized and treatment duration is relatively short (6 doses over 17 days). During the short-term treatment, we have not observed resistance even in doublet treated group as it was expected - a major shortcoming of short-term treatment. Futher experimentation with lower doses and longer treatment are indicated to assess true clinical applicability and reduce side effects. For example, low doses (<30 % of what is required for full inhibition of cell viability) combination of EGFR+RAF+MEK+ERK inhibitors in lung cancer preclinical model resulted durable responses without associated toxicity [47].

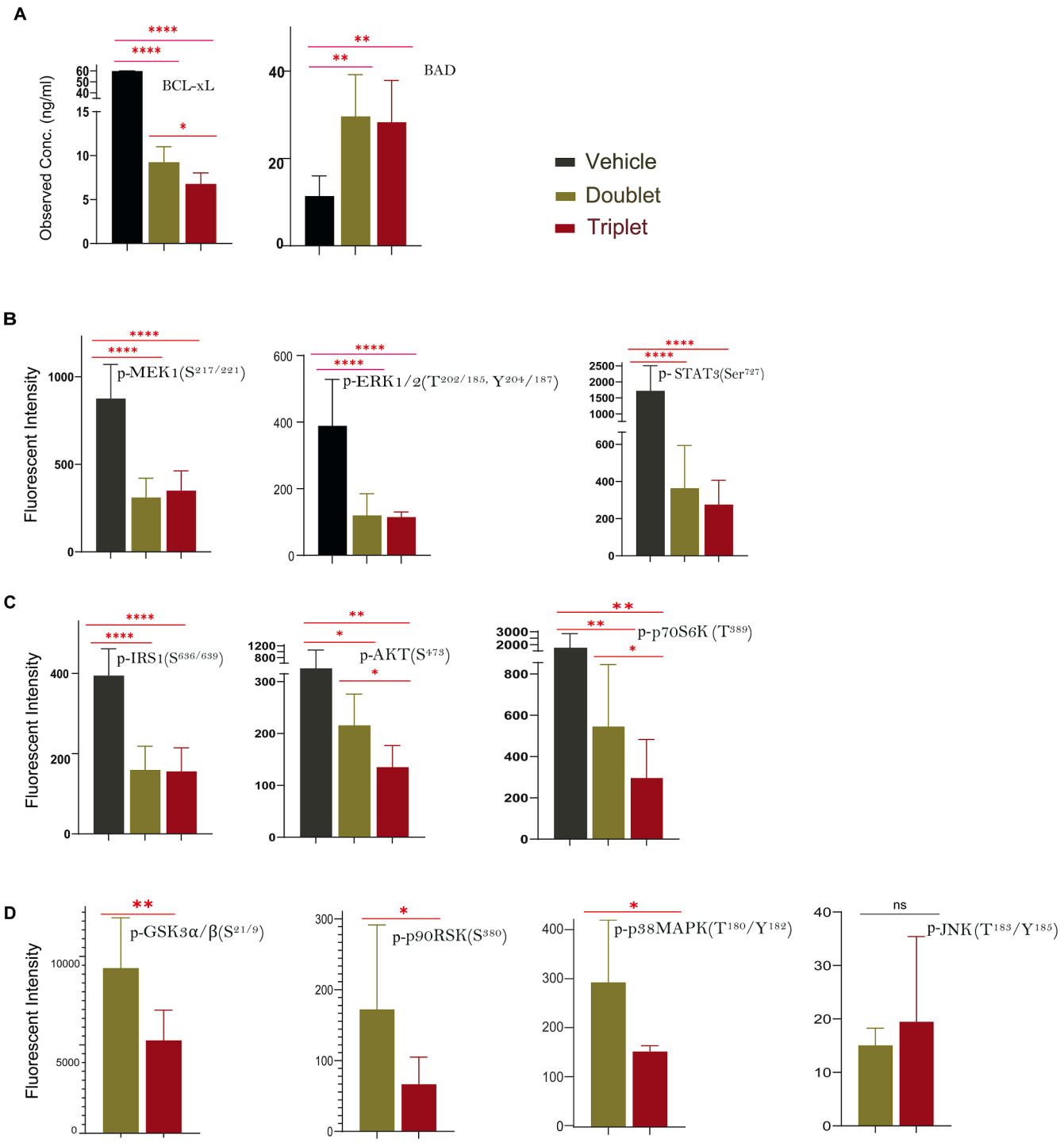


Fig. 6. Doublet and triplet treatments affecting molecules in MAPK and PI3K pathways. PDAC-lm1 subcutaneous xenograft tumors were treated with vehicle, a doublet regimen of trametinib/DT2216, or a triplet regimen of trametinib/DT2216/afatinib. Three mice from each group were sacrificed 4 hours after dose 3 administration and tumors were collected and analyzed using the three immune-plex kits, (A) Expression levels of BCL-xL and BAD by concentration of ng/ml, (B) Phosphorylation status (p-) of p-MEK1, p-ERK1/2, and the substrate of p-STAT3, (C) Phosphorylation status (p-) of p-IRS1, p-AKT, and p-p70S6K, and (D) Phosphorylation status (p-) of p-GSK3α/β, p-p90RSK, p-P38 MAPK, and p-JNK. All phosphorylation sites are as labeled and fluorescent intensity (y-axis) indicate levels of phosphorylation. Bar graphs for mean ±/ SEM. *$q < 0.05$, **$q < 0.01$, ****q < 0.0001, ns = not statistically significant.

Conclusion

We demonstrated improved efficacy of pan-EGFR inhibition with afatinib to further diminish both MAPK and PI3K/AKT survival signaling from the MEK/BCL inhibition via enhanced targeting of AKT downstream cascades of GSK3 and uncovered a second route of p38 MAPK pathway activation. Whether these observations are purely due to the simple effect of additional afatinib or the combined effects of the three compounds needs further investigation. Our data support the theory of the salvage feedback of EGFR signaling during the MEK inhibition treatment and suggest a rescue treatment strategy. Moreover, exploring the complex signaling pathway in combination could offer breakthrough therapeutics for pancreatic cancer.

CRediT authorship contribution statement

Song Han: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gerik W. Tushoski-Alemán:** Writing – review & editing, Methodology, Investigation, Data curation. **Peiyi Zhang:** Writing – review & editing, Methodology, Investigation. **Guangrong Zheng:** Writing – review & editing, Methodology, Investigation. **Daohong Zhou:** Writing – review & editing, Methodology, Investigation, Data curation. **Zhiguang Huo:** Writing – review & editing, Formal analysis. **Jonathan Licht:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Thomas J. George:** Writing – review & editing, Resources, Investigation, Data curation, Conceptualization. **Carmen Allegra:** Writing – review & editing, Supervision, Project administration, Investigation, Data curation, Conceptualization. **Jose G. Trevino:** Writing – review & editing, Supervision, Conceptualization. **Steven J. Hughes:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

P.Z., G.Z., and D.Z. are inventors of the following patent and pending patent application for use of BCL-xL PROTACs as senolytic and antitumor agents: 1. COMPOUNDS THAT INDUCE DEGRADATION OF ANTI-APOPTOTIC BCL-2 FAMILY PROTEINS AND THE USES THEREOF (Patent number: 10807977, Status: Granted, Applicant/Assignee: BioVentures, LLC, Inventors: Guangrong Zheng, Daohong Zhou, Xuan Zhang, Yingying Wang, Jianhui Chang). 2. THERAPEUTIC AGENTS AND METHODS OF TREATMENT (Patent number: 2020218367, Status: Pending, Applicant/Assignee: University of Florida Research Foundation, Inventors: Guangrong Zheng, Daohong Zhou, Pratik Pal, Xingui Liu, Dinesh Thummuri, Wenyi Hu, Peiyi Zhang, Dongwen Lyu, Yaxia Yuan, and Xuan Zhang). G.Z., and D.Z. are co-founders of and have equity in Dialectic Therapeutics, which develops BCL-xL/2 PROTACs to treat cancer. A.C. is the scientific advisory board for Dialectic that is developing DT2216 PROTAC.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2024.101070.

References

[1] RL Siegel, AN Giaquinto, A. Jemal, Cancer statistics, 2024, CA Cancer J. Clin. 74 (1) (2024) 12–49, https://doi.org/10.3322/caac.21820.
[2] T Conroy, F Desseigne, M Ychou, O Bouche, R Guimbaud, Y Becouarn, et al., FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer, N. Engl. J. Med. 364 (19) (2011) 1817–1825, https://doi.org/10.1056/NEJMoa1011923.
[3] SA Long, AM Amparo, G Goodhart, SA Ahmad, AM. Waters, Evaluation of KRAS inhibitor-directed therapies for pancreatic cancer treatment, Front. Oncol. 14 (2024) 1402128, https://doi.org/10.3389/fonc.2024.1402128.
[4] RB Corcoran, KA Cheng, AN Hata, AC Faber, H Ebi, EM Coffee, et al., Synthetic lethal interaction of combined BCL-XL and MEK inhibition promotes tumor regressions in KRAS mutant cancer models, Cancer Cell 23 (1) (2013) 121–128, https://doi.org/10.1016/j.ccr.2012.11.007.
[5] RB Corcoran, KT Do, JE Kim, JM Cleary, AR Parikh, OO Yeku, et al., Phase 1/2 study of combined BCL-xL and MEK inhibition with navitoclax and trametinib in KRAS or NRAS mutant advanced solid tumors, Clin. Cancer Res. (2024), https://doi.org/10.1158/1078-0432.CCR-23-3135.
[6] A Prahallad, C Sun, S Huang, F Di Nicolantonio, R Salazar, D Zecchin, et al., Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR, Nature 483 (7387) (2012) 100–103, https://doi.org/10.1038/nature10868.
[7] ML Janmaat, FA Kruyt, JA Rodriguez, G. Giaccone, Response to epidermal growth factor receptor inhibitors in non-small cell lung cancer cells: limited antiproliferative effects and absence of apoptosis associated with persistent activity of extracellular signal-regulated kinase or Akt kinase pathways, Clin. Cancer Res. 9 (6) (2003) 2316–2326.
[8] JM Balko, BR Jones, VL Coakley, EP. Black, MEK and EGFR inhibition demonstrate synergistic activity in EGFR-dependent NSCLC, Cancer Biol. Ther. 8 (6) (2009) 522–530, https://doi.org/10.4161/cbt.8.6.7690.
[9] A Xiao, M. Fakih, KRAS G12C Inhibitors in the Treatment of Metastatic Colorectal Cancer, Clin. Colorectal. Cancer 23 (3) (2024) 199–206, https://doi.org/10.1016/j.clcc.2024.05.004.
[10] S Kopetz, A Grothey, R Yaeger, E Van Cutsem, J Desai, T Yoshino, et al., Encorafenib, Binimetinib, and Cetuximab in BRAF V600E-Mutated Colorectal Cancer, N. Engl. J. Med. 381 (17) (2019) 1632–1643, https://doi.org/10.1056/NEJMoa1908075.
[11] AH Ko, T Bekaii-Saab, J Van Ziffle, OM Mirzoeva, NM Joseph, A Talasaz, et al., A Multicenter, Open-Label Phase II Clinical Trial of Combined MEK plus EGFR Inhibition for Chemotherapy-Refractory Advanced Pancreatic Adenocarcinoma, Clin. Cancer Res. 22 (1) (2016) 61–68, https://doi.org/10.1158/1078-0432.CCR-15-0979.
[12] MH Wong, A Xue, RC Baxter, N Pavlakis, RC. Smith, Upstream and Downstream Co-inhibition of Mitogen-Activated Protein Kinase and PI3K/Akt/mTOR Pathways in Pancreatic Ductal Adenocarcinoma, Neoplasia 18 (7) (2016) 425–435, https://doi.org/10.1016/j.neo.2016.06.001.
[13] RM Perera, N. Bardeesy, Ready, set, go: the EGF receptor at the pancreatic cancer starting line, Cancer Cell 22 (3) (2012) 281–282, https://doi.org/10.1016/j.ccr.2012.08.019.
[14] K Pham, D Delitto, AE Knowlton, ER Hartlage, R Madhavan, DH Gonzalo, et al., Isolation of Pancreatic Cancer Cells from a Patient-Derived Xenograft Model Allows for Practical Expansion and Preserved Heterogeneity in Culture, Am. J. Pathol. 186 (6) (2016) 1537–1546, https://doi.org/10.1016/j.ajpath.2016.02.009.
[15] Y He, R Koch, V Budamagunta, P Zhang, X Zhang, S Khan, et al., DT2216-a Bcl-xL-specific degrader is highly active against Bcl-xL-dependent T cell lymphomas, J. Hematol. Oncol. 13 (1) (2020) 95, https://doi.org/10.1186/s13045-020-00928-9.
[16] Y Jia, L Han, CL Ramage, Z Wang, CC Weng, L Yang, et al., Co-targeting BCL-XL and BCL-2 by PROTAC 753B eliminates leukemia cells and enhances efficacy of chemotherapy by targeting senescent cells, Haematologica 108 (10) (2023) 2626–2638, https://doi.org/10.3324/haematol.2022.281915.
[17] M Kerstjens, SS Pinhancos, PG Castro, P Schneider, P Wander, R Pieters, et al., Trametinib inhibits RAS-mutant MLL-rearranged acute lymphoblastic leukemia at specific niche sites and reduces ERK phosphorylation in vivo, Haematologica 103 (4) (2018), https://doi.org/10.3324/haematol.2017.174060 e147-e50.
[18] J Witkowski, S Polak, Z Rogulski, D. Pawelec, In Vitro/In Vivo Translation of Synergistic Combination of MDM2 and MEK Inhibitors in Melanoma Using PBPK/PD Modelling: Part I, Int. J. Mol. Sci. 23 (21) (2022), https://doi.org/10.3390/ijms232112984.
[19] X Mao, Z Chen, Y Zhao, Y Yu, S Guan, SE Woodfield, et al., Novel multi-targeted ErbB family inhibitor afatinib blocks EGF-induced signaling and induces apoptosis in neuroblastoma, Oncotarget. 8 (1) (2017) 1555–1568, https://doi.org/10.18632/oncotarget.13657.
[20] L Booth, JL Roberts, M Tavallai, T Webb, D Leon, J Chen, et al., The afatinib resistance of in vivo generated H1975 lung cancer cell clones is mediated by SRC/ERBB3/c-KIT/c-MET compensatory survival signaling, Oncotarget. 7 (15) (2016) 19620–19630, https://doi.org/10.18632/oncotarget.7746.
[21] D Bates, M Mächler, BM Bolker, SC. Walker, Fitting Linear Mixed-Effects Models Using lme4, J. Stat. Softw. 67 (1) (2015) 1–48, https://doi.org/10.18637/jss.v067.i01.
[22] S Gysin, SH Lee, NM Dean, M. McMahon, Pharmacologic inhibition of RAF–>MEK–>ERK signaling elicits pancreatic cancer cell cycle arrest through induced expression of p27Kip1, Cancer Res. 65 (11) (2005) 4870–4880, https://doi.org/10.1158/0008-5472.CAN-04-2848.
[23] AK Srivastava, S Jaganathan, L Stephen, MG Hollingshead, A Layhee, E Damour, et al., Effect of a Smac Mimetic (TL32711, Birinapant) on the Apoptotic Program and

Apoptosis Biomarkers Examined with Validated Multiplex Immunoassays Fit for Clinical Use, Clin. Cancer Res. 22 (4) (2016) 1000–1010, https://doi.org/10.1158/1078-0432.CCR-14-3156.
[24] J Wang, S Weng, Y Zhu, H Chen, J Pan, S Qiu, et al., PKCiota induces differential phosphorylation of STAT3 to modify STAT3-related signaling pathways in pancreatic cancer cells, J. Cell Commun. Signal. 17 (4) (2023) 1417–1433, https://doi.org/10.1007/s12079-023-00780-9.
[25] H Chen, A Bian, W Zhou, Y Miao, J Ye, J Li, et al., Discovery of the Highly Selective and Potent STAT3 Inhibitor for Pancreatic Cancer Treatment, ACS. Cent. Sci. 10 (3) (2024) 579–594, https://doi.org/10.1021/acscentsci.3c01440.
[26] YP Huang, L Zhou, XL Meng, BQ Yu, HT Wang, YF Yang, et al., IRS-1 regulates proliferation, invasion and metastasis of pancreatic cancer cells through MAPK and PI3K signaling pathways, Int J Clin Exp Patho 11 (11) (2018) 5185–5193.
[27] A. Tzatsos, Raptor binds the SAIN (Shc and IRS-1 NPXY binding) domain of insulin receptor substrate-1 (IRS-1) and regulates the phosphorylation of IRS-1 at Ser-636/639 by mTOR, J. Biol. Chem. 284 (34) (2009) 22525–22534, https://doi.org/10.1074/jbc.M109.027748.
[28] P Jaaks, EA Coker, DJ Vis, O Edwards, EF Carpenter, SM Leto, et al., Effective drug combinations in breast, colon and pancreatic cancer cells, Nature 603 (7899) (2022) 166–173, https://doi.org/10.1038/s41586-022-04437-2.
[29] JS Lopez, U. Banerji, Combine and conquer: challenges for targeted therapy combinations in early phase trials, Nat. Rev. Clin. Oncol. 14 (1) (2017) 57–66, https://doi.org/10.1038/nrclinonc.2016.96.
[30] WL Cheng, PH Feng, KY Lee, KY Chen, WL Sun, N Van Hiep, et al., The Role of EREG/EGFR Pathway in Tumor Progression, Int. J. Mol. Sci. 22 (23) (2021), https://doi.org/10.3390/ijms222312828.
[31] A Tesoriere, A Dinarello, F. Argenton, The Roles of Post-Translational Modifications in STAT3 Biological Activities and Functions, Biomedicines. 9 (8) (2021), https://doi.org/10.3390/biomedicines9080956. ARTN 956.
[32] JM Blando, S Carbajal, E Abel, L Beltran, C Conti, S Fischer, et al., Cooperation between Stat3 and Akt Signaling Leads to Prostate Tumor Development in Transgenic Mice, Neoplasia 13 (3) (2011) 254–U93, https://doi.org/10.1593/neo.101388.
[33] V Drubay, N Skrypek, L Cordiez, R Vasseur, C Schulz, N Boukrout, et al., TGF-betaRII Knock-down in Pancreatic Cancer Cells Promotes Tumor Growth and Gemcitabine Resistance. Importance of STAT3 Phosphorylation on S727, Cancers. 10 (8) (2018), https://doi.org/10.3390/cancers10080254.
[34] P He, A Bian, Y Miao, W Jin, H Chen, J He, et al., Discovery of a Highly Potent and Orally Bioavailable STAT3 Dual Phosphorylation Inhibitor for Pancreatic Cancer Treatment, J. Med. Chem. 65 (22) (2022) 15487–15511, https://doi.org/10.1021/acs.jmedchem.2c01554.
[35] M Zhang, X Hu, Y Kang, Z Wang, W Zhou, C Liu, et al., SHP1 Decreases Level of P-STAT3 (Ser727) and Inhibits Proliferation and Migration of Pancreatic Cancer Cells, J. Environ. Pathol. Toxicol. Oncol. 40 (1) (2021) 17–27, https://doi.org/10.1615/JEnvironPatholToxicolOncol.2020035980.
[36] K Bouzakri, M Roques, P Gual, S Espinosa, F Guebre-Egziabher, JP Riou, et al., Reduced activation of phosphatidylinositol-3 kinase and increased serine 636 phosphorylation of insulin receptor substrate-1 in primary culture of skeletal muscle cells from patients with type 2 diabetes, Diabetes 52 (6) (2003) 1319–1325, https://doi.org/10.2337/diabetes.52.6.1319.
[37] Z Liu, QS Fu, Y Wang, L Cui, WQ Zhang, Y Teng, et al., Synergy between vinorelbine and afatinib in the inhibition of non-small cell lung cancer progression by EGFR and p53 signaling pathways, Biomed. PharmacOther 134 (2021), https://doi.org/10.1016/j.biopha.2020.111144. ARTN 111144.
[38] TL Fitzgerald, K Lertpiriyapong, L Cocco, AM Martelli, M Libra, S Candido, et al., Roles of EGFR and KRAS and their downstream signaling pathways in pancreatic cancer and pancreatic cancer stem cells, Adv. Biol. Regul. 59 (2015) 65–81, https://doi.org/10.1016/j.jbior.2015.06.003.
[39] S Stanciu, F Ionita-Radu, C Stefani, D Miricescu, S Stanescu II, M Greabu, et al., Targeting PI3K/AKT/mTOR Signaling Pathway in Pancreatic Cancer: From Molecular to Clinical Aspects, Int. J. Mol. Sci. 23 (17) (2022), https://doi.org/10.3390/ijms231710132.
[40] A Kaefer, J Yang, P Noertersheuser, S Mensing, R Humerickhouse, W Awni, et al., Mechanism-based pharmacokinetic/pharmacodynamic meta-analysis of navitoclax (ABT-263) induced thrombocytopenia, Cancer Chemother Pharmacol. 74 (3) (2014) 593–602, https://doi.org/10.1007/s00280-014-2530-9.
[41] S Khan, X Zhang, D Lv, Q Zhang, Y He, P Zhang, et al., A selective BCL-X(L) PROTAC degrader achieves safe and potent antitumor activity, Nat. Med. 25 (12) (2019) 1938–1947, https://doi.org/10.1038/s41591-019-0668-z.
[42] Y He, X Zhang, J Chang, HN Kim, P Zhang, Y Wang, et al., Using proteolysis-targeting chimera technology to reduce navitoclax platelet toxicity and improve its senolytic activity, Nat. Commun. 11 (1) (2020) 1996, https://doi.org/10.1038/s41467-020-15838-0.
[43] N Hondo, M Kitazawa, M Koyama, S Nakamura, S Tokumaru, S Miyazaki, et al., MEK inhibitor and anti-EGFR antibody overcome sotorasib resistance signals and enhance its antitumor effect in colorectal cancer cells, Cancer Lett. 567 (2023) 216264, https://doi.org/10.1016/j.canlet.2023.216264.
[44] C Demirkiran, M Greenman, S Bellone, B McNamara, TMP Hartwich, D Manavella, et al., Preclinical in vitro and in vivo activity of the RAF/MEK clamp avutometinib in combination with FAK inhibition in uterine carcinosarcomas, Gynecol. Oncol. 187 (2024) 12–20, https://doi.org/10.1016/j.ygyno.2024.04.010.
[45] A Noll, C Myers, MC Biery, M Meechan, S Tahiri, A Rajendran, et al., Therapeutic HDAC inhibition in hypermutant diffuse intrinsic pontine glioma, Neoplasia 43 (2023) 100921, https://doi.org/10.1016/j.neo.2023.100921.
[46] ME Lacouture, M Anadkat, A Jatoi, T Garawin, C Bohac, E. Mitchell, Dermatologic Toxicity Occurring During Anti-EGFR Monoclonal Inhibitor Therapy in Patients With Metastatic Colorectal Cancer: A Systematic Review, Clin. Colorectal. Cancer 17 (2) (2018) 85–96, https://doi.org/10.1016/j.clcc.2017.12.004.
[47] J Boshuizen, DS. Peeper, Rational Cancer Treatment Combinations: An Urgent Clinical Need, Mol. Cell 78 (6) (2020) 1002–1018, https://doi.org/10.1016/j.molcel.2020.05.031.