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Enhancing the evaluation of PI3K inhibitors through 3D melanoma models

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Abstract

Targeted therapies for mutant *BRAF* metastatic melanoma are effective but not curative due to acquisition of resistance. PI3K signaling is a common mediator of therapy resistance in melanoma; thus, the need for effective PI3K inhibitors is critical. However, testing PI3K inhibitors in adherent cultures is not always reflective of their potential *in vivo*. To emphasize this, we compared PI3K inhibitors of different specificity in two- and three-dimensional (2D, 3D) melanoma models and show that drug response predictions gain from evaluation using 3D models. Our results in 3D demonstrate the anti-invasive potential of PI3K inhibitors and that drugs such as PX-866 have beneficial activity in physiological models alone and when combined with BRAF inhibition. These assays finally help highlight pathway effectors that could be involved in drug response in different environments (e.g. p4E-BP1). Our findings show the advantages of 3D melanoma models to enhance our understanding of PI3K inhibitors.

Keywords

melanoma; PI3K inhibition; mutant BRAF; resistance; 3D models; PX-866

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INTRODUCTION

Melanoma is the most lethal skin cancer and the treatment of advanced disease remains a challenge despite encouraging results with targeted and immune therapies (Shah and Dronca, 2014). Therapeutic hurdles come in multiple forms owing to the heterogeneity and plasticity of the disease; however, common signaling nodes play important roles in melanoma biology and tumor growth (Vultur et al., 2014). One such example is the MAPK pathway; its inhibition has led to positive results in patients, resulting in the approval of vemurafenib, dabrafenib and trametinib in *BRAF*-mutant metastatic melanomas (Menzies and Long, 2014; Flaherty et al., 2010). Unfortunately, drug resistance occurs even in initially responding patients (Poulikakos et al., 2010; Villanueva, 2010; Villanueva et al., 2013; Nazarian et al., 2010). One important mechanism for MAPK inhibitor resistance involves the activation of the PI3K pathway by signaling rewiring or through genetic alterations (Villanueva, 2010; Villanueva et al., 2013; Shi et al., 2014; Shull et al., 2012).

Mutations in phosphatidylinositol 3-kinase genes do not occur frequently in melanoma prior to therapy (Omholt et al., 2006); however, the activity of the PI3K-related pathway is often elevated, commonly through the mutation, deletion, or methylation of the tumor suppressor PTEN (Hollander et al., 2011; Shull et al., 2012). Strengthening the role of PI3K signaling in melanoma pathobiology, GEM models were established in which expression of BRAFV600E cooperates with silencing of PTEN or PI3KCA mutations for melanomagenesis; these models display distinct responses to PI3K inhibitors depending on genetic background and compound specificity (Marsh Durban et al., 2013; Deuker et al., 2015). These models were also used to demonstrate the importance of PI3K inhibitors in preventing MEK1/2 drug resistance in mutant *BRAF* melanoma (Deuker et al., 2015). Recently, mouse models also established the role of AKT1 in melanoma metastasis, notably to the brain (Cho et al., 2015). Finally, the importance of PI3K signaling is demonstrated by Brooks and colleagues who showed that G2-phase decatenation checkpoint-defective melanomas (reported in ~67% of melanoma cell lines) depend on PI3K for survival (Brooks et al., 2014). Thus, PI3K inhibition has the potential to inhibit melanoma cell survival, metastasis, and drug resistance.

Six classes of PI3K pathway inhibitors have so far been developed, these are: pan-class I PI3K inhibitors, PI3K isoform-specific inhibitors, dual PI3K/mTOR inhibitors, AKT inhibitors, active-site mTOR inhibitors, and rapamycin analogues (Fruman and Rommel, 2014). Given the complexity of PI3K signaling and downstream effects of this pathway (reviewed in (Davies, 2012)), careful preclinical evaluations must be conducted to understand the strengths and limitations of PI3K targeting agents. A strength to consider is that clinical benefits have been reported for PI3K drugs (Sarker et al., 2015). Unfortunately, it is still unclear which compounds are the most effective (for example in different melanoma genetic backgrounds or in combination therapies), and which can show clinical efficacy with limited toxicity.

Here, we sought to understand the role of the tumor microenvironment in modulating response to PI3K inhibitors in melanoma using advanced preclinical models that increasingly recapitulate tumor architecture and heterogeneity. Our models include adherent

cell cultures (treatment naïve and BRAF inhibitor-resistant), three-dimensional (3D) spheroids grown in collagen, human skin reconstructs, and xenografts. We show that PI3K inhibitor evaluation using 3D models is advantageous given the effects of these drugs on invasion and that certain inhibitors can be more effective in 3D than 2D culture. Our data suggest the need to evaluate PI3K inhibitors in more tissue-like contexts to better understand their therapeutic potential.

RESULTS

Adherent melanoma cell line response to PI3K inhibitors is not predicted by AKT inhibition alone

To identify melanoma cell lines that could be most sensitive to PI3K inhibition, a panel of genetically distinct melanoma cell lines with known BRAF, NRAS, PTEN, and AKT mutation status (Table 1) was exposed to 10 μ M of BAY80-6946 (PI3K α/β inhibitor), PX-866 (pan-PI3K inhibitor), AZD6482 (PI3K β inhibitor) or GDC-0941 (PI3K α/δ inhibitor), and total cell count was assessed following a 72h treatment. Drug response in adherent 2D melanoma cultures varied. Changes were most pronounced in BAY80-6946 treated cells followed by GDC-0941 in most cell lines tested, while PX-866 and AZD6482 treatment caused less than a 50% decrease in cell numbers (Figure 1A). To understand what PI3K/MAPK-associated signaling changes occur with response, we then examined the levels of phosphorylated AKT (pAKTSer473, pAKTThr308), ribosomal protein S6 (pS6Ser235/236), 4E-binding protein 1 (p4E-BP1Thr37/46), and ERK1/2 (pERKThr202/Tyr204). Signaling changes were compared to LY294002, a pan-PI3K and mTOR inhibitor previously shown to cause cell cycle arrest in melanoma cell lines (Marone et al., 2009; Smalley et al., 2006). LY294002 was also used as a control since it was proposed that mTOR inhibitors lack genotype specificity (Will et al., 2014). Upon a 72h treatment, all PI3K inhibitors were able to reduce pAKT levels; however, they did not affect pS6 or p4E-BP1 equally (Figure 1B). BAY80-6946 was most effective in decreasing pS6 and p4E-BP1 levels in both cell lines investigated and this is consistent with a previous study showing that BAY80-6946 can inhibit mTOR activity at nanomolar concentrations (Will et al., 2014). In contrast, the other PI3K inhibitors were able to reduce p4E-BP1 levels but not completely, and pS6 reduction was variable across cell lines. pERK levels remained unchanged. cPARP levels also did not change with any PI3K inhibitor tested indicating a largely cytostatic effect occurring following treatment. Interestingly, we observed that pAKT levels were not fully decreased in all cases as seen for BAY80-6946 treated WM983B cells and LY94002 showing residual signal. These observations suggest that understanding the effects of PI3K inhibitors on adherent cells could benefit from assessing signaling effectors beyond AKT.

Enhanced understanding of PI3K inhibitor effects in collagen-embedded melanoma spheroids

Multicellular 3D tumor spheroids provide tools to model cell heterogeneity and microenvironmental elements of *in vivo* growth (Thoma et al., 2014). It has also been suggested that evaluating cells in 3D reveals more realistic gene expression profiles and drug responses (Hongisto et al., 2013). Given that adherent melanoma cultures do not accurately replicate tumor architecture or heterogeneity, we sought to examine if PI3K inhibitors could

affect melanoma cells equally in 3D models. Melanoma cells grown as 3D spheroids embedded in collagen were treated for 72h with PI3K inhibitors of different specificity including AZD6482, PX-866, BAY80-6946, GDC0941; these were assessed for viability and invasion using the Live/Dead assay (Invitrogen, Grand Island, NY, USA). We observed that compared to controls, all PI3K inhibitor treated spheroids were less invasive when grown in collagen and displayed increased cell death (Figure 1C). We also noted that the most effective PI3K inhibitors in 2D cultures were no longer as clearly ranked. We then hypothesized that certain PI3K inhibitors could be underestimated based on adherent culture observations, just as others have been previously overestimated such as LY294002, which loses activity in 3D (Smalley et al., 2006). To test this, we further investigated the PI3K inhibitors that were less effective in 2D culture. We seeded the same number of melanoma cells as adherent cultures or 3D collagen-embedded spheroids, treated these for 72h with PX-866 or AZD6482, and evaluated response using the alamarBlue assay. PX-866 showed a significant decrease in proliferation in 3D spheroids embedded in collagen compared to 2D adherent cultures (Figure 1D). We observed a similar response with AZD6482 (Suppl. Fig. 1A). These results indicate that PI3K inhibitors could be underestimated if only evaluated in standard adherent cell cultures.

Since PX-866 was not as effective as GDC0941 or BAY80-6946 against melanoma cells grown as 2D cultures (all evaluated at 10 μ M), we examined the potential of PX-866 to inhibit the PI3K pathway according to dose and treatment time. PX-866 inhibited pAKT at a concentration of 0.5 μ M, 1h post-treatment, and this inhibition was still present (though less pronounced) at 72h. This was assessed in PTEN wild type (WM983B) and PTEN null (1205Lu) cells (Suppl. Fig.1B). Levels of pS6 (Ser235/236) did not decrease with treatment; however, levels of p4E-BP1 (Thr37/46) were lower at 72h in both cell lines tested. An upregulation of total 4E-BP1 levels was also detected following treatment, but this was not maintained at 72h in both cell lines. Our findings suggest that in adherent cultures, PX-866 affects AKT at early time-points, but effects on p4E-BP1 activity are time and cell-line dependent.

Given our detection of cytotoxic and anti-invasive effects in PX-866-treated 3D melanoma spheroids, we next examined if similar changes occurred in 2D but at lower levels. We did not observe significant cell death or proliferation changes in 2D melanoma cultures treated with PX-866 as assessed by cleaved PARP immunoblots or by propidium iodide staining (Figure 1B, Suppl. Fig.1C). However, we did observe a significant reduction in cell migration as assessed by a transwell migration assay, an observation that was also seen in 3D spheroids grown in collagen (Suppl. Fig.1D; Suppl. movies 1-2). These findings suggest that PX-866 inhibits melanoma cell migration in different culture settings; however, effects on proliferation and survival can depend on the microenvironment.

PX-866 reduces tumor growth in 3D melanoma models and *in vivo*

Because the effects of PX-866 in 3D spheroids could be model-dependent, we synthesized human-derived skin reconstructs *in vitro* using 1205Lu melanoma cells (used as single cell suspensions) and observed that even in a skin-like microenvironment that displays healthy fibroblasts and keratinocytes, PX-866 is able to decrease melanoma cell numbers and

invasion, as indicated by the S100 staining of melanoma cells (Figure 2A). A model xenograft system using our metastatic cell line 1205Lu in NOD-SCID-IL2- γ -null (NSG) mice next showed that PX-866 reduces tumor growth as a single agent once 200mm³ tumors are established (Figure 2B). There was a significant difference in the growth rates between the control and PX-866 (10mg/kg, q2d) treated groups ($p=0.0384$) with the average growth slope for the control group being 0.1042, and the average growth slope for the PX-866 group being 0.0377. When tumor lysates were collected on the last treatment day, we observed that the PX-866 treated group had lower levels of pAKTSer473, increased total AKT, but variable phosphorylation of 4E-BP1 and S6 (Figure 2C). Due to mouse loss and possible toxicity, we could not increase the dose of PX-866 to achieve tumor regression or complete reduction of pAKT or p4E-BP1. However, in the 3D melanoma spheroid setting, we could dramatically reduce pAKTSer473 levels (also associated with higher total AKT levels) and p4E-BP1 (Thr37/46) levels compared to what is observed in 2D cultures using the same dose (Figure 2D). Changes in p70S6K were minimal in accordance with the high pS6 levels also seen in treated adherent cultures (Figure 1B). These findings suggest that despite the limited effects of PX-866 on adherent cultures (even in the presence of low pAKT), more complex 3D melanoma models respond to this drug and full pathway blockade is a desirable outcome.

PX-866 and PLX4720 prevent tumor growth *in vivo*

Analysis of BRAFV600E and PI3K signaling in melanoma cell lines previously showed that both pathways cooperate to regulate protein synthesis through AKT-independent, mTORC1-dependent effects on p70S6K, S6, and 4EBP1 phosphorylation (Silva et al., 2014). These studies also showed that single-agent inhibition of mTOR decreased cell proliferation as potently as inhibiting BRAFV600E and PI3K signaling. Thus, we tested if the effects of PX-866 could be further enhanced in 3D melanoma models in the presence of the BRAF inhibitor PLX4720. Invasion was significantly affected by the presence of PX-866 alone; thus, in combination with PLX4720 differences were difficult to assess. However, an increase in cell death was observed when both PX-866 and PLX4720 were present (Figure 3A). This was next investigated *in vivo* using mutant BRAF 1205Lu melanoma cells that were previously shown to be partially responsive to PLX4720 (Paraiso et al., 2010). For treatment, we selected a PLX4720 (200mg/kg) diet in order to achieve physiological drug doses (Grippio et al., 2014), while the PX-866 2mg/kg dose was selected to prevent any toxicity. We injected the *BRAF* mutant metastatic cell line 1205Lu in NOD-SCID-IL2- γ -null (NSG) mice, then started a 2 week treatment once 200mm³ tumors were established. As shown in Figure 3B, treatment of tumor-bearing mice with the single agents did not halt 1205Lu tumor growth; however, significant arrest ($p<0.001$) was observed with a combination of PLX4720 and PX-866, without causing detectable toxic effects. Following 14 days of treatment, tumor size and weights of treated mice were also lower than untreated mice (Suppl. Fig. 2A). Tumor weight changes were significant in PX-866 vs combination therapy groups ($p<0.005$), and were marginally significant in PLX4720 vs combination therapy groups ($p=0.078$); note that the combination treatment of 2D melanoma cultures with PLX4720 and increasing doses of PX-866 up to 20 μ M had minor effects on proliferation (Suppl. Fig. 2B). Our data show that PI3K inhibitors such as PX-866 with

limited single agent activity at low doses can enhance the anti-tumor activity of PLX4720 in a melanoma xenograft model.

To investigate *in vivo* pathway inhibition following treatment, phosphorylated and total levels of AKT and ERK were measured in tumor samples (Figure 3C, Suppl. Fig. 2C). We observed that following 14 days of treatment, clear changes in pAKT and pERK could not be detected in the single agent treated tumors; however, the combination of both inhibitors showed reduced pAKT levels. Changes in cPARP levels were also not detected in this combination treatment group, suggesting a largely cytostatic effect being observed by day 14. High pERK levels in all treatment groups could contribute to this observation. Since we have previously shown the importance of insulin-like growth factor 1 receptor (IGF1R) and PI3K signaling for BRAF inhibitor-mediated drug resistance (Villanueva, 2010), we examined IGF1R levels in 1205Lu adherent cells. Using flow cytometry, we observed the enrichment of IGF1R negative cells in the presence of PX-866 and PLX4720 as single agents, with a significant change ($p=0.0008$) when both compounds were combined (Figure 3D, Suppl. Fig. 2D). Taken together, these data suggest that PI3K and MAPK inhibitors can contribute to lower IGF1R and pAKT levels in mutant *BRAF* and *PTEN* cells beyond single agent activity.

PX-866 is effective against BRAF inhibitor resistant cells grown as collagen-embedded spheroids

Using our 3D melanoma models, we observed that cytotoxicity can be achieved with PX-866 in mutant BRAF melanoma cells. Therefore, we investigated whether cells with acquired resistance to BRAF inhibitors could also respond to PX-866, especially given the reported importance of PI3K signaling in mediating resistance (Villanueva, 2010; Paraiso et al., 2011; Shi et al., 2014). BRAF inhibitor-resistant cell lines (BR), were previously generated by our group using chronic treatment of mutant BRAFV600E melanoma cells with SB-590885. These cells are cross-resistant to other BRAF-selective inhibitors and their resistance mechanisms involve flexible signaling through the different RAF isoforms to reactivate MAPK signaling, and enhanced IGF-1R/PI3K signaling (Villanueva, 2010). As shown for the parental cell lines, response of BR cells to different PI3K inhibitors in adherent 2D melanoma culture varied and was most pronounced following BAY80-6946 and GDC-0941 treatment, while PX-866 and AZD6482 were less effective (Figure 4A). Interestingly, 451Lu cells that responded equally to all PI3K inhibitors (less than 40% proliferation inhibition), showed increased sensitivity to BAY80-6946 and GDC-0941 once rendered BRAF inhibitor resistant. This is consistent with the acquired dependency of these melanoma cells on the PI3K pathway following BRAF inhibitor resistance. The same observation however, could not be extended to WM983B cells and their BR counterparts that share a similar BRAF/NRAS/PTEN genetic background as 451Lu (Table 1).

While PX-866 was less effective than other PI3K inhibitors in 2D BR cultures, the alamarBlue assay interrogating the same number of cells shows that 3D BR spheroids are significantly more sensitive to PX-866 (Figure 4B). Using Live/Dead cell staining, we further show that PX-866-treated BR spheroids are smaller, they display cytotoxicity, and they are less invasive (Figure 4C). However, unlike their parental cell lines, BR cells hardly

display enhanced cell death in 3D when PX-866 is combined with PLX4720, consistent with the heavier reliance of BR cells on PI3K signaling. Western blot analyses confirmed that PX-866 as a single agent can increase cell death in 3D BR spheroid samples as indicated by higher levels of cleaved PARP in 3D but not 2D samples (Figure 4D, Suppl. Fig. 3). These findings suggest that PI3K inhibitors such as PX-866 with limited activity in 2D adherent melanoma cultures can still provide beneficial anti-melanoma effects in 3D models of BRAF inhibitor resistant melanoma.

The microenvironment plays a role in melanoma response to PX-866

To start dissecting why melanoma cells show enhanced response in 3D growth conditions following PX-866 treatment, we sought to compare signaling profiles from untreated 1205Lu cells grown as adherent 2D cultures, 3D spheroids in collagen, and tumors in xenografts. For this purpose, we used a receptor tyrosine kinase (RTK) antibody array featuring PI3K and MAPK signaling effectors (this array was also used to inform future drug combinations). Changes between samples were detected in 14 targets and appeared to be signaling node-dependent and not universally up- or down-regulated in one growth environment compared to another (Figure 5A). Of interest, Src levels were found elevated in adherent 1205Lu cells, which could also contribute to their resistance to BRAF inhibition (Girotti et al., 2015). Examination of effectors of the PI3K and MAPK pathways, such as phosphorylated AKT, ERK, and ribosomal protein S6 indicated that their activity is similar and reduced in 3D melanoma spheroids and tumor samples compared to adherent 2D cultures (Figure 5B). This was further confirmed by western blot analyses using different samples for each growth condition (Figure 5C). Additionally, we examined IGF1R levels in adherent, spheroid, and tumor 1205Lu samples and show that the microenvironment (unlike treatment with PLX4720 and PX-866) does not alter its expression. Taken together, our data suggest that lower levels of pAKT, pERK, and pS6 can be found in cells from more complex 3D melanoma models, which could enhance pathway blockade and potentiate the anti-tumor effects of PI3K inhibitors.

DISCUSSION

The PI3K pathway is important for melanoma pathobiology, therapy resistance, and is of concern given its role in metastasis (Cho et al., 2015; Brastianos et al., 2015; Atefi et al., 2014). Because this pathway is also regulated by the tumor microenvironment, it is not clear how PI3K inhibitors are affected in the 3D tissue setting. Previous studies showed that ovarian cancer spheroid cells display distinct responses to PI3K/mTOR inhibition depending on their matrix attachment (Muranen et al., 2012). Here we examined the effects of PI3K inhibitors of different specificity on 3D models of melanoma featuring elements of the TME. We observed that the most effective PI3K inhibitors in 2D cultures were no longer as easily identified in 3D spheroids embedded in collagen. This is not surprising given that cell-to-matrix adhesion, hypoxia, and nutrients such as glucose and amino acids can regulate the PI3K/mTOR pathway (Jewell and Guan, 2013; Guo and Giancotti, 2004). We note that all PI3K inhibitors tested reduced melanoma spheroid invasion in collagen, concordant with the important role of AKT in melanoma metastasis (Cho et al., 2015). Interestingly, we observed that two compounds with limited effects in 2D culture displayed enhanced activity in the 3D

setting (PX-866 and AZD6482); this raises the possibility that certain PI3K inhibitors may be underestimated based on adherent culture results alone. This needs careful exploration given that 3D disease models can be more reflective of the drug sensitivity profile found in patients (Katsila et al., 2014).

We focused further experiments on PX-866 because of its previous reported effects in 3D models of glioblastoma, prostate, breast, and colon cancer (Howes et al., 2007; Ihle et al., 2004). A Phase I trial in patients with advanced solid tumors showed that the drug is well tolerated and associated with a best overall response by Response Evaluation Criteria in Solid Tumors (RECIST) of stable disease in 22% of patients on an intermittent drug schedule, and 53% on a continuous drug schedule (Hong et al., 2012); thus, showing clinical merit. On the other hand, preclinical evaluations have shown that in adherent melanoma cultures, PX-866 [1 μ M] maximally inhibited pAKT in two different PTEN-null human melanoma cell lines, but resulted in minimal cell cycle changes or cell death (Deng et al., 2012). Our results are in agreement with these observations that despite low pAKT levels, the response of 2D adherent cultures to PX-866 is limited and does not reveal obvious clinical benefits. Interestingly, Deuker et al. showed that GDC0941 can inhibit downstream effectors of mTORC1 such as p4EBP1 and pS6 in BRAFV600E/PTENnull human melanoma cell lines and this compound was one of the most effective PI3K inhibitors in our 2D melanoma studies (Deuker et al., 2015). Meanwhile, we observed that PX-866 decreased levels of p4E-BP1 (Thr37/46) but at later time points than pAKT and not as strongly as GDC0941 or BAY80-6946. The reduced effects of PX-866 on mTOR effectors could have spared melanoma cells in 2D culture compared to other inhibitors. In our spheroid models however, PX-866 can achieve pronounced p4E-BP1 inhibition and this could contribute to its enhanced activity in that context.

Through studies on skin reconstructs and a xenograft model generated from a mutant BRAF/mutant PTEN melanoma cell line, we observed that PX-866 induces significant melanoma cell growth reduction in more physiological models. PI3K signaling is involved in nutrient sensing and response to hypoxia, indicating the possibility that PI3K inhibitors could be differentially effective in a tissue context where heterogeneity is more readily achieved (Sinclair et al., 2008; Semenza, 2013). Although tested in one cell line only (1205Lu), our RTK array data indicate that spheroids and tumor samples can share similar MAPK and PI3K signaling activity; this points to the potential of 3D melanoma models to enhance our understanding of PI3K inhibitors prior to *in vivo* studies. In addition, models that display invasive phenotypes could be highly informative for PI3K inhibitor studies on metastasis.

Crosstalk between the MAPK and PI3K pathways is to be taken into consideration when developing targeted therapy-based strategies. In addition to PI3K signaling conferring resistance to BRAF inhibitors, previous work indicates that AKT and mTOR inhibition can further activate ERK signaling (Will et al., 2014; Villanueva, 2010). In our 3D melanoma models, enhanced cytotoxicity was detected in 1205Lu spheroids when PX-866 was combined with PLX4720 and reduced tumor growth was detected *in vivo*; however, we suspect that tumor regression was not achieved due to the lack of complete or maintained PI3K and ERK pathway inhibition (Howes et al., 2007). A transient pathway inhibition by PI3K drugs is no less of biologic value and can induce apoptosis (Will et al., 2014). While

time course studies are difficult *in vivo* to monitor tumor and pathway adaptation over time, we can at least conclude from our studies that a PI3K inhibitor with limited activity in adherent cells can show *in vivo* activity and enhance the anti-melanoma effects of PLX4720. In a study by Herkert et al., single agent PI3K β inhibitor activity is observed *in vivo* despite limited activity *in vitro*, but combined inhibition of PI3K β , IGFR, and MAPK signaling led to complete and sustained pathway blockade and induction of cell death in PTEN^{LOF}/BRAF^{MUT} melanomas (Herkert et al., 2015). Interestingly, we also detected lower IGF1R levels following the combination of PX-866 and PLX4720 compared to either single agent. Given that 1205Lu cells are only partially responsive to BRAF inhibition, an added PI3K/IGFR targeting strategy could be more effective. A schematic of MAPK/PI3K/IGFR signaling is provided in Suppl. Fig. 4 to highlight the usefulness of targeting multiple effectors of these pathways to prevent resistance. We also suggest that certain PI3K inhibitors could be underestimated against established BRAF inhibitor resistant melanomas as we observed PX-866 to be more effective in a 3D matrix-supported setting than 2D culture.

We cannot at this time conclude which PI3K inhibitors are the most effective and least toxic for *in vivo* studies; however, in order to facilitate this process, we suggest that 3D culture models are useful and will better inform *in vivo* observations or combination studies. This will be of importance for more selective PI3K inhibitors with improved toxic profiles and less general cytotoxicity. The value of 3D models in drug development is increasing as they are amenable to high-throughput screening (HTS) (Fennema et al., 2013). We envision that melanoma studies conducted in 3D models could be as fruitful as those conducted in breast tissue and cancer, where 3D cultures are already used to dissect complex signaling interactions (Vargo-Gogola and Rosen, 2007). Overall, our findings highlight the need for more tissue-relevant melanoma models to fully and accurately evaluate the potential of small molecule inhibitors and determine the most reliable biomarkers of response; they also support the therapeutic potential of combining both MAPK and PI3K pathway inhibitors in melanomas that are treatment naïve or resistant to MAPK inhibition.

METHODS

Cell culture and reagents

Human melanoma cell lines have been previously described (Satyamoorthy et al., 2003; Iliopoulos et al., 1989). Cells were cultured in DMEM supplemented with 5% fetal bovine serum and grown at 37°C in 5% CO₂. The consistency of cellular genotypes and cell line identities were confirmed by DNA fingerprinting, using Coriell's microsatellite kit. PX-866 was provided by Oncothyreon (Seattle, WA, USA); PLX4720 was supplied by Plexxikon/Roche (Berkeley, CA, USA). GDC-0941, BAY80-6946, AZD6482, LY294002 were purchased from Selleckchem (Houston, TX, USA). All compounds were stored at -20°C in DMSO as 10mM stocks.

Proliferation assays

Cell numbers following PI3K inhibitor addition were assessed by seeding 2×10⁴ cells/well in a 24 well plate, allowing cells to adhere overnight, and adding drug 12h later. After

treatment, cells were counted with a haemocytometer. Proliferation comparisons between 2D and 3D cultures were conducted by seeding 5000cells/well in 96-well plates as either adherent cultures or 3D spheroids in collagen. Following drug treatment, wells were evaluated by alamarBlue addition (Invitrogen, Grand Island, NY, USA), and absorbance was measured as per the suppliers' instructions. Percent proliferation was normalized to the absorbance of DMSO-treated cells.

Western blots and antibody array

Proteins were extracted as described in (Vultur et al., 2008), and 50µg of cell extract were resolved on a 10% polyacrylamide-SDS gel before being transferred onto a polyvinylidene membrane (BioRad, Hercules, CA, USA). All primary antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). The secondary antibodies IRDye 700 and IRDye 800 were obtained from Li-Cor (Lincoln, NE, USA). Membranes were imaged and analyzed using the Odyssey Infrared Imaging System and software (Li-Cor). Relative band intensities are derived from 3 separate experiments. To identify the relative levels of phosphorylated RTKs across different melanoma models, the PathScan RTK Signaling Antibody Array Kit was used (#7949; Cell Signaling Technologies), using the manufacturer's instructions.

Collagen-embedded melanoma spheroids

Melanoma spheroids were generated according to previous work (Smalley and Herlyn, 2005; Smalley et al., 2008). Briefly, 5000cells/well in 96-well plates were allowed to grow and coalesce on a non-adherent agar layer for 72h before extraction and re-suspension in a collagen type I mixture. Spheroids were either lysed for western blot analyses or stained with the Live/Dead cell assay (Invitrogen) then imaged using a Nikon Inverted TE2000 microscope (Melville, NY, USA). Images were analyzed using the Image Pro software (Media Cybernetics, Rockville, MD, USA). For red signal quantitation purposes, unmodified images were analyzed using the same parameters and signal values were obtained using the formula: (Fluorescence value-area count)/Background x 100. Quantitation of spheroid invasion was performed by measuring a mask for the total area covered by all the cells of a given spheroid minus the mask for the cell-dense core of each spheroid. The migration assay was performed as previously described with modifications found in Suppl. Information (Vultur et al., 2013).

Human skin reconstructs

Human skin reconstructs were generated as described previously (Fukunaga-Kalabis et al., 2006; Vultur et al., 2013). Briefly, culture tray inserts (Organogenesis, Canton, MA, USA) were coated with 1 ml bovine collagen I mixture (Organogenesis) then layered with 3 ml collagen I mixture containing 7.5×10^4 fibroblasts. After 7 days of incubation at 37°C, melanoma cells were mixed with keratinocytes (1:5 ratio) and seeded on top of the fibroblast mixture. All cells were cultured in skin reconstruct media for 5 days, then raised to the air—liquid interface (Vultur et al., 2013). Two weeks later, skin reconstructs were harvested for imaging or fixed in 10% neutral buffered formalin for 4-6 hours before being processed by routine histological methods. Drug treatment of skin reconstructs involved the pretreatment

of melanoma cells prior to their addition to the reconstruct as well as drug addition in the media 72h before skin harvest.

***In vivo* studies**

All animal experiments were performed in accordance with The Wistar IACUC protocol 111954 in NOD/LtSscidIL2R γ null mice (NSG). Mice were each inoculated s.c. with 1×10^5 1205Lu human melanoma cells in a 1:1 suspension of matrigel (BD Matrigel™ Basement Membrane Matrix, Growth Factor Reduced, Becton Dickinson) and complete media. Treatment started at an average tumor volume of 200 mm³. For single agent assessment, mice were randomized into two groups: (i) vehicle control, (ii) PX-866 every other day (10mg/kg p.o.). For drug combination assessments, mice were randomized into four groups and treated with: (i) vehicle control, (ii) PLX4720 200mg/kg diet, (iii) PX-866 every other day (2mg/kg p.o.) or (iv) combination of PLX4720 and PX-866. Hydroxypropyl- β -cyclodextrin (1%) in distilled water (Cyclodextrin Technologies Development, Inc, La Jolla, CA, USA) served as vehicle control. Tumor growth was measured every 2-3 days using a caliper and volumes calculated according to the formula $V = (W \times D \times H) / 2$ [mm³]. Tumor samples were snap frozen in liquid nitrogen for subsequent protein analyses.

Flow cytometry

Cell suspensions (3×10^5 cells/sample) were incubated for 30min at 4°C with primary mouse anti-human IGF1R antibody PE-conjugated or PE conjugated isotype control antibody (Becton Dickinson) according to manufacturer instructions. Data were acquired by LSRII[®] (Becton Dickinson) and analyzed using FlowJo, V 10.0.0 (Tree Star, Ashland, OR, USA). Histograms were generated after gating of single live cell and markers were set on isotypic controls. Graphs are representative of n=3 experimental replicates.

Statistical analyses

The analysis of variance (ANOVA) or a t-test was used to evaluate mean differences between groups. Tukey's procedure was used to compare means when the ANOVA was significant. When variances were unequal, Welch's ANOVA or t-statistic was used. For between group comparisons in Figures 1A and 4A, F-test of equal variance was carried out to determine the option for the t-test using equal or unequal variance test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SIGNIFICANCE

The PI3K pathway plays an important role in melanoma pathobiology and resistance to current therapies. Using models of melanoma that recapitulate elements of the tumor microenvironment, we examined how PI3K inhibitors have different effects depending on culture conditions and what pathways and effectors are modulated in response to treatment. We also identified compounds that display enhanced anti-tumor effects in more tissue-like contexts (3D cultures), indicating inhibitors could be underestimated if evaluated in adherent cultures alone. We propose that tissue-like disease models enhance our understanding of PI3K inhibitors and inform their use for maximum therapeutic effect.

Figure 1. Melanoma cell line response to PI3K inhibitors in 2D and 3D is not predicted by AKT inhibition alone

(A) Adherent melanoma cultures were treated with the indicated PI3K inhibitors ([10µM], 72h treatment) and drug response was assessed by cell counts. Error bars represent SE from 3 independent experiments. Comparisons of mean between groups were done using ANOVA and Tukey's procedure; p-values are shown in the table below. (B) Lysates of WM983B and 1205Lu cells treated for 72h with the indicated inhibitors (all [10µM]) were analyzed by immunoblotting. Levels of phosphorylated AKT (Ser473 and Thr308), ERK1/2 (Thr202/Tyr204), S6 (Ser235/236), 4E-BP1 (Thr37/46) as well as total protein and cleaved PARP (cPARP) were assessed. Hsp90 is the loading control. (C) Collagen-embedded melanoma spheroids were treated for 72h, with 10µM of the indicated drugs, before staining. Green fluorescence indicates metabolically active (live) cells, red fluorescence indicates membrane compromised (dead) cells. Scale bar represents 300 microns. Quantitation of red signal intensity is shown on the right. Error bars represent SE from 3 independent experiments; representative images are shown. Statistical analyses used ANOVA and Tukey's procedure; p-values are shown in the table below. (D) Equal number of cells were seeded as adherent 2D melanoma cultures or 3D spheroids in collagen before treatment with PX-866 ([10µM], 72h) and alamarBlue assessment. Error bars represent SE from 3 independent experiments. Welch's t-test assuming unequal variances shows significant proliferation decrease in 3D samples ($p < 0.001$).

Figure 2. PX-866 displays anti-melanoma effects in 3D melanoma cultures and *in vivo*

(A) Melanoma cells were pre-treated with PX-866 48h prior to incorporation into skin reconstructs and were again treated for 72h prior to reconstruct fixation. Staining for the melanoma marker S100 (red) and for nuclei, DAPI (blue), is shown. Scale bar represents 10 microns. (B) NSG mice were xenotransplanted with 1205Lu cells and tumors were allowed to grow above 200mm³. Mice were then treated with PX-866 (10mg/kg, orally provided every other day). Tumor growth rate differences between treatment groups were significant ($p=0.0384$). Error bars represent SE, $n=5$ mice/group. // = Mouse loss on the last treatment day. (C) Western blot analyses of mouse tumor lysates from (B) collected on the last treatment day. Levels of total and phosphorylated AKT (Ser473, Thr308), S6 (Ser235/236), 4E-BP1 (Thr37/46) protein as well as cleaved PARP (cPARP) and Hsp90 (loading control) were examined. (D) Lysates from 3D melanoma spheroids treated with PX-866 for 72h were interrogated for levels of phosphorylated AKT (Ser473), p70S6K (Thr389), 4E-BP1 (Thr37/46), for total protein, and for Hsp90 (loading control).

Figure 3. PX-866 displays anti-melanoma activity in combination with the BRAF inhibitor PLX4720 *in vivo*

(A) Collagen-embedded melanoma spheroids were treated for 72h with PX-866, PLX4720, or their combination. Spheroids were stained for live (green) and dead (red) cells. Scale bar represents 300 microns. Quantitation graphs of cell invasion area (top), and red signal intensity (bottom) in spheroids is shown on the right. Error bars represent SE from 3 independent experiments, representative images are shown. One-way ANOVA analysis shows significantly decreased invasion ($p < 0.005$), and significantly increased red signal intensity ($p < 0.05$) in the presence of PX-866 for the two cell lines. (B) NSG mice were xenotransplanted with 1205Lu melanoma cells and tumors were treated with PX-866 (2mg/kg, orally provided every other day), PLX4720 (200mg/kg, through diet), or with the combination of both drugs. Tumor volumes were measured every two days; statistical analyses used one-way ANOVA. Three comparisons are shown for vehicle control, PLX4720, and PX-866 each versus the combination treatment ($p < 0.001$ for all comparisons). Error bars represent SE, $n = 10$ mice/group. Representative images of tumors from each treatment group are shown (mm scale). (C) Western blot analyses of mouse tumor lysates from (B) collected on day 14. Levels of phosphorylated AKT (Ser473), ERK1/2 (Thr202/Tyr204), total protein, cleaved PARP (cPARP) and Histone H3 (loading control) were examined. (D) IGFR expression on 1205Lu cells (by FACS) treated with DMSO, PLX4720, PX-866, or a combination of PLX4720 and PX-866. Bars represent the average fold increase in IGFR negative cells from triplicate samples. The combination treatment significantly increased IGFR-negative cells compared to the untreated cells ($p = 0.0008$); PX-866 as a single agent did not ($p = 0.99$).

Figure 4. PX-866 is effective against 3D cultures of BRAF inhibitor-resistant (BR) melanoma cells

(A) Parental and BR melanoma cells (2D) were treated with the indicated PI3K inhibitors ([10μM], 72h) and drug response was assessed by cell counts. Error bars represent SE from 3 independent experiments. Means between groups were compared using ANOVA and Tukey's procedure; p-values are shown in the table below. (B) Equal number of BR cells were seeded as adherent 2D melanoma cultures or 3D spheroids in collagen before treatment with PX-866 ([10μM], 72h) and alamarBlue assessment. Error bars represent SE from 3 independent experiments. There was significantly lower levels of proliferation in 3D compared to 2D; Welch's t-test assuming unequal variances ($p < 0.001$ for both cell lines). (C) Collagen-embedded BR melanoma spheroids were treated for 72h with PX-866, PLX4720, or a combination of both. Spheroids were stained for live (green) cells, and dead (red) cells. Experiments were conducted in triplicate and representative images are shown. Scale bar represents 500 microns. Quantitation of spheroid invasion area and red signal intensity is also shown. Error bars represent SE from 3 independent experiments. Statistical analyses used one-way ANOVA. Invasion was significantly decreased ($p < 0.05$) and red signal intensity was significantly increased ($p < 0.05$) in the presence of PX-866 for both cell lines. (D) Western blot analyses of 3D spheroid-derived BR melanoma lysates (top) show a decrease in phosphorylated AKT (Ser473) and an increase in PARP cleavage (cPARP). Lysates from adherent 2D-grown BR melanoma cell lysates (bottom) indicate no consistent

changes in cleaved PARP levels. Average cPARP band intensity from 3 separate experiments is shown. Hsp90 is the loading control.

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Figure 5. Melanoma-relevant pathways affected by the microenvironment
(A) 1205Lu melanoma cells were grown as confluent 2D adherent cells, 3D spheroids in collagen, or xenograft tumors. Whole-cell lysates were incubated on a PathScan RTK signaling antibody array where each RTK antibody is spotted in duplicate. RTK dots showing different intensities among samples are encased in white, numbered, and listed on the right. Values were normalized according to positive and negative controls for each panel. (B) RTK array fluorescence relative intensity for AKT, ERK, and S6 indicate similarities between spheroids and xenograft samples. (C) Western blot analyses were conducted on 2D adherent cells, 3D spheroids, and xenograft tumor samples distinct from those interrogated in (A). Expression levels of pAKT (Ser473), pERK1/2 (Thr202/Tyr204), pS6 (Ser235/236), and total levels of IGF1R were examined. Hsp90 served as the loading control.

Table 1

Melanoma cell line basic genetic information (BRAF, NRAS, PTEN/AKT mutation status).

Cell Line	Stage	BRAF	NRAS	PTEN/AKT
WM983B	MET	V600E	WT	WT
1205Lu	MET	V600E	WT	MUT-PTEN (W274X Ex8)/HemiDel
451Lu	MET	V600E	WT	WT

Abbreviations: MET, metastasis; HemiDel, hemizygous deletion; MUT, mutation; WT, wild type; N/A, not available.