Combined PI3K/mTOR and MEK Inhibition Provides Broad Antitumor Activity in Faithful Murine Cancer Models

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Abstract

Purpose: Anticancer drug development is inefficient, but genetically engineered murine models (GEMM) and orthotopic, syngeneic transplants (OST) of cancer may offer advantages to in vitro and xenograft systems.

Experimental Design: We assessed the activity of 16 treatment regimens in a RAS-driven, Ink4a/Arf-deficient melanoma GEMM. In addition, we tested a subset of treatment regimens in three breast cancer models representing distinct breast cancer subtypes: claudin-low (T11 OST), basal-like (C3-TAg GEMM), and luminal B (MMTV-Neu GEMM).

Results: Like human RAS-mutant melanoma, the melanoma GEMM was refractory to chemotherapy and single-agent small molecule therapies. Combined treatment with AZD6244 [mitogen-activated protein-extracellular signal-regulated kinase (MEK) inhibitor] and BEZ235 [dual phosphoinositide-3 kinase (PI3K)/mammalian target of rapamycin (mTOR) inhibitor] was the only treatment regimen to exhibit significant antitumor activity, showed by marked tumor regression and improved survival. Given the surprising activity of the “AZD/BEZ” combination in the melanoma GEMM, we next tested this regimen in the “claudin-low” breast cancer model that shares gene expression features with melanoma. The AZD/BEZ regimen also exhibited significant activity in this model, leading us to testing in even more diverse GEMMs of basal-like and luminal breast cancer. The AZD/BEZ combination was highly active in these distinct breast cancer models, showing equal or greater efficacy compared with any other regimen tested in studies of over 700 tumor-bearing mice. This regimen even exhibited activity in lapatinib-resistant HER2+ tumors.

Conclusion: These results show the use of credentialed murine models for large-scale efficacy testing of diverse anticancer regimens and predict that combinations of PI3K/mTOR and MEK inhibitors will show antitumor activity in a wide range of human malignancies. Clin Cancer Res; 18(19); 5290–303. ©2012 AACR.
clinical outcomes seen in patients (12). Despite these promising series, there has not been a comprehensive assessment of GEM models versus traditional preclinical efficacy testing. The GEMM approach until recently has been hampered by a variety of factors relating to experimental logistics, intellectual property, and other nonscientific concerns (covered in ref. 2). As these impediments to GEMM testing have been largely resolved, and others have turned to the large-scale testing of novel and traditional therapeutics in credentialed and faithful murine models of human cancers.

We believe RAS-driven tumors (e.g., melanoma, carcinomas of colon, pancreas, and lung) represent a particular clinical need. As mutations of K-, N-, or H-RAS occur in 15% to 30% of all human cancers (see Compilation of Somatic Mutations in Cancer, ref. 13), RAS activation represents the foremost "undrugged" tumor-driver in cancer biology. Moreover, RAS mutation is associated with adverse outcomes in several tumor types, and targeted approaches for mutant RAS are lacking. For example, in melanoma, although mutations of B-RAF are more common (43%), mutations of N-, K-, and H-RAS are also frequent in human disease (19%, 2%, and 1%, ref. 14), and RAS-mutant tumors exhibit a worsened prognosis compared with RAF-mutant disease (15). For these reasons, we initially elected to focus on Ras-mutant tumors, particularly melanoma where easy serial assessment offers advantages for large-scale drug testing. Toward that end, we used a previously established GEMM of RAS-driven melanoma (Tyr-H-RasG12V Ink4a/Arf−/−; herein referred to as "TRIA"; ref. 16) that is favorable for therapeutic testing (see Materials and Methods).

In this model, we tested all cytotoxic agents reported to have single-agent activity in melanoma, as well as novel therapeutics that have been of interest in this disease. In particular, we included agents thought to be "RAS-specific" alone or in combination, as well as combined therapy with AZD6244/BEZ235, a regimen previously shown to have activity in murine models of RAS-driven lung cancer (17). Given the unexpected results in murine melanoma, we further studied the efficacy of AZD6244/BEZ2235 in a related breast cancer model, and then even more diverse breast cancer GEMMs. These results in more than 700 tumor-bearing mice suggest combined inhibition of phosphoinositide-3 kinase (PI3K)/mammalian target of rapamycin (mTOR) and mitogen-activated protein–extracellular signal-regulated kinase kinase (MEK) inhibitors. These data have significant implications for the clinical development of MEK and PI3K/mTOR inhibitors and predict that this combination will have broad clinical activity across disparate human malignancies.

Translational Relevance

We describe a comprehensive assessment of 16 anti-cancer therapeutic regimens in multiple engineered murine model (GEMM) and orthotopic, syngeneic transplant (OST) cancer model. Aggregate analysis from these studies shows that, in contrast to standard preclinical efficacy testing (e.g., xenograft models), these faithful models exhibit a high predictive accuracy of the clinical efficacy of known chemotherapeutic agents used in melanoma and breast cancer. Moreover, testing of novel agents showed extraordinary anticancer activity from combined therapy with phosphoinositide-3 kinase (PI3K)/mammalian target of rapamycin (mTOR) and mitogen-activated protein–extracellular signal-regulated kinase kinase (MEK) inhibitors. These data have significant implications for the clinical development of MEK and PI3K/mTOR inhibitors and predict that this combination will have broad clinical activity across disparate human malignancies.
Compounds

Compounds were obtained from commercial sources, by custom synthesis, or rarely under material transfer agreement from an industry partner. The MP1U has focused on conventional chemotherapeutic agents and small molecule agents rather than monoclonal antibodies because of interspecies differences in tumor epitopes ABT-888, AZD-6244, and FTS (S-trans,trans-farnesylthiosalicylic acid) were synthesized by the Center for Integrative Chemical Biology and Drug Discovery (CICBDD) at the University of North Carolina. Carboplatin (Hospira, Inc.), cyclophosphamide (Hospira, Inc.), doxorubicin (Bedford Laboratories), etoposide (Teva Parenteral Medicines, Inc.), and paclitaxel (Ivax Pharmaceuticals, Inc.) were obtained from their respective manufacturers and handled as per standard practice. Erlotinib (Genentech, Inc.), lapatinib (GlaxoSmithKline), sunitinib (Pfizer, Inc.), temozolomide (Merck) were obtained from clinical commercial sources. BEZ-235 and lonafarnib was obtained under material transfer agreements (MTA) with Novartis AG and Schering-Plough (now Merck).
published datasets were analyzed on 3 different Agilent transcriptional similarities. Samples within each of these were combined into a single dataset and compared for GSE27101; refs. 24 and 26) and melanoma (GSE34866) Murine model cluster of drug treatment.

Response criteria

Tumor volume was calculated from 2-dimensional measurements. The percent change in volume at 14 and 21 days was used to quantify response. SD, PR, and CR were defined as per RECIST criteria. Survival was measured from first day of drug treatment.

Murine model cluster

Murine models of mammary carcinoma (GSE3165, GSE27101; refs. 24 and 26) and melanoma (GSE34866) were combined into a single dataset and compared for transcriptional similarities. Samples within each of these published datasets were analyzed on 3 different Agilent array platforms (22K, 4 × 44K, or 4 × 180K). Using normalization methods described to correct for platform effects between 22K and 4 × 44K platforms (26), we used 10 microarrays (5 MMTV-c-neu and 5 C3(1)-Tag) from each array type (30 microarrays total) to calculate a median, probe-level normalization factor using R v2.12.2. These arrays were chosen based on their high correlation to each other within each array type when clustered using a previously defined intrinsic gene list (24) to eliminate outliers from the array type correction. Principle component analysis (PCA) was used to verify proper normalization of the platforms. Unpublished MMTV-c-neu and C3(1)-Tag tumors used for platform correction were collected and microarray processed using methods previously described (24, 26). These arrays were uploaded to the Gene Expression Omnibus under series GSE35722 and to the University of North Carolina Microarray Database (27).

To identify transcriptional similarities between murine models of mammary carcinoma and melanoma, an unsupervised cluster analysis was carried out using any probe with a log2 absolute expression value greater than 2 on at least 3 microarrays (2,584 probes) using Cluster v3.0 (28). The data were viewed using Java Treeview v1.1.5r2 (29).

Cell culture and western blotting

Tumor-derived C3Tag and TRIA cell lines were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Tumor-derived T11 cells were grown in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were lysed on ice for 20 minutes in lysis buffer containing 50 mmol/L HEPES (pH 7.5), 0.5% Triton X-100, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L sodium fluoride, 2.5 mmol/L sodium orthovanadate, 1× protease inhibitor cocktail (Roche), and 1% each of phosphatase inhibitor cocktails 2 and 3 (Sigma). Cell lysates centrifuged for 15 minutes (13,000 rpm) at 4°C and the supernatant was collected. Proteins from cell lysates were separated by SDS-PAGE chromatography, transferred to nitrocellulose membranes, and probed with the indicated primary antibodies. Antibodies recognizing pAKT (S473), pERK1/2 (T202/Y204), and phospho-MEK1/2 antibody (S217/S221) were obtained from Cell Signaling Technology. The antibody for ERK2 was obtained from Santa Cruz Biotechnology. HRP-anti-rabbit secondary antibody was obtained from Jackson Immunoresearch Laboratories. Western blots were incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and exposed to film.

Statistics

Unless otherwise noted, comparisons are made with t test or 1-way ANOVA, with Bonferroni correction for multiple comparisons where appropriate. P values of less than 0.05 are considered significant. Error bars represent ±SEM.

Results

Large-scale GEMM testing

We tested 11 distinct single agents in 16 treatment regimens in the TRIA model, and a subset of these treatment
regimens were also tested in models of breast cancer representing distinct clinical subtypes in the UNC Mouse Phase I Unit (MP1U). In a contemporaneous study, additional treatment regimens were tested in the 3 breast cancer models (Usary and colleagues, submitted), and the most active regimens from that study were compared with AZD/BEZ in this work. All FDA-approved and experimental compounds used in this work are described in Supplementary Table S1. Relevant features of all MP1U testing are:

1. The use of credentialed GEMMs. For the breast models, credentialing was accomplished with expression profiling (see refs. 24 and 26). For melanoma, model choice was guided by RAS-dependence and other experimental features (see Materials and Methods).

2. The use of large (10–25) cohorts of tumor-bearing animals per therapeutic cohort, with assessment of both novel and approved agents in each disease type. Large-scale testing of combinations in the breast models will be reported elsewhere (Usary et al., submitted).

3. The serial assessment for tumor response as opposed to measures of nonprogression (e.g., tumor growth inhibition, TGI), as TGI does not mimic acceptable measures of efficacy in humans. The 2 primary endpoints described in this work are percent change in tumor volume at day 21 and overall survival (both calculated as described in the Materials and Methods).

Choice of murine model for efficacy testing in this work was initially guided by RAS-dependence, but given the activity of a single regimen, we subsequently turned to more diverse GEM models.

**Drug testing in Ras-mutant melanomas**

Current treatment approaches for advanced melanoma are largely ineffective, with no targeted agents for RAS-mutant disease. Temozolomide provides a meager 12% response rate (30), and even newly approved ipilimumab offers only a modest 3.5-month survival advantage (31). To test different regimens in a GEM model of human RAS-driven melanoma, we generated cohorts of 10 to 20 inbred male TRIA mice. Mice were serially assessed for tumors, and treatment regimens were initiated in tumors at a minimum size of 25 mm³. In total, 15 single-agent or combination regimens and total body irradiation (single dose TBI; 7.5 Gy) were tested (Fig. 1A). Regimens of cytotoxic therapeutics with activity in human melanoma such as temozolomide and carboplatin–paclitaxel were not effective at inducing tumor response by 21 days of therapy. Similarly, small molecule agents such as sunitinib, lapatinib, and potential anti-RAS approaches [S-trans, trans-farnesylthiosalicylic acid (FTS) and lonafarnib, a farnesyltransferase inhibitor] were ineffective in this model as single agents. Response rates of the tested agents in the TRIA model using a modified version of Response Evaluation Criteria In Solid Tumor (RECIST) correlated with the antitumor efficacy of these agents in human melanoma (Table 1). Therefore, like human RAS-mutant melanoma, this model was highly refractory to therapy and treatment studies in this GEMM correlate with an agent’s activity in human disease.

Therapeutic response, defined as tumor regression and survival improvement, was observed with only one treatment regimen in the TRIA GEMM. The co-administration of AZD6244 (an MEK inhibitor) and BEZ235 (a dual PI3K/mTOR inhibitor) resulted in significant tumor regression at 21 days in the majority of mice (Fig. 1A). Overall, the dual AZD/BEZ treatment cohort had a 27.5% (95% CI = −4.5–59.4) median increase in tumor volume at 21 days compared with 217.5% (95% CI = 151.6–283.4) increase in the untreated cohort (P < 0.0001). Neither AZD nor BEZ exhibited significant activity at day 21 as a single agent. A “waterfall plot” of best response (21 days or later) showed that AZD/BEZ treatment resulted in partial response (PR) or stable disease (SD) in 12 of 19 animals (63%) (Fig. 1B). This observation suggests that inhibition of single RAS downstream effector pathways, MEK or PI3K, in isolation is not sufficient to induce tumor regression, but concomitant MEK and PI3K/mTOR inhibition can cooperatively produce tumor regression in most RAS-driven melanomas.

The regimens determined to be most active in the 21-day response assay were given in long-term survival experiments (Fig. 1C). Although every agent thus far tested in the MP1U that produces a reduction in tumor growth at 21 days also enhances survival, the converse is not true. Therefore, an overall survival endpoint was assessed on therapy given that some agents showing only modest activity at 21 days still produce a significant survival benefit (see, e.g., carboplatin-

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Mean day 0 tumor volume (mm³)</th>
<th>Mean day 21 tumor volume (mm³)</th>
<th>RECIST response rate (CR + PR + SD)</th>
<th>Reported human response rates for melanoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>83</td>
<td>322</td>
<td>0%</td>
<td>14%–23%</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>89</td>
<td>217</td>
<td>21%</td>
<td>14–18%</td>
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<tr>
<td>Paclitaxel</td>
<td>62</td>
<td>182</td>
<td>21%</td>
<td>19%–47%</td>
</tr>
<tr>
<td>Carboplatin/paclitaxel</td>
<td>41</td>
<td>97</td>
<td>38%</td>
<td>15% (10%–17%)</td>
</tr>
<tr>
<td>Temozolomide</td>
<td>88</td>
<td>266</td>
<td>10%</td>
<td>33%</td>
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<td>115</td>
<td>32%</td>
<td>Unknown</td>
</tr>
<tr>
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<td>53</td>
<td>29</td>
<td>63%</td>
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</table>
paclitaxel; Fig. 1A and C). Intravenous carboplatin–paclitaxel and oral single-agents sunitinib, AZD, and BEZ all led to modest improvements in survival compared with untreated animals, with single-agent BEZ the best of this group increasing the duration of median survival by 70% (21–36 days, \( P < 0.001 \)). In accord with the 21-day response data, however, AZD/BEZ treatment afforded far greater clinical benefit than any other regimen, increasing median survival by 205% compared with untreated animals (21–64 days, \( P < 0.001 \)). These data show that the marked antitumor activity observed at 21 days or later (Fig. 1B) translates into a tripling of median survival in mice with established, high-grade melanomas.

**Testing of AZD/BEZ in a model of claudin-low breast cancer**

After identifying the potent activity of the AZD/BEZ drug combination in melanoma, we turned to a model of “claudin-low” breast cancer (25). This choice was motivated by the unexpected finding that claudin-low breast models show expression features in common with melanoma. For example, unsupervised analysis of 13 murine mammary carcinoma models (24, 26) and 4 murine melanoma models showed that the melanoma samples clustered with claudin-low tumors (Fig. 2A). As seen in human claudin-low samples (25), murine melanomas exhibited low expression of claudins (e.g., Cldn3, Cldn4, Cldn7) and cytokeratins (e.g., Krt5, Krt14, Krt17) and increased expression of genes associated with epithelial-to-mesenchymal transition [e.g., Snail1/2, Zeb2, Cldn1 (E-cadherin); Fig. 2B and C]. Both murine tumor types were also characterized by increased expression of inflammatory/immune genes compared with other breast models (e.g., Il6, Il33, Cd2; Fig. 2D). Melanomas differed from claudin-low breast tumors in the expression of melanocyte lineage genes such as Pax3 as well as transcripts expressed in melanoma as opposed to breast cancer [Gas7 and Cdh2 (N-cadherin)]. Using a recently described metric to calculate a “differentiation score” in breast cancer (higher score = more differentiated; ref. 25), melanoma, and claudin-low models showed similarly low scores (Fig. 2F), suggestive of expression profiles more similar to mammary stem cells than differentiated luminal cells. Given these shared transcriptional features with the TRIA model, we elected to further test AZD/BEZ in the claudin-low breast cancer model.

We have not identified a faithful GEM model of the claudin-low breast cancer subtype that only gives rise to just this tumor subtype, therefore have taken to the analysis of genomically selected orthotopic, syngeneic transplant (OST) models. The T11 model derives from the serial orthotopic transplantation of a murine breast tumor derived from a p53-null mouse into a syngeneic p53 competent recipient, and features sporadic, somatic K-Ras mutation (data not shown). Tumors from the T11 model display an RNA expression pattern characteristic of the human claudin-low disease (Fig. 2 and ref. 26), and are extremely aggressive, with the majority of untreated animals surviving less than 21 days from the time of enrollment in the therapy studies. Therefore, a 14-day rather than 21-day response endpoint was used to assess activity in this model. As in the TRIA melanoma model, AZD/BEZ was highly active in the T11 OST model (Fig. 3A compares AZD/BEZ with 4 of the most active other 16 tested regimens, see also Usary et al., submitted). Although no CRs were seen in the T11 model, AZD/BEZ treatment caused an SD + PR rate of 62% (Fig. 3B) and more than doubled median survival compared with untreated animals (from 15 to 36 days, \( P < 0.0001 \); Fig. 3C). Compared to other active regimens (sunitinib and carboplatin–paclitaxel) and single-agent AZD or BEZ, the combination was superior in terms of response and survival prolongation. Therefore, combined PI3K/mTOR and MEK inhibition afforded significant clinical benefit in a murine transplantation model of the claudin-low breast cancer subtype.

**Testing of AZD/BEZ in diverse GEM breast cancer models**

Given the activity of this regimen in RAS-driven melanoma and claudin-low breast cancer, we determined the efficacy of this regimen in 2 other breast cancer GEMMs: representing the basal-like (or BBC, C3-TAg) and luminal (MMTV-c-neu) subtypes of breast cancer. These models have been previously credentialed by unbiased gene expression analysis that indicated maximal similarity to their respective human breast cancer subtypes (BBC and luminal) from a large panel of other GEM breast models (24). The C3-TAg model (19) harbors a standard transgenic allele with a breast-specific promoter driving overexpression of the simian virus 40 Large T Antigen (TAG), which inactivates the p53 and RB tumor suppressors. This model is faithful to human BBC, which frequently features combined RB and p53 loss (32–36), and C3-TAg and human BBC show common patterns of gene expression by RNA expression profiling (24, 32). Of relevance to AZD/BEZ testing, tumors from this model frequently (~30%), but not always, acquire K-Ras amplification with progression (37), and haploid loss of K-Ras delays progression in this model (38). Therefore, a fraction of tumors in this GEMM likely require persistent RAS activation for tumor maintenance, and we reasoned differential sensitivity to AZD/BEZ might correlate with K-Ras activation status.

We were surprised, therefore, to note potent activity for the dual AZD/BEZ treatment in nearly all tumors from this BBC model. As in the TRIA model, AZD/BEZ treatment resulted in significant tumor regression and improved survival compared with untreated mice or mice treated with other active therapeutic regimens (Fig. 4A–C). A waterfall plot of best response (21 days or later) showed SD + PR + CR (SD, stable disease; PR, partial response; CR, complete response) in 16 of 17 treated mice, indicating a 94% response rate, with almost half of the treated mice exhibiting CR. This high response rate translated into a marked survival benefit with median survival increasing by 115% compared with untreated animals (33–71 days, \( P < 0.0001 \)). As in the TRIA model, the response rate and survival advantage of AZD/BEZ was superior to any other regimen tested in this model (of \( N = 15 \) regimens; Usary...
Figure 2. Transcriptional analysis of murine models of mammary carcinoma and melanoma. A, an unsupervised cluster of all probes with a log₂ absolute expression value greater than 2 on at least 3 microarrays (2,584 probes). The colored bars correspond to the region of the cluster for the gene lists in parts C to E. B, highlighted is the location in the dendrogram of the C3-TAg, MMTV-c-neu, claudin-low (including T11 OST tumors), and melanoma tumors (including TRIA tumors). Below are markers of the claudin-low subtype as defined by Prat and colleagues (25). C, a cluster of genes that are downregulated in both claudin-low and melanoma tumors. D, a cluster of genes that are upregulated in tumors from 6 different claudin-low and 4 different melanoma models. E, a melanoma specific gene cluster. F, a rank of median differentiation scores for tumors from the indicated breast and melanoma models.
et al., submitted; see also Fig. 4A and C). For example, the next most active regimens (sunitinib or carboplatin–paclitaxel) only modestly extended survival (64% and 33%) compared with AZD/BEZ. Similarly, neither AZD nor BEZ was nearly as active in this model as a single-agent therapy compared with combined therapy (Fig. 4A and C). These data indicate AZD/BEZ induces significant tumor response and extends survival in a credentialed GEM model of BBC, regardless of the presence or absence of K-Ras amplification.

Given this lack of general correlation between AZD/BEZ efficacy and Ras mutation in the C3-TAg model, we considered a model that does not harbor Ras mutation or amplification: the MMTV-c-neu GEMM. This mouse model of luminal breast cancer features transgenic expression of the rat ortholog of human HER2 driven by an ER-enhanced breast promoter (MMTV; ref. 20). Treatment with AZD/BEZ was also highly active in this model (Fig. 5A–C), with a PR + CR rate of 100% (12 of 12), with 5 CRs. Several regimens (e.g., carboplatin–paclitaxel, lapatinib) induced responses and significantly prolonged survival in this model, as did single-agent treatment with AZD and BEZ (Fig. 5C, all \( P < 0.001 \) in pairwise comparison with untreated mice). As expected, this model was exquisitely sensitive to lapatinib (100% CR rate; Fig. 5A and C), a HER2 kinase inhibitor that is FDA-approved for HER2-amplified breast cancer in humans. Remarkably, however, the AZD/BEZ combination was better in terms of response than all other regimens except lapatinib (Fig. 5A), and equivalent to or better than

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Figure 3. Combined PI3K/mTOR/MEK inhibition is the most effective treatment regimen in claudin-low breast cancer model. T11 mice were stratified by tumor size and randomly assigned to a treatment cohort. A, combined AZD/BEZ was the most effective treatment regimen as measured by percent change in tumor volume at day 14. B, waterfall plot distribution of tumor response from combined AZD/BEZ compared to the average tumor size of untreated animals on day 14. AZD/BEZ response represents the best response seen on day 14 or beyond. Negative values indicate tumor shrinkage. C, combined AZD/BEZ treatment prolonged median survival from 15 to 32 days; MS, median survival.
lapatinib in terms of survival prolongation, inducing a striking 400% increase in median survival (29–173 days, \( P < 0.0001 \); Fig. 5C). Therefore, combined PI3K/mTOR and MEK inhibition offers equivalent activity in HER2-driven GEMM (expressing wild-type RAS) compared to a FDA-approved targeted anti-HER2 agent.

**Cross-talk between PI3K and MEK signaling**

Although AZD6244 and BEZ235 both showed only modest single-agent activity in the models studied, the combined AZD/BEZ regimen was highly effective in each of these models. In an effort to understand the molecular basis for this synergy, cell lines derived from the tumor models were treated *in vitro* with AZD6244 (1 \( \mu \text{mol/L} \)), BEZ235 (250 \( \text{nmol/L} \)), or combination AZD/BEZ (1 \( \mu \text{mol/L} \)/250 \( \text{nmol/L} \)). Tumor-derived cell lines from TRIA, T11, and C3-TAg tumors were studied; the MMTV-c-neu model was not considered as cell lines could not be derived from this model. Cell lines were treated for 24 hours with the indicated compounds, and analyzed by Western blot for

![Figure 4](link)

**Figure 4.** Basal-like breast cancer model is exquisitely sensitive to combined PI3K/mTOR/MEK inhibition. C3-TAg mice were stratified by tumor size and randomly assigned to a treatment cohort. A, combined AZD/BEZ was the most effective treatment regimen, resulting in a median percent change in tumor volume at day 21 of \(-84\%\). B, waterfall plot distribution of tumor response from combined AZD/BEZ compared with the average tumor size of untreated animals on day 21. AZD/BEZ response represents the best response seen on day 21 or beyond. Negative values indicate tumor shrinkage. C, combined AZD/BEZ treatment prolonged median survival from 33 to 71 days; MS, median survival.
target inhibition (Fig. 6). Expression of pERK was low in all 3 cell lines, and was not affected by single-agent AZD6244 treatment. Single-agent AZD6244 treatment produced an increase in phosphorylation of AKT at serine 473, a site phosphorylated by mTOR. In contrast, single-agent BEZ235 treatment decreased S473 phosphorylation of AKT, as expected and consistent with inhibition of mTOR, but also led to a marked increase in phosphorylation of ERK1/2. Cotreatment with AZD6244 and BEZ235 inhibited phosphorylation of both AKT and ERK1/2, indicating effective target inhibition. The surprising activation of AKT by MEK inhibitors and ERK activation by PI3K inhibitors has been reported in other cell types (39–43), and reflects compensatory feedback loops between these pathways. We believe these complex inhibitory networks explain the modest activity of PI3K and MEK inhibitors as single agents in the multiple murine models thus far tested, and also explain the potent synergy of the combined AZD/BEZ regimen in vivo, which is seen in our in vivo results.

**Dual PI3K/MEK inhibition in lapatinib-resistant HER2⁺ breast cancers**

We next turned to an assessment of the AZD/BEZ regimen in the setting of resistant disease. Given the potent activity of
AZD/BEZ in the MMTV-c-neu model, we decided to evaluate the efficacy of this regimen in tumors that had become completely refractory to lapatinib therapy. MMTV-c-neu tumors will always respond completely to initial lapatinib therapy (Fig. 5A), but then resistant tumors will develop on therapy causing progression and death within 150 days on treatment (Fig. 5C). Given this observation, a model to study lapatinib-resistant disease was developed as shown in Fig. 7A. In brief, at the time of progression and while on continuous lapatinib therapy, resistant tumors were harvested and orthotopically passaged into the mammary fat pad of syngeneic female mice. Tumors were allowed to establish to a size of at least 5 mm in any one direction before the initiation of lapatinib rechallenge or second-line treatment. As expected, retreatment with lapatinib in mice transplanted with lapatinib-resistant tumors was not

![Figure 6](image_url)

**Figure 6.** Inhibition of AZD6244 and BEZ235 targets in tumor-derived cell lines. To evaluate the molecular response to AZD6244, BEZ235, or combined AZD/BEZ treatment, tumor-derived cell lines from TRIA, T11, and C3Tag tumors were treated for 24 hours with AZD6244 (1 µmol/L) and/or BEZ235 (250 nmol/L) and evaluated by Western blot analysis for target inhibition. Single-agent AZD led to PI3K activation (as evidenced by increased S473 phosphorylation of AKT), whereas single-agent BEZ led to ERK activation (as evidenced by increased phosphorylation of ERK1/2). Combined treatment with AZD/BEZ produced combined inhibition of the PI3K/mTOR and MEK/ERK pathways.

AZD/BEZ in the MMTV-c-neu model, we decided to evaluate the efficacy of this regimen in tumors that had become completely refractory to lapatinib therapy. MMTV-c-neu tumors will always respond completely to initial lapatinib therapy (Fig. 5A), but then resistant tumors will develop on therapy causing progression and death within 150 days on treatment (Fig. 5C). Given this observation, a model to study lapatinib-resistant disease was developed as shown in Fig. 7A. In brief, at the time of progression and while on continuous lapatinib therapy, resistant tumors were harvested and orthotopically passaged into the mammary fat pad of syngeneic female mice. Tumors were allowed to establish to a size of at least 5 mm in any one direction before the initiation of lapatinib rechallenge or second-line treatment. As expected, retreatment with lapatinib in mice transplanted with lapatinib-resistant tumors was not
effective (Fig. 7B and C). Remarkably, however, combined AZD/BEZ treatment was highly active in lapatinib-resistant disease, resulting in median tumor reduction of 57% (Fig. 5B, \( P = 0.007 \)) and increasing survival 290% (from 29 to 113 days, \( P < 0.001 \), Fig. 7C) compared with lapatinib retreatment. These data show that AZD/BEZ therapy can be effective in tumors chosen for therapeutic refractoriness to other agents.

To further evaluate the mechanism of lapatinib resistance, we isolated tumors from mice that were lapatinib naïve, responding to lapatinib treatment, lapatinib resistant, and lapatinib resistant responding to AZD/BEZ treatment. These tumors were harvested directly from mice while on therapy, and then analyzed by Western blot for target inhibition. This analysis showed that lapatinib-resistant tumors exhibited increased phosphorylation of AKT S473 (mTOR target), MEK1/2, and ERK1/2 (Fig. 7D). Treatment of lapatinib-resistant tumors with AZD/BEZ led to phosphorylation levels of these proteins equivalent to that seen in lapatinib-sensitive tumors on lapatinib (compare lanes 4 and lanes 2 of Fig. 7D). These data show that lapatinib resistance occurs in the setting of MEK/ERK and PI3K/mTOR activation, explaining the efficacy of the AZD/BEZ regimen even in lapatinib-resistant disease.

Discussion

In this work, we show that therapeutic testing in GEM and OST models can be carried out in a medium-throughput manner at reasonable expense to identify unexpected therapeutic single-agent and combination regimens. We also show potent antitumor activity of combined therapy with PI3K/mTOR inhibitors and MEK inhibitors in a diverse array of credentialed GEM and OST models. We showed impressive activity of this regimen in the RAS-driven melanoma model. Based on a shared gene expression between melanoma and claudin-low breast tumors, we tested AZD/BEZ in the T11 OST model, which also harbors an activating mutation of K-Ras. Given significant activity in this treatment-refractory and aggressive model, we then turned to 2 additional breast GEM models representing BBC (C3-TAg mice) and luminal breast cancer (MMTV-c-neu), which are not obligately RAS-driven. We observed broad and reproducible antitumor activity of the AZD/BEZ regimen in all of these disparate models, in accord with prior studies in GEM models of NSCLC (17, 44). In aggregate, these studies predict broad antitumor efficacy of combined MEK and PI3K/mTOR inhibitors in human cancer patients.

This large experience in animal models points to some of the strengths of GEM testing versus conventional measures to assess preclinical efficacy. For example, certain regimens (e.g., carboplatin–paclitaxel and sunitinib in TRIA mice) clearly reduced tumor growth rates, and therefore would score highly in traditional tumor-growth inhibition (TGI) assays. When assessed by more rigorous standards, however, these regimens were not capable of inducing tumor regression (Fig. 1A) and produced only modest enhancement of long-term survival (Fig. 1C). Our results using tumor regression and long-term survival correspond better to the efficacy of these agents in human patients (Table 1) than predictions based on TGI in xenograft systems. Also in contrast to xenograft models, we have generally observed significant heterogeneity among tumors within any given model in terms of rate of growth and response rates to active regimens. We believe this more faithfully models the human setting and provides an opportunity to understand primary therapeutic resistance to active agents, an area of intense ongoing study.

We believe that the implications of this work for human therapeutics are considerable, as testing of MEK and PI3K/mTOR inhibitors has already begun in human cancer patients. Our data showing compensatory activation of ERK signaling by PI3K/mTOR inhibitors and compensatory activation of AKT by MEK inhibitors explains the observed therapeutic synergy of the AZD/BEZ combination, and suggest that single-agent trials of these agents should rapidly give way to combination trials in certain tumor types. Of note, there are approximately 17 ongoing early phase clinical trials evaluating 14 unique combinations of MEK and PI3K/mTOR inhibitors, and preliminary results for a few of these novel combinations have been promising (clinicaltrials.gov; ref. 45). In addition, our data suggest trials of combined PI3K/mTOR and MEK inhibitors should be opened in a variety of cancers, and in particular should focus on RAS-mutant cancers for which no effective targeted approaches exist. Our data also predict that resistance to combined MEK and PI3K/mTOR inhibition will develop within months of starting therapy (see Figs. 1C, 3C, and 4C), emphasizing the immediate need to study acquired resistance in human clinical specimens as well as murine models. Toward that end, these models will provide an excellent platform to study both de novo and acquired resistance to AZD/BEZ and related combinations. The finding in lapatinib-resistant tumors of ERK/AKT activation and significant sensitivity to the AZD/BEZ regimen (Fig. 7B–D) is particularly encouraging. This result suggests that mechanisms of resistance to HER2 kinase inhibitors still require activation of persistent downstream HER2 effectors such as PI3K, mTOR, and/or MEK. This observation is analogous to the finding that resistance to B-RAF inhibitors in melanoma often requires downstream activation of MEK (reviewed in ref. 46). Similarly, although lapatinib resistance in HER2-driven breast cancer is imperfectly understood, resistance is frequently associated with increased activation of the PI3K pathway (reviewed in ref. 47). Therefore, our results suggest that the AZD/BEZ combination can be active in tumors resistant to other targeted kinase inhibitors, and also suggest the possibility that early combination of these agents (e.g., with lapatinib) may limit primary refractory disease and development of secondary resistance.

A critical question based on these results relates to the toxicity of this regimen. Because of practical concerns, we do not conduct comprehensive toxicologic assessment of regimens in the MP11. We did conduct the serial assessment of animal weights on all treated mice, as well as monitoring of blood counts in a subset of treated animals, and by these...
measures, toxicity of this regimen was manageable. In some mice, brief treatment breaks were given because of weight loss, and often we observed tumor regrowth during these treatment breaks, further emphasizing the activity of this regimen. Gratifyingly, the limited amount of human phase I testing thus far accomplished with combined MEK and PI3K inhibitors suggests these combinations are not too toxic for human use (48). In summary, our work predicts broad clinical activity of the combination of MEK and PI3K/mTOR inhibitors in a large variety of human malignancies with expected tolerable levels of toxicity.

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

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Please enter any additional contributions here: Molecular analysis of drugs in tumors and cells via western blot (J. S. Duncan).

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Combined PI3K/mTOR and MEK Inhibition Provides Broad Antitumor Activity in Faithful Murine Cancer Models


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