Dual PI3K/mTOR Inhibition in Colorectal Cancers with APC and PIK3CA Mutations

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Abstract

Therapeutic targeting of the PI3K pathway is an active area of research in multiple cancer types, including breast and endometrial cancers. This pathway is commonly altered in cancer and plays an integral role in numerous vital cellular functions. Mutations in the PIK3CA gene, resulting in a constitutively active form of PI3K, often occur in colorectal cancer, though the population of patients who would benefit from targeting this pathway has yet to be identified. In human colorectal cancers, PIK3CA mutations most commonly occur concomitantly with loss of adenomatous polyposis coli (APC). Here, treatment strategies are investigated that target the PI3K pathway in colon cancers with mutations in APC and PIK3CA. Colorectal cancer spheroids with ApC and Pik3ca mutations were generated and characterized confirming that these cultures represent the tumors from which they were derived. Pan and alpha isomer-specific PI3K inhibitors did not induce a significant treatment response, whereas the dual PI3K/mTOR inhibitors BEZ235 and LY3023414 induced a dramatic treatment response through decreased cellular proliferation and increased differentiation. The significant treatment responses were confirmed in mice with Apc and Pik3ca-mutant colon cancers as measured using endoscopy with a reduction in median lumen occlusion of 53% with BEZ235 and a 24% reduction with LY3023414 compared with an increase of 53% in controls (P < 0.001 and P = 0.03, respectively). This response was also confirmed with 18F-FDG microPET/CT imaging.

Implications: Spheroid models and transgenic mice suggest that dual PI3K/mTOR inhibition is a potential treatment strategy for APC and PIK3CA-mutant colorectal cancers. Thus, further clinical studies of dual PI3K/mTOR inhibitors are warranted in colorectal cancers with these mutations. Mol Cancer Res; 15(3): 317–27. ©2016 AACR.

Introduction

Colorectal cancer is a leading cause of cancer-related death in the United States, accounting for nearly 50,000 deaths annually (1). Although significant progress has been made over the last 20 years, improved treatment options and biological markers predictive of response are clearly needed. Several driver mutations are important in tumor initiation, progression, metastasis, and the response to some therapeutic agents. These mutations include adenomatous polyposis coli (APC; present in 80% of colorectal cancers), TP53 (50%), KRAS (35%–45%), PIK3CA (20%–30%), and BRAF (10%), among others (2). Changing the way in which colorectal cancer is treated to a more precision-based approach will require a better understanding of the utility of the molecular profile in selecting therapies.

Targeting molecular subtypes of colorectal cancer continues to promote growing enthusiasm. Over the past few years, clinical trials have demonstrated benefit for targeting subtypes of colorectal cancer, including BRAF-mutant, HER2-amplified, and microsatellite-unstable colorectal cancers (3–5). The PIK3CA-mutant colorectal cancer population has been an additional cohort of great interest for precision-medicine strategies secondary to the array of agents in clinical development targeting the PI3K pathway (6–8). The PIK3CA gene encodes the p110α catalytic subunit of PI3K and is commonly mutated in multiple human cancers, including breast, colon, and endometrial cancers (9). These mutations, which result in a constitutively active kinase, are found in three hotspots: E542K, E545K, and H1047R with the H1047R mutation being the most common across all cancer types (10). Due to the presence of oncogenic activation of the PI3K pathway in many cancers and its importance in many essential cellular functions, targeting the PI3K pathway has become a key focus for the treatment of cancer.

In a retrospective analysis of patients treated across multiple early phase clinical trials, PIK3CA-mutant cancers were shown to have an increased response rate to inhibitors targeting the PI3K/AKT/mTOR signaling cascade (11). However, resistance to PI3K inhibitors in colorectal cancer patients has been encountered in early-phase clinical trials, leaving researchers to explore the
mechanisms behind this resistance (11–14). Concomitant mutations, including those in APC, have been implicated in the resistance of PIK3CA-mutant colorectal cancers (15).

Further studies into the patient population most likely to benefit from these therapies are needed as the current early-phase clinical studies are pre-selecting for PIK3CA-mutant cancers, but are not taking other concomitant mutations into consideration. Here, we examine the response of PIK3CA-mutant colon cancers to agents targeting the PI3K signaling cascade and examine whether the concurrent loss of APC leads to resistance to these therapies.

Materials and Methods

Mouse husbandry

All animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committee at the University of Wisconsin (Madison, WI) following the guidelines of the American Association for the Assessment and Accreditation of Laboratory Animal Care. Mice were housed in the animal facilities of the University of Wisconsin (Madison, WI) following the guidelines by the Institutional Animal Care and Use Committee at the University of Wisconsin (Madison, WI).

Colorectal cancer cell isolation and spheroid culture

Colon cancer cells were harvested from AP mice by obtaining biopsy samples using a murine endoscope. These tumors were rinsed with sterile PBS and placed on a gelatin chopper block (17). The tumor tissue was digested with collagenase and dispase at 37°C. The cells were pelleted and the supernatant discarded. The resulting cell solution was combined with Matrigel at a 1:1 ratio. The cells were plated by placing 50 μL droplets of the 1:1 cell suspension:Matrigel mixture into the wells of a 24-well culture plate and incubated for 2 minutes at 37°C. The resulting cell solution was combined with Matrigel at a 1:1 ratio. The cells were plated by placing 50 μL droplets of the 1:1 cell suspension:Matrigel mixture into the wells of a 24-well culture plate and incubated for 2 minutes at 37°C. Plates were inverted for an additional 13 minutes at 37°C. Plates were then turned upright, and the Matrigel covered with feeding media consisting of ADF supplemented with murine EGF to a final concentration of 5 ng/mL. Spheroids were passaged at least once prior to therapeutic investigations. Spheroids beyond 9 passages were not utilized. Therapeutic investigations were performed by exchanging feeding media containing the desired concentration of each agent over the spheroids entrapped in the Matrigel. BE235 (LC Laboratories), NVP-BYL719 (ChemieTek), GDC0941 (LC Laboratories), and LY3023414 (Eli Lilly and Co) were each dissolved in dimethyl sulfoxide to make 10 mmol/L stocks. These agents were then diluted 1:1,000 in culture media prior to diluting to the desired concentration of 50 ng/mL. Spheroids were passaged at least once prior to therapeutic investigations. Spheroids beyond 9 passages were not utilized.

Immunoblotting

Spheroid and colon tissue samples were collected and flash frozen. After 24 hours, protein was extracted and immunoblotting performed as previously described (50). The membranes were blocked with 5% nonfat dry milk for 1 hour and then probed with primary antibodies against phospho PI3K (Ser473, #4691, Cell Signaling Technology), pAKT (Ser473, #4060, 1:100, Cell Signaling Technology), CTNNB1 (#8480, 1:200, Cell Signaling Technology), phospho ERK 1/2 (Thr202/Tyr204, #4370, 1:400, Cell Signaling Technology), total AKT (#4691, Cell Signaling Technology), total 4EBP1 (9644, Cell Signaling Technology), and phospho ribosomal protein S6 (RPS6) (Ser235/236, #2217, 1:500, Cell Signaling Technology). Anti-GAPDH (#8884, Cell Signaling Technology) or total eEF2 (#2332, Cell Signaling Technology) in BSA at 1:1,000. Anti-GAPDH (#8884, Cell Signaling Technology), total 4EBP1 (9644, Cell Signaling Technology), and phospho ribosomal protein S6 (RPS6) (Ser235/236, #4858, 1:50, Cell Signaling Technology) were blocked with 5% nonfat dry milk for 1 hour and then immunoblotted as previously described (50). The membranes were blocked with 5% nonfat dry milk for 1 hour and then probed with primary antibodies against phospho PI3K (Ser473, #4691, Cell Signaling Technology), pAKT (Ser473, #4060, Cell Signaling Technology), phospho 4EBP1 (Thr37/46, #2855, Cell Signaling Technology), phospho EEF2 (Thr56, #2855, Cell Signaling Technology), phospho AKT (Ser473, #4060, 1:100, Cell Signaling Technology), phospho AKT (Ser473, #4060, 1:100, Cell Signaling Technology), and total AKT (Thr202/Tyr204, #4370, #4379, 1:400, Cell Signaling Technology). The membranes were blocked with 5% nonfat dry milk for 1 hour and then probed with primary antibodies against phospho PI3K (Ser473, #4691, Cell Signaling Technology), total 4EBP1 (9644, Cell Signaling Technology), and total AKT (Thr202/Tyr204, #4370, #4379, 1:200, Cell Signaling Technology). The membranes were blocked with 5% nonfat dry milk for 1 hour and then probed with primary antibodies against phospho PI3K (Ser473, #4691, Cell Signaling Technology), total 4EBP1 (9644, Cell Signaling Technology), and total AKT (Thr202/Tyr204, #4370, #4379, 1:200, Cell Signaling Technology). The membranes were blocked with 5% nonfat dry milk for 1 hour and then probed with primary antibodies against phospho PI3K (Ser473, #4691, Cell Signaling Technology), total 4EBP1 (9644, Cell Signaling Technology), and total AKT (Thr202/Tyr204, #4370, #4379, 1:200, Cell Signaling Technology).

Histology and immunohistochemistry

Murine colon cancers were excised and fixed in 10% buffered formalin for 24 to 48 hours. Tissues were then stored in 70% ethanol until sectioned. Colorectal cancers were embedded in paraffin, and cut into 5 μm sections. Every tenth section was stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed as previously described (20). The primary antibodies included: Ki67 (#12202, 1:400, Cell Signaling Technology), phospho ERK 1/2 (Thr202/Tyr204, #4370, 1:400, Cell Signaling Technology), CTNNB1 (#8480, 1:200, Cell Signaling Technology), pAKT (Ser473, #4060, 1:100, Cell Signaling Technology), and phospho ribosomal protein S6 (RPS6) (Ser235/236, #4858, 1:50, Cell Signaling Technology).

In vivo pharmacologic treatments

BE235 was formulated in 90% polyethylene glycol and 10% 1-methyl-2-pyrrolidinone. Mice were dosed via oral gavage at 35 mg/kg/day. The study was performed using an in vivo mouse PK model and an 8-week continuous dosing regimen. LY3023414 was formulated in 1% hydroxyethyl cellulose and given to mice at 20 mg/kg via oral gavage every 24 hours for 14 days.

Quantification of tumor response

Dual hybrid 18F-fluorodeoxyglucose (FDG) micro PET/CT imaging and murine endoscopy were utilized to determine treatment response. To interrogate the response of the murine colorectal cancers in AP mice, 18F-FDG microPET/CT imaging was utilized, as previously described (49). Analysis and images were generated using Inveon Research Workplace (Siemens). Baseline and posttreatment PET scans were normalized to injected dose, dose decay, activity, and weight. Murine colonoscopies were performed on each subject at days 1, 7, and 14 of the study. Using these images, percent lumen occlusion values for each tumor at each colonoscopy date with ImageJ, as previously described (18, 19).

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Spheroid H&E staining and immunofluorescence

Spheroids were plated onto plastic 22 mm circular coverslips and grown until mature. Coverslips were then fixed in cold 2% paraformaldehyde for 15 minutes and rinsed in PBS. H&E stains were performed by moving the fixed coverslips through hematoxylin, water, acid alcohol, water, saturated lithium carbonate, water, 70% alcohol, alcoholic eosin, followed with an ethanol dehydration series and xylenes before being mounted onto a blank slide.
In preparation for immunofluorescence assays, spheroids were plated onto glass 22 mm circular coverslips and grown from 24 to 96 hours before appropriate treatments were applied. Twenty-four hours after treatment, coverslips were fixed in cold 2% paraformaldehyde for 15 minutes and rinsed in PBS. Immunofluorescence assays were performed by placing each cover slip into a humidity chamber and blocking with 5% BSA in TBS with 0.05% tween20 for 1 hour at room temperature. Coverslips were then washed in TBS. Primary antibodies were diluted in PBS and incubated overnight at 4 °C. After incubation, coverslips were rinsed in TBS, and the secondary antibody (Alexa Fluor 488 goat anti-rabbit A11008, 1:1000, Thermo Fisher Scientific) was applied for those treated with nonconjugated antibodies. Coverslips were then washed in TBS and mounted to slides using 15 μL of Prolong Gold DAPI mounting media (P36931, Thermo Fisher Scientific) and sealed using clear nail polish.

Results

Murine colon cancers with Apc and Pik3ca mutations can be cultured as spheroids for translational investigations

Improved preclinical modeling will enhance the ability to develop precision-medicine strategies. The capacity to investigate subtypes of colorectal cancer is limited with adherent cell culture techniques. The majority of the currently available human colorectal cancer cell lines are KRAS mutated, despite this mutation being present in only 38% of cancers (21). These cell lines were selected for their ability to be grown in adherent cultures and not based upon their mutation profile. To investigate a subtype of colorectal cancer not represented by the widely available cell lines, we have previously created the AP mouse model (see Materials and Methods) to examine for potential synergy between loss of APC and a constitutively active PI3K in the intestine (16). In the setting of both mutant Pik3ca and Apc, an increased number of tumors developed; these lesions were larger in size and progressed to invasive cancer at a higher rate than in mice with loss of APC alone. More than 80% of the colon tumors in this model at 50 days of age were invasive adenocarcinomas.

To overcome the limitations of classic adherent techniques, multiple investigators are now taking advantage of three-dimensional systems to culture otherwise difficult-to-grow tissues (17, 22). These cultures are representative of the tumors from which they were generated and are a valuable tool for translational research (23). We have now generated murine colon cancer spheroids from AP mice (Fig. 1A–C). The cancer cells in these cultures form hollow spheres (Fig. 1D), develop in-folding as they mature (Fig. 1E), and can even produce crypt-like structures (Fig. 1E–G). These spheroids maintain key characteristics of the tumors from which they were harvested (Fig. 1H). AP spheroids grown on coverslips demonstrate morphology similar to that of AP cancers. In addition, nuclear CTNNB1 (β-catenin), phosphorylation of ribosomal protein S6 (RPS6), and Ki67 staining are similar (Fig. 1H).

Change in diameter can be measured as a marker of cellular proliferation in spheroid cultures (Fig. 1I and Supplementary Fig. S1). Variation in sphere growth was seen depending on the baseline size of the spheres (Supplementary Fig. S1). There was an overall slowing of the relative rate of growth in the largest spheroids. In preparation for translational investigations, a standard change point approach was utilized to determine the baseline spheroid size range most appropriate for further studies (Supplementary Fig. S2). AP spheroids were plated and allowed to mature. A total of 177 spheres were measured at baseline and again at 48 hours using ImageJ. The relative change in diameter was compared across spheroids based on their initial size. A single change point was detected at a baseline size of 283 pixels (~643 μm; Supplementary Fig. S2). Only spheres less than this cutoff were used in further analysis to allow comparisons across treatments.

Apc and Pik3ca-mutant colon cancer spheres are resistant to NVP-BYL719, a PI3K alpha isomer-specific inhibitor, and GDC0941, a pan-PI3K inhibitor

De novo resistance to agents targeting the PI3K pathway has been encountered in clinical trials of patients with PIK3CA-mutant cancers (12–14). To investigate the potential for resistance in Apc and Pik3ca-mutant colorectal cancers, AP spheroids were treated with NVP-BYL719 at 100, 200, or 400 nmol/L concentrations or vehicle control. Treatments were performed by exchanging culture media overlying the Matrigel without disrupting the cells within (Fig. 1C). Despite treatment with these concentrations of NVP-BYL719, spheroid size continued to increase over the 48-hour treatment period (Fig. 2A) with a median increase in size of 47% in those spheres treated with 400 nmol/L NVP-BYL719 compared with a 97% increase in those treated with control (P < 0.001, Wilcoxon rank sum test; Fig. 2B). To determine if pan-PI3K inhibition could overcome resistance to the alpha isomer–specific agents and lead to a decrease in spheroid size, AP spheroids were treated with GDC0941 (Fig. 2A and B). Median increases in spheroid diameter of 85%, 108%, and 68% were observed with 100, 200, and 400 nmol/L concentrations of GDC0941, respectively, compared with a 109% increase with control treatment.

Dual PI3K/mTOR inhibition overcomes resistance in Apc and Pik3ca-mutant spheroids

Inhibition of mTOR results in antiproliferative signaling and a treatment response in Apc-mutant tumors (18, 24, 25). We hypothesized that owing to the antineoplastic effects of mTOR inhibition in this setting, treatment of AP spheroids with a dual PI3K/mTOR inhibitor would result in an improved response rate. As described above, cells were treated with BEZ235, a PI3K, and a mTORC1/mTORC2 inhibitor, at concentrations of 100, 200, or 400 nmol/L or vehicle control over 48 hours. All concentrations investigated dramatically reduced the spheroid growth rate compared with control (Fig. 2A and B). At the 200 and 400 nmol/L concentrations of BEZ235, changes in median spheroid size of 4% and ~17%, respectively, were observed. To confirm that this was a class effect, a second dual PI3K/mTOR inhibitor, LY3023414, was examined. Similar to results seen with BEZ235, a reduction in spheroid growth was identified in response to treatment with LY3023414 (3%–11%, and ~15% at 200, 400, and 600 nmol/L, respectively, compared with 105% for controls; Fig. 2A and B).

BEZ235 and LY3023414 result in inhibition of key regulators of the PI3K signaling pathway

Signaling downstream of mTOR is important for anabolic cellular metabolism promoting growth and proliferation (26).
Figure 1.
Moderately differentiated adenocarcinomas form within the colon of AP mice (A and B) and can be cultured using three-dimensional techniques (C). These cancers can be grown as spheroids that form hollow spheres (D) and can even develop crypt-like structures (E–G). In spheroid culture, AP spheres retain many characteristics of AP cancers, including a similar morphology, nuclear localization of CTNNB1 (β-catenin), phosphorylation of RPS6, and expression of Ki67 (H). Proliferation of these spheres can be monitored over time with serial imaging (I). Size bars, A, 1 cm; D and E, 500 μm; H, 200 μm; I, 500 μm. B is a 5x enlargement of the area outlined in A; F is a 5x enlargement of E; and G is a 2x enlargement of F.
Inhibitors of the PI3K pathway were investigated in AP spheroids. Spheres were allowed to mature and then treated with NVP-BYL719 or GDC0941 for 48 hours. Only a modest reduction in the growth rate was observed with each of these agents (A and B). The dual PI3K/mTOR inhibitors BEZ235 and LY3023414 were also tested over 48 hours and resulted in a significant reduction in the diameter of AP spheroids (A and B). AP spheroids were treated with NVP-BYL719 or BEZ235, and protein was isolated for immunoblotting (C). After 3 hours, both agents reduced phosphorylation of AKT and RPS6. Only BEZ235 inhibited phosphorylation of 4EBP1. After 24 hours, a significantly greater inhibition of phosphorylation of AKT, RPS6, and 4EBP1 was observed with BEZ235 compared with NVP-BYL719. Interestingly, induction of phosphorylation of EEF2 was noted with both agents. LY3023414 was also shown to inhibit phosphorylation of AKT, RPS6, and 4EBP1 in AP spheroids. Size bar for A, 500 μm.
The downstream mediators RPS6 and 4EBP1 are critical for therapeutic response following treatment with agents targeting the PI3K pathway (27, 28). AP spheroids were treated with NVP-BYL719 and BEZ235 (Fig. 2C). At the 3- and 24-hour time points, a modest reduction in phosphorylation of AKT was observed with NVP-BYL719 compared with control. However, persistent levels of phosphorylated RPS6 and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) were identified in those spheres treated with BEZ235 (Fig. 2C). Interestingly, an induction of phosphorylation of eukaryotic elongation factor 2 (EEF2) occurred following treatment with both NVP-BYL719 and BEZ235 (Fig. 2C). In addition, reductions in the phosphorylation of AKT, RPS6, and 4EBP1 were observed following treatment with LY3023414 (Fig. 2C).

**Spheroid cultures can be utilized to determine heterogeneity in the therapeutic response**

Tumor heterogeneity is a considerable concern when developing precision-medicine strategies. The described spheroid technique allows for the treatment of multiple established clonal, or at least oligoclonal, units derived from an individual cancer. Response measurement can be obtained for each sphere before and after treatment. In addition to the overall change in spheroid diameter, individual spheres can be classified based on their response: progressing (25% or greater increase in spheroid diameter), stable (between a 25% increase and a 30% decrease in diameter), or responding (30% or greater decrease in spheroid size). The response rates for the AP spheroids across the four treatments (NVP-BYL719, GDC0941, BEZ235, and LY3023414) are presented in Supplementary Table S1. A significant increase in the percent of spheroids responding to treatment was observed with dual PI3K/mTOR inhibition. At the 400 nmol/L concentration, the response rate was 31.7% with BEZ235 and 20.3% with NVP-BYL719 and GDC0941 (Fig. 3A). The benefit rate (responding and stable spheres) was also significantly improved with BEZ235 and LY3023414 (P < 0.001; B). The rate of progression was also examined in those spheres that were in the progressing category. Those spheres treated with the dual PI3K/mTOR inhibitors that were progressing still grew to a lesser extent (P = 0.003, Wilcoxon rank sum test; C).

**Figure 3.** Spheroid cultures can be utilized to determine the heterogeneity in response. AP spheroids were treated with NVP-BYL719, GDC0941, BEZ235, and LY3023414 for 48 hours. Individual spheres were categorized based on their change in diameter (responding, >30% reduction; stable, <30% reduction to <25% growth; and progression, >25% increase). A significant improvement in the response rate was observed with the dual PI3K/mTOR inhibitors, BEZ235 and LY3023414, compared with NVP-BYL719 and GDC0941 (P = 0.006, χ² test; A). The benefit rate (responding and stable spheres) was also significantly improved with BEZ235 and LY3023414 (P < 0.001; B). The rate of progression was also examined in those spheres that were in the progressing category. Those spheres treated with the dual PI3K/mTOR inhibitors that were progressing still grew to a lesser extent (P = 0.003, Wilcoxon rank sum test; C).

Dual PI3K/mTOR inhibition induced an in vivo treatment response in colon cancers possessing Apc and Pik3ca mutations

To confirm the results of the spheroid studies, AP mice were generated as previously described (16). Colonoscopy was
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performed to identify colon cancers at 40 to 50 days of age. Those mice possessing cancers able to be followed endoscopically were randomized to receive BEZ235 35 mg/kg/day or vehicle control by oral gavage daily for 14 days. A total of 23 tumors were able to be monitored endoscopically (12 treated with BEZ235 and 11 controls). Dual hybrid FDG microPET/CT imaging was performed at baseline immediately prior to treatment. To assess response, imaging was repeated at least 18 hours following the last treatment dose. To detect a change in tumor size over time endoscopically, percent lumen occlusion was measured as previously described (25, 29). The characteristics of the mice treated are described in Supplementary Table S2. In mice receiving control, a median increase in lumen occlusion of 53% was detected (Fig. 4A and B). This is compared with a median decrease in lumen occlusion of 53% in those mice treated with BEZ235 (P < 0.001, Wilcoxon rank sum test). A similar response was seen on microPET/CT imaging (Fig. 4C) following BEZ235 treatment with a median decrease in tumor volume of 23% and a median decrease in tumor SUV of 24% (Fig. 4D). At necropsy, no difference in the total tumor number of lesions was noted within the colon and distal segment of the small intestine (Supplementary Table S2). A trend toward a decrease in tumor diameter was observed (Supplementary Table S2), though the variation in tumor size limits the detection of a response by this method. Notable differences in tumor thickness were observed. This was most profound in the small intestinal tumors (Supplementary Fig. S3).

In vivo reduction in phosphorylation of AKT and RPS6 in the colon and small intestinal cancers was confirmed 6 hours after a single dose of BEZ235 (Fig. 4E). After 14 days of administration, persistent inhibition of phosphorylation of RPS6 was seen (Fig. 4E). Interestingly, phosphorylation of AKT in the colon cancers was no longer inhibited at this time point (Fig. 4E). In addition, 24 hours following withdrawal of BEZ235, an increase in activation of the PI3K pathway is observed (Supplementary Fig. S4).

Similarly, LY3023414 also demonstrated considerable antitumor activity in AP mice. LY3023414 was administered at a dose of 20 mg/kg/day by oral gavage over 14 days. A total of 7 tumors were able to be followed endoscopically. Pre- and posttreatment colonoscopies demonstrated a median reduction in lumen occlusion of 24% compared with controls with a median increase of 53% (Fig. 4A and B). FDG microPET/CT imaging demonstrated a

**Figure 4.**
BEZ235 and LY3023414 result in colon tumor response in vivo. AP mice were treated with BEZ235 (35 mg/kg/day), LY3023414 (20 mg/kg/day), or vehicle control daily by oral gavage. Endoscopy was performed prior to and after 14 days of treatment (A). A significant reduction in tumor size was observed in those mice treated with BEZ235 (P < 0.001) and LY3023414 (P = 0.03; B). FDG microPET/CT was also performed before and after treatment with BEZ235 and LY3023414 (C). Reductions in the total tumor volume and avidity (SUV) were observed with BEZ235 (P = 0.016 for volume and P = 0.2 for SUV, Wilcoxon rank sum test; D) and LY3023414 (P = 0.01 for volume and P = 0.02 for SUV; D). Tumors were collected from AP mice and flash frozen prior to protein isolation for immunoblotting. After 6 hours of treatment with BEZ235, a reduction in the phosphorylation of AKT and RPS6 was observed in both colon and the distal small intestine (S4; E). After 14 days of treatment, phosphorylation of AKT was similar to controls in the colon tissue, but still decreased in the S4 tumors (E). RPS6 (S6) phosphorylation was decreased in both the colon and S4 cancers after 14 days of treatment with BEZ235 (E). A reduction in the phosphorylation of AKT and RPS6 after 14 days of treatment with LY3023414 was observed in AP colon cancers (E).
mice develop pale colons secondary to blood loss from their intestinal cancers. The paleness of the colon can be observed on endoscopy as a surrogate marker of anemia. We developed a method to quantify the degree of paleness using the murine endoscope. Individual images of the colon are split into red and green channels (Fig. 5A). With the threshold set at 0 to 254 to exclude white pixels, measurements of the mean gray values (mgv) are obtained for the red and green channels using ImageJ. The EAS is calculated as the ratio of the red mgv to the green mgv. A worsening in the EAS, correlating to an increase in the paleness of the colon, was seen in the majority of AP mice treated with control (Fig. 5B). An improvement in EAS was observed in the majority of mice treated with BEZ235 and LY3023414, indicating a decrease in the paleness of the colon (compared with controls, \( P < 0.006 \) for BEZ235 and 0.005 for LY3023414; Fig. 5B).

Dual PI3K/mTOR inhibition decreases cell proliferation and increases differentiation

To examine the potential mechanisms of sensitivity of Apc and Pik3ca-mutant colon cancer cells to dual PI3K/mTOR inhibition, AP spheroids were grown on coverslips and treated with NVP-BYL719, BEZ235, LY3023414, or control. Decreased proliferation, as determined by Ki67 immunofluorescence, was observed with the dual PI3K/mTOR inhibitors compared with control (\( P < 0.05 \), Wilcoxon rank sum test; Fig. 6A and B). A decrease in cleaved caspase-3 staining was also observed in cell populations treated with BEZ235 and LY3023414 compared with control (\( P = 0.04 \), Fisher exact test; Fig. 6B). No significant differences were observed in staining for DAPI or keratin 20 (Fig. 6C).
ERK1/2 phosphorylation persists in colorectal cancers. Following 14 days of treatment with BEZ235, LY3023414, or control, AP colon tumors were excised and fixed in formalin. Immunohistochemical staining was performed to access the induction of phosphorylation of ERK1/2 with AP tumors. Minimal phosphorylation of ERK1/2 was seen within cancers from the different treatment groups. Interestingly, peritumoral phosphorylation of ERK1/2 was seen in the BEZ235- or LY3023414-treated mice. Size bar, 1 mm. Right panels are 4X magnifications of the indicated regions.

Supplementary Fig. S5). No significant decrease in proliferation was observed with NVP-BYL719. No significant changes in cleaved caspase 3 were identified (Fig. 6B). Keratin 20 immunofluorescence was performed as a marker of differentiation. Increased keratin 20 expression in the BEZ235- and LY3023414-treated spheres was observed with minimal keratin 20 in the control and NVP-BYL719–treated spheres.

Phosphorylation of ERK1/2 is not induced in AP cancers following dual PI3K/mTOR inhibition, though peritumoral ERK1/2 phosphorylation persists.

Activation of ERK1/2 has been identified as a mechanism of resistance to PI3K and mTOR inhibitors (30, 31). Following treatment of AP mice with colon tumors with BEZ235, LY3023414, or control for 14 days, as above, tissues were harvested and fixed in formalin. Tumor sections were stained for phosphorylation of ERK1/2. In mice treated with control, minimal phosphorylation of ERK1/2 was observed within the cancers cells (Fig. 7). The hyperplastic epithelium immediately surrounding the tumors demonstrated significant phosphorylation of ERK1/2. In AP colon tumors treated with either BEZ235 or LY3023414, no change in phosphorylation of ERK1/2 was noted within the cancer cells. Interestingly, the peritumoral phosphorylation of ERK1/2 was also maintained in the setting of dual PI3K/mTOR inhibition.

Discussion

The PI3K/AKT/mTOR pathway is the most commonly altered signaling pathway in cancer (32). Targeting cancers with activating mutations in this pathway is an area of interest; however, identification of those cancer patients likely to benefit from PI3K pathway inhibitors has remained elusive. In an attempt to limit toxicities, especially hyperglycemia, multiple pharmaceutical companies have focused on targeting this signaling axis proximally with pan or isomer-specific PI3K inhibitors, such as NVP-BYL719. Unfortunately, limited clinical activity has been discovered with these agents in patients with metastatic colorectal cancer (15, 33).

Clinical activity has been observed in clinical trials examining inhibitors along the PI3K pathway in cancers with activation of the PI3K pathway (11). This pathway is commonly activated secondary to mutations in PIK3CA, including approximately 20% of metastatic colorectal cancers (2). In PIK3CA-mutant colorectal cancers, other driver mutations are often present simultaneously. RAS/RAF signaling has already been implicated as a potential mechanism of resistance in the setting of PI3K pathway inhibition, and mutations in KRAS, NRAS, and BRAF are present in approximately 50% of colorectal cancers with PIK3CA mutations (34, 35). In addition, alterations in WNT signaling are present in the vast majority of PIK3CA-mutant colorectal cancers (36). Targeting the PI3K pathway in PIK3CA-mutant cancers with loss of APC is the focus of the above investigations.

Loss of APC could result in resistance through activation of the downstream PI3K pathway at mTOR. Phosphorylation of RPS6 in ApcMin/+ adenomas and response to mTOR inhibition has been described in the setting of loss of APC (29). We hypothesized that inhibition of PI3K alone would be insufficient to induce a response in these cancers secondary to downstream phosphorylation of RPS6 and 4EBP1 as a consequence of WNT pathway activation. In our studies here, alpha isomer–specific and pan-PI3K inhibition alone was insufficient to induce a treatment response in Apc and Pik3ca-mutant colon cancer spheroids. This lack of response was correlated with persistent phosphorylation of RPS6 and 4EBP1.

mTOR signaling is able to promote protein synthesis on multiple levels (37). Assembly of the eukaryotic initiation factor 4F (eIF4F) complex promotes the recruitment of the ribosome and subsequent translation initiation. 4EBP1 represses translation at the initiation step by preventing assembly of the eIF4F complex.
Once activated, mTORC1 phosphorylates 4EBP1 priming it for phosphorylation at additional sites. These phosphorylation events lead to 4EBP1 release from eIF4E and subsequent initiation of translation. In addition to the initiation step, the S6 kinases regulate translation elongation by phosphorylating and inhibiting the EEF2 kinase (EEF2K), an important negative regulator of EEF2. mTOR signaling results in phosphorylation of EEF2K inhibiting its phosphorylation of EEF2, thus promoting translation elongation.

Dual PI3K/mTOR inhibition of AP spheroids results in decreased proliferation and differentiation likely mediated at least in part due to the inhibition of protein synthesis. Key proteins, such as CMYC and CCND1, are necessary for the proliferative properties of WNT and PI3K signaling (38, 39). Previously mTORC1 inhibition has been shown to be sufficient to prevent adeno-na formation in the setting of loss of APC with growth arrest and decreased protein synthesis seen in ramapycin-treated APC-deficient enterocytes (40). This was thought to be mainly the result of rapamycin treatment on EEF2K signaling and inhibition of protein elongation. In our studies here, in the setting of invasive adenocarcinomas of the colon with loss of APC and a constitutively active PI3K, a reduction in phosphorylation of 4EBP1, and thus prevention of translation initiation, appears to be a better predictor of treatment response than the phosphorylation status of EEF2. This is supported by other studies indicating the importance of the regulation of translation initiation (38, 41, 42). Here, we demonstrated the dual PI3K/mTOR inhibition results in suppression of phosphorylation of RPS6 and 4EBP1 which is correlated with a decrease in proliferation and increase in cellular differentiation. Further studies will need to be completed to examine whether mTORC1/2 inhibition alone is sufficient for this treatment response.

Dual PI3K/mTOR inhibitors demonstrate tolerability and activity in early-phase clinical trials. PF-05212384 (intravenous), XL765 (oral), BEZ235 (oral sachet), and LY3023414 (oral) are all dual PI3K/mTOR inhibitors that have completed phase I clinical trials (43–46). Similar toxicities were seen across these agents, including asthenia, rash, hyperglycemia, and nausea. The PF-05212384 phase 1 clinical trial demonstrated three partial responses in patients with PTEN null non–small cell lung cancer, PTEN-null granulosa cell cancer of the ovary and endometrial cancer (43). Eight patients had stable disease greater than 6 months, including 2 patients with colorectal cancer. In response to XL765, 48% of patients developed stable disease, including 12 patients with stable disease for ≥12 weeks (43). In a phase I clinical trial of single-agent BEZ235, 10 patients had stable disease for ≥16 weeks, including 4 patients with colorectal cancer (45). Partial responses to LY3023414 were observed in a patient with PIK3R1-mutant endometrial cancer and a patient with an unselected colorectal cancer (46). Further clinical trials exploring the antitumor activity of dual PI3K/mTOR inhibitors in patients with colorectal cancers are warranted. Patient selection is important for maximizing the benefit. Our studies indicate that APC and PIK3CA-mutant colorectal cancers are likely to respond to these agents. Concomitant mutations should be assessed, including KRAS, NRAS, and BRAF, as these additional mutations might alter the effectiveness of this treatment strategy. The population of APC and PIK3CA-mutant colorectal cancers is 15% to 20% of all colorectal cancers, and half of this population is free of additional KRAS, NRAS, and BRAF mutations (34, 35). Molecular profiling should be completed on fresh biopsies or at least done in conjunction with cell-free DNA analyses. This is important as mutational changes have been observed in response to both cytotoxic chemotherapy and anti-epidermal growth factor receptor therapies (47). In summary, dual PI3K/mTOR inhibition is a promising treatment strategy for PIK3CA-mutant colorectal cancer for further clinical development and for building upon in combination regimens.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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