PI3K-AKT Signaling Is a Downstream Effector of Retinoid Prevention of Murine Basal Cell Carcinogenesis

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Abstract
Basal cell carcinoma (BCC) is the most common human cancer. We have demonstrated previously that topical application of the retinoid prodrug tazarotene profoundly inhibits murine BCC carcinogenesis via retinoic acid receptor-γ-mediated regulation of tumor cell transcription. Because topical retinoids can cause adverse cutaneous effects and because tumors can develop resistance to retinoids, we have investigated mechanisms downstream of tazarotene’s antitumor effect in this model. Specifically we have used (i) global expression profiling to identify and (ii) functional cell-based assays to validate the phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway as a downstream target pathway of tazarotene’s action. Crucially, we have demonstrated that pharmacologic inhibition of this downstream pathway profoundly reduces murine BCC cell proliferation and tumorigenesis both in vitro and in vivo. These data identify PI3K/AKT/mTOR signaling as a highly attractive target for BCC chemoprevention and indicate more generally that this pathway may be, in some contexts, an important mediator of retinoid anticancer effects. Cancer Prev Res; 7(4); 407–17. ©2014 AACR.

Introduction
Basal cell carcinomas (BCC) comprise approximately one third of all cancers in the United States with an incidence that is increasing by 4% to 5% per year and with a cost to Medicare alone of $400 million/year (1). Inappropriate hyperactivation of hedgehog signaling is the pivotal abnormality that underlies BCC carcinogenesis in both sporadic and inherited (basal cell nevus [Gorlin] syndrome; BCNS; OMIM #109400) cases and generally is caused by mutational inactivation of the tumor suppressor gene PATCHED 1 (PTCH1) or by gain-of-function mutations in the hedgehog pathway effector SMOOTHENED (SMO; ref. 2). Like PTCH1+/− patients with BCNS Ptch1+/− mice exposed to clinically relevant environmental mutagens develop multiple BCCs, thus providing an accurate and practical model of human BCCs (3, 4).

Endogenous retinoids have a major role in skin homeostasis (5), and pharmacologic doses of oral retinoids can inhibit BCC carcinogenesis in humans, albeit with significant systemic toxicities (6). In the epidermis, the major retinoid receptors are retinoic acid receptors (RAR) α and γ and retinoid X receptor (RXR) α. Previously we found that topical application of tazarotene (Tazorac), an anti-acne retinoid prodrug that is converted into tazarotenic acid and specifically activates RARE and γ (7), robustly prevents BCCs in Ptch1+/− mice (8, 9). In addition, a pharmacologic pan-RAR antagonist that inhibits physiologic RAR signaling in skin increases the BCC burden (8). These observations suggest that physiological retinoid signaling in the skin restrains BCC carcinogenesis and are consistent with the observation that long-term topical tazarotene cures 30% to 50% of sporadic human BCCs (10). Approximately 60% of untreated visible BCCs arising in Ptch1+/− mice have partial or total loss of RARγ expression, suggesting that receptor loss may underlie the resistance of some BCCs to tazarotene. We have assessed the chemopreventive efficacy of topical tazarotene in a phase II clinical trial (11). Although topical tazarotene may be found to be an effective agent for long-term chemoprevention of human BCCs under some circumstances, some patients are deterred from using it optimally because topical retinoids irritate the skin, and this irritation may account for some of the disparity between the robust results in mice and the comparatively disappointing effects in humans.

Therefore, we have investigated downstream pathways mediating tazarotene’s anti-BCC chemopreventive efficacy to search for novel therapeutic strategies that (i) might be tolerated better and (ii) might overcome retinoid resistance.
Materials and Methods

Global gene changes with tazarotene

BCC cell line treatment and RNA extraction. We cultured ASZ001 cells as described previously (12). Cells at 80% confluency were incubated in serum-free media (154-CF medium containing 0.05 mmol/L CaCl₂ and 1 × penicillin/streptomycin) for 2 hours. Working concentrations of tazarotene and dimethyl sulfoxide (DMSO) were prepared as follows: for tazarotene, the powder was dissolved in 100% DMSO at a stock concentration of 10 mmol/L and diluted to 10 μmol/L working concentration in 154-CF medium containing 0.05 mmol/L CaCl₂ and 1 × penicillin/streptomycin. We then incubated cells with 10 μmol/L tazarotene or 0.1% DMSO for 10 or 24 hours in a 5% CO₂ incubator. Four replicates were carried out for each treatment, and the 3 replicates with the best RNA integrity were analyzed with Affymetrix gene expression arrays. We analyzed a further replicate in which ASZ001 cells were incubated for 48 hours in 0.1% DMSO or 10 μmol/L tazarotene using the Wst-1 cell-proliferation assay (Roche Applied Science) to confirm cell-proliferation inhibition by 10 μmol/L tazarotene. After the incubation, cells were washed in PBS and harvested in 1 mL of TRIzol for RNA extraction (Invitrogen). Total RNA was repurified using RNasy RNA isolation columns (Qiagen), and RNA integrity was confirmed with the RNA Bioanalyzer (Ambion/Applied Biosystems). Two micrograms of total RNA were used for cRNA amplification using the MessageAmp II-Biotin Kit (Ambion/Applied Biosystems). Briefly, reverse transcription of total RNA was carried out using an oligo (dT) primer bearing a T7 promoter using ArrayScript. Second-strand synthesis using the cDNA was carried out and purified for use as a template for in vitro transcription in a reaction containing biotin-modified UTP and T7 RNA polymerase (Ambion/Applied Biosystems). Biotin-labeled, amplified RNA (aRNA) was then purified for gene expression analysis on Mouse Genome 430A 2.0 Array GeneChips (Affymetrix), at the Gladstone Genomics Core Facility. Assessment of hybridization quality was also performed (Gladstone Genomics Core Facility) using the Bioconductor software affyPLM, which fitted a specified robust linear model to the probe-level data. Preprocessing of the data was performed using an bioconductor robust multiarray analysis algorithm to correct for background; the data were normalized using the quantile method (13) to generate lists of statistically significant, differentially expressed genes (comparing tazarotene and DMSO vehicle control groups). After correcting for the false discovery rate, Partek Genomics Suite was used for principal component analysis and hierarchical clustering, and differentially expressed genes that were significantly differentially expressed were further analyzed using bioinformatic software from Ingenuity Systems and Stratagene.

Generation of stably-transfected Myr-HA-AKT1 and HA-AKT1 cell lines

One million log-phase ASZ001 cells were transfected with pLNCCX/Myr-HA-AKT1 and pLNCCX-HA-AKT1 (ref. 14; Addgene #903 and #901, respectively), using program T29 of the Amaxa Nucleofection system (Amaxa-Lonza; ref. 12). As a negative control, plasmid #903 was digested with restriction enzymes to remove the anti-hemagglutinin (HA) tag and most of the AKT1 open reading frame, generating pLNCCX. Cells were then mixed with 154-CF complete media and replated. Media was removed after 24 hours to remove the dead cells, and living cells were allowed to recover for 1 to 2 days and then at 70% confluency were passaged and replated at a lower density. After 4 days, ASZ001 cells containing the pLNCCX-Myr-HA-AKT1, pLNCCX-HA-AKT1, or pLNCCX were selected using G418 (Life Technologies) at 1 mg/mL (a dose that killed nontransfected ASZ001 cells) to generate myr-HA-AKT1, HA-AKT1, and pLNCCX cell lines, respectively. After at least 1 month of G418 selection, cells were expanded for cell-proliferation experiments.

In vivo pharmacologic phosphoinositide 3-kinase inhibitor drug efficacy testing

Mice. Ptch1+/− K14-CreERT² p53f/f mice treated with tamoxifen (0.1 mg/day) for 3 days at age 1.5 months and 4 Gray (Gy) of X-rays at age 8 weeks were given drugs by oral gavage 5 days/week from age 13 weeks until age 21 weeks when a dorsal skin biopsy (1 cm × 1 cm) was obtained (9). Mice were then monitored for the first visible BCC, and visible BCC burden was compared at age 28 weeks. Mice that died or were euthanized for unrelated causes were censored in the study.

In vivo drug treatments. Small molecule phosphoinositide 3-kinase (PI3K) inhibitors GDC-0941 (a gift from Genentech-Roche), XL147, and XL765 (gifts from Exelixis) were given by gavage at 50 mg/kg every day, 100 mg/kg, and 30 mg/kg twice a day, respectively.

Tazarotene-resistant allograft assessment. A tazarotene-resistant clone was established by challenging BCC allografts with high dose of tazarotene orally (10 mg/kg daily, 5 days per week) for 3 weeks followed by a low dose of tazarotene (2 mg/kg daily, 5 days per week) for 8 weeks. One resistant clone was then further transplanted into 3 naïve NOD/SCID mice (2 sites per mouse). When allografts became palpable (age 7 weeks), one mouse each received treatment with vehicle, tazarotene (5 mg/kg daily, 5 days per week), or XL765 (30 mg/kg twice daily, 5 days per week). Tumor volume was assessed for each mouse.

Results

Tazarotene induces gene expression changes in murine BCC cells in vitro

To explore tazarotene’s anti-BCC mechanism of action, we began by assessing global gene expression changes induced by tazarotene in ASZ001 cells, a line derived from a Pch1+/− mouse BCC. We treated log-phase cells in serum-free conditions for 10 or 24 hours in triplicate with 0 or 10 μmol/L tazarotene, a concentration that inhibits their proliferation by at least 50% after 48 hours and does not inhibit the proliferation of either the immortalized murine nontumorigenic keratinocyte cell line C5N or of a murine medulloblastoma cell line (12). We selected these timepoints for analysis based on detectable increases in
expression of Crabp2, a direct RA target gene (12) at 10 hours of incubation and the first detectable reduction of cell proliferation at 24 hours of incubation with 10 μmol/L tazarotene (data not shown). We extracted RNA and converted it to cDNA, which we then hybridized to Mouse Genome 430A 2.0 Array GeneChips (Affymetrix) containing 20,000 probes representing 14,000 transcripts. Each incubation time point and treatment group was hybridized at the same time (i.e., hybridized under similar conditions).

Preprocessing of the raw data from the arrays indicated that for each experimental group (i.e., tazarotene or DMSO treated) the technical replicates were similar to one another and of good quality (data not shown). The quantile-normalized array data for treatments with vehicle and tazarotene and for both incubation timepoints were compared by principal component analysis using the Partek Genomics Suite software (Fig. 1A), which indicated a high degree of concordance between the replicates and demonstrated that tazarotene treatment significantly perturbed the gene expression profiles of ASZ001 cells at both 10 and 24 hours, compared with DMSO treatment, at both time points (Fig. 1B). After adjustment for the false discovery rate (FDR), comparison to the 10 or 24 hour DMSO control gene sets generated statistically significant (P < 0.05) lists of differentially expressed genes (Fig. 1C). Tazarotene treatment at 10 hours gave 279 differentially expressed genes and expressed sequence tags (EST), which after disregarding replicate probes generated a list of 240 differentially expressed genes of which 193 were upregulated and 47 were downregulated (P < 0.05). Tazarotene treatment at 24 hours yielded 649 differentially expressed genes, excluding gene/EST replicates, of which 146 were upregulated and 503 were downregulated (P < 0.05). The top 30 gene probes (including replicates) with the most up- and downregulated expression at 10 hours are listed in Supplementary Tables S1 and S2, respectively, as are the top differentially expressed gene probes after 24 hours tazarotene treatment (Supplementary Tables S3 and S4, respectively). The greater number of upregulated than of downregulated genes at 10 hours is consistent with a direct transcriptional activator effect of RARs, which fully dissociate from corepressors/silencing mediators and bind to coactivators in the presence of a retinoid hormone agonist such as tazarotenic acid to activate retinoid-target genes (15, 16). Indeed, known RA target genes such as Tgm2, Dhrs3, and Rai3 were upregulated after 10-hour tazarotene incubation (Fig. 1D, and data not shown). At 24 hours however, more genes were downregulated than upregulated, likely as a result of the secondary effects of tazarotene treatment. We designated genes whose expression was altered by 10 hours as "early" and those whose expression was altered at 24 hours but not at 10 hours as "late." We used qPCR to confirm the altered expression of a selection of the 10-hour differentially expressed genes—Fst, Tgm2, Trib3, Eif4ebp1 (4E-BP1), Gadd45a, Ndrg1, and Dtx4 (Fig. 1D). To investigate whether tazarotene specifically downregulates hedgehog signaling, we searched the differentially expressed gene lists for known direct hedgehog target genes (i.e., genes that contain the consensus Gli binding site) such as Gli1, Pit1, Hhip1, Nmyc1, Cnd1, Cnd2, Grem1, Fst, and Pthlh (17). Of these genes, only Fst and Pthlh were downregulated at 10 hours (Fig. 1D, and data not shown). Fst was also downregulated at 24 hours. Other genes that are strongly associated with hedgehog signaling are FoxM1, Cnd1, and Gas1. We found that FoxM1 expression was downregulated after 24 hours of tazarotene treatment and not at 10 hours, suggesting it is an indirect target of tazarotene signaling. Gas1 was downregulated at 10 and 24 hours whereas Cnd1 was not represented. However, other cyclins—Ccnb2, Ccnb1, and Ccnb2—were downregulated at 24 hours (data not shown), suggesting that these late differentially expressed genes are indirect targets of tazarotene-mediated signaling and that at least part of tazarotene’s anti-BCC efficacy is via blocking of cell-cycle progression at the G2–M checkpoint. Differentially expressed genes such as Tgm2, Dtx4, Eif4ebp1, Fst, and Trib3 were represented more than once in the differentially expressed gene lists (i.e., by replicate probes on the microarrays), suggesting that these genes are likely to be "real" targets of tazarotene-mediated signaling (Fig. 1D, left graph). However, 10 μmol/L tazarotene treatment of both ASZ001 and the murine medulloblastoma cell line Med1 (a cell line whose proliferation is not altered by 10 μmol/L tazarotene treatment) upregulated Tgm2 expression in both cell lines, that is irrespective of whether or not tazarotene inhibited cell proliferation (data not shown). This suggested that Tgm2 upregulation was not related to tazarotene’s antiproliferative effects, but rather to direct effects of retinoid transcriptional activation of target genes involved in other skin biofunctions. Therefore, because (i) the numbers of differentially expressed genes from tazarotene treatment at both 10 and 24 hours were relatively high, and (ii) changes in individual differentially expressed genes such as Tgm2 suggest biologic processes that may be irrelevant to tazarotene’s anticancer effects, we used bioinformatic software to identify tazarotene-altered pathways and functions. Bioinformatic analyses of the 10- and 24-hour differentially expressed genes using Stratagenes’s Pathway Architect software indicated that the differentially expressed genes with the most connections to other differentially expressed genes in their respective lists were associated with VEGF (Supplementary Table S5) and insulin-like growth factor-insulin receptor/phosphatidylinositol 3-kinase/AKT (IGF-IR/Pi3K/AKT) signaling (Supplementary Tables S5 and S6). Of note, the IGF-IR/Pi3K/AKT pathway was represented in the analyses of both 10- and 24-hour differentially expressed genes lists. Gene network analyses using the Ingenuity Systems software suggested 3 top networks at 10 hours, one of which, again, was the IGF-IR/Pi3K/AKT pathway, indicating that the latter may be a central downstream functional “node” involved in BCC inhibition by tazarotene (Fig. 2). The other 2 “top” networks identified by Ingenuity Systems software were cholesterol metabolism and the mitogen-activated protein kinase pathway, the latter of which has been found to interact with hedgehog signaling (refs. 18 and 19; data not shown). Bioinformatic functional clustering analyses of the
Figure 1. Global gene expression analysis of tazarotene-treated ASZ001 cells indicated that the expression of a relatively large number of genes was altered significantly. A, principal component analysis of the Affymetrix array data showed that treatment with 10 μmol/L tazarotene for 10 or 24 hours significantly altered the gene expression profile of ASZ001 in a manner that was distinct from treatment with 0.1% DMSO for 10 and 24 hours (\( P < 0.05 \)). The red triangles and squares represent genes from replicate samples treated with 0.1% DMSO for 10 and 24 hours, respectively, whereas the blue triangles and squares represent genes from replicates treated with 10 μmol/L tazarotene for 10 and 24 hours, respectively. (Continued on the following page.)
late differentially expressed genes using Ingenuity Systems software suggested that many of these were linked to biologic processes such as cancer, cell cycling, and DNA recombination, and repair (P < 0.0001; Supplementary Table S7).

Also, a “top canonical pathway” cluster analysis using the same software suggested that the 24-hour differentially expressed genes affected by tazarotene treatment were associated with metabolism and cell-cycle processes (P < 0.0001; Supplementary Table S8), which are known to be regulated by IGF-IR/PI3K/AKT signaling (20). Moreover, the observation that differentially expressed genes such as Trib3, Eif4ebp1, and Igfbp3, all of which are negative regulators of the IGF-IR/PI3K/AKT pathway, were upregulated by tazarotene suggests that at least part of tazarotene’s anti-BCC effect is via inhibition of the IGF-IR/PI3K/AKT pathway with consequent decrease in cell growth and proliferation.

B. heatmap analyses of the standardized array data (using Partek Genomics Suite) showed that the 10 and 24 hours tazarotene-treated samples had similar gene expression profiles, which were distinct from the 10 and 24 hours DMSO-treated samples: expression levels of 4292 genes were significantly altered with 10 μmol/L tazarotene treatment compared with treatment with 0.1% DMSO (P < 0.05, ANOVA test followed by FDR correction). C, using Bioconductor software, MA-plot analyses of all gene probes showed that a relatively large number of genes were differentially expressed between tazarotene- and DMSO-treated samples. The genes whose expression was statistically significant altered (P < 0.05) sat as outliers (red dots) from the majority of the gene probes (gray dots) that were either not, or only modestly, differentially expressed. For lists of the most differentially expressed genes, see Supplementary Tables S1 to S4. D, graphical presentation of a number of statistically significant gene probes from the 10-hour Bioconductor gene set (left graph), and taqman real-time qPCR validation of the altered expression with tazarotene treatment (right graph; P < 0.05). The different colored bars for a gene in the microarray expression data graph indicated that replicates gene probes that were significantly differentially expressed (left graph; P denotes an Affymetrix gene probe). For Tgm2, in tazarotene-treated ASZ001 cells four differentially expressed gene probes were represented in the 10-hour Bioconductor list in a statistically significant manner (P < 0.05). Taqman PCR confirmed the differential expression of the selected 10-hour differentially expressed genes, whose expression is shown as fold change compared with the DMSO-treated ASZ001 samples.
Overexpression of AKT1 in ASZ001 cells reduces the in vitro anti-BCC effect of tazarotene

To investigate whether BCCs that arise in our Ptch1+/−K14-CreER2 p53<sup>flox/flox</sup> mice have activated PI3K/AKT signaling, we assessed expression of phosphorylated (Ser473; activated) AKT (p-AKT) immunohistochemically. We detected activated AKT at low-to-moderate levels in 50% (4/8) of visible IR-induced BCCs in these mice (Fig. 3A), a finding similar to that of the moderate levels of p-AKT activity detected in human BCCs (21). Similarly, we also detected activated AKT signaling in our ASZ001 BCC cell line (Fig. 3A).

To test whether tazarotene inhibits proliferation at least in part by blocking IGF-IR/PI3K/AKT signaling, constructs containing HA-tagged wild-type AKT1 (HA-AKT1) or myristoylated AKT1 (myr-HA-AKT1) were selected with G418 to generate stable HA-AKT1 expressing cell lines (left). FACS analyses demonstrated that both stable cell lines generated expressed heterogeneous levels of AKT1 as measured by the HA-tag levels and indicated that only 10% and 12% of cells that were stably transfected with the AKT1 constructs expressed high levels of HA-myr-AKT1 and HA-AKT1, respectively (middle table). Slightly higher levels of AKT1 in the HA-AKT1 cell line compared with the negative control cell line (pLNCX) were confirmed by Western blotting with antibodies against AKT1. Western blotting for tubulin (on the same blot) indicated that similar amounts of protein were loaded (right panel). C, 10 μmol/L tazarotene treatment of these cell lines for 48 hours showed that the cell lines overexpressing AKT1 were partially resistant to tazarotene compared with the negative control cell line, pLNCX (P < 0.05, Student t test). D, FACS analysis of the HA-AKT1 cell line after 48-hour incubation in 10 μmol/L tazarotene or 0.1% DMSO vehicle showed that tazarotene enriched the population of HA-AKT1 cells as measured by HA-tag levels, suggesting that AKT1 overexpressing cells were more resistant to tazarotene treatment (P < 0.05, Student t test).
PI3K-AKT Signaling Is Target of Retinoid Inhibition of BCCs

PI3K inhibitor that inhibits PI3K activity via competitive inhibition of an ATP binding site on the p85 subunit of PI3K. LY294002 not only binds to class I PI3Ks and other PI3K-related kinases, inhibiting PI3K-dependent production of the second messenger PIP3, but also to novel targets unrelated to the PI3K family (22). XL147 is a potent, orally bioavailable, specific inhibitor of class I PI3K kinases α, β, δ, and γ, which also binds the ATP-binding pocket of the catalytic domain of class I PI3Ks and at nanomolar concentrations inhibits PI3K phosphorylation and consequent PI3K-dependent production of the second messenger PIP3 (23). Similarly, XL765 is also a bioavailable, highly selective, potent inhibitor of all four class I PI3K isoforms with IC50 values at nanomolar concentrations in biochemical assays. However, in contrast to XL147, XL765 also inhibits mTOR and DNA-PK with IC50 values in the nanomolar range: in cellular assays, XL765 can inhibit nutrient stimulated mTOR-dependent signaling by inhibiting mTOR-dependent phosphorylation of key PI3K pathway components including AKT, the AKT substrates PRAS40 and GSK3β, p70S6K, and the p70S6K substrate S6, and 4EBP1 (24). XL765 treatment causes tumor regression, with associated decreased cellular proliferation and angiogenesis and increased apoptosis in various cancer models (25). We found that all 3 pharmacologic inhibitors caused dose-dependent reduction of ASZ001 cell proliferation (Fig. 4). XL147 and XL765 seemed to be more effective than LY294002 in their inhibition of ASZ001, significantly reducing proliferation with an IC50 dose at approximately 5 μmol/L and with a more pronounced inhibition at 10 μmol/L (P < 0.0001). The IGF-IR inhibitor AG1024 (Sigma-Aldrich) also inhibited ASZ001 cell proliferation at 10 μmol/L (data not shown). These data are similar to those we observed for tazarotene treatment of ASZ001 cells (12) and are consistent with the idea that much of tazarotene’s anti-BCC efficacy is mediated via inhibition of this pathway.

Pharmacologic PI3K inhibitors inhibit BCC proliferation in vivo

Having showed that inhibition of PI3K/AKT signaling can reduce BCC cell proliferation in vitro, we investigated the chemopreventive efficacy of XL147 and XL765 as well as of the additional PI3K inhibitor GDC-0941 (Genentech), a highly selective pan class I PI3K inhibitor that also has favorable pharmacokinetic and toxicologic properties (26), in vivo in Pch1+/− K14-CreER2 p53fl/fl/null mice. We administered the oral dose of each drug recommended by the manufacturers (Genentech and Exelixis). Initially, we confirmed that these doses caused no significant changes in weight and appearance in normal mice when given from ages 6 to 12 weeks (data not shown). We then administered the drugs for approximately 8 weeks starting at age 9 weeks (after one dose of 4 Gy X-ray irradiation at age 8 weeks), after which a dorsal skin biopsy was taken and processed for microscopic BCC analyses. As a positive control for this study, we treated 3 Pch1+/− K14-CreER2 p53fl/fl/null mice topically with tazarotene and confirmed its efficacy in reducing BCC number and size (data not shown).
shown). Of the 3 PI3K inhibitors used in this study, we found that only XL765 inhibited both microscopic BCC number (P < 0.001) and size (P < 0.05; Fig. 5A and B) by a statistically significant amount.

Next, we assessed whether the PI3K inhibitors had a sustained effect on tamoxifen/ionizing radiation-induced BCC growth in Ptch1+/− K14-CreER2 p53fl/fl mouse (that had been treated with ionizing radiation for mutagenesis and with tamoxifen to activate Cre) and a Kaplan–Meier graph showed the time to the appearance of the first visible BCCs. Only XL765-treated mice had a delay in the first visible BCC appearance, although it was not statistically significant. XL147 and GDC-0941 did not seem to have any effect on the rate of BCC tumorigenesis. D and E, mice were assessed from age 28 weeks to determine the visible BCC number and size, respectively. Again, although different control vehicles were used, there were not significantly different from each other in terms of BCC numbers and size (data not shown), therefore the vehicle groups were combined. Only XL765 treatment reduced visible BCC number and volume by statistically significant amounts at the doses tested (P < 0.05, Student t test). Mean and SEMs are shown. C, a Kaplan–Meier graph showed the time to the appearance of the first visible BCCs. Only XL765-treated mice had a delay in the first visible BCC appearance, although it was not statistically significant. XL147 and GDC-0941 did not seem to have any effect on the rate of BCC tumorigenesis. D and E, mice were assessed from age 28 weeks to determine the visible BCC number and size, respectively. Again, although different control vehicles were used, there were not significantly different from each other in terms of BCC numbers and size (data not shown), therefore the vehicle groups were combined. Only XL765 treatment reduced visible BCC number and volume by statistically significant amounts at the doses tested (P < 0.05, Student t test). Mean and SEMs are shown.

**PI3K inhibitor slows growth of Tazarotene-resistant BCC allografts**

To assess whether PI3K inhibitors might reduce growth of tazarotene-resistant BCCs, we utilized our allograft model in NOD/SCID mice. We treated orally a mouse bearing an allograft made from a BCC from a Ptch1+/− K14-CreER2 p53 fl/fl mouse (that had been treated with ionizing radiation for mutagenesis and with tamoxifen to activate Cre) initially with oral tazarotene, initially at a high dose (10 mg/kg 5 days/week) for 3 weeks and then with a lower dose (2 mg/kg 5 days/week) for 8 weeks. We then transplanted cells from a tazarotene-resistant tumor into 3 mice and compared the growth rates when the individual recipient mice

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<th>XL147</th>
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<td><strong>A</strong> Microscopic BCC number</td>
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<td><strong>B</strong> Microscopic BCC size</td>
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<td><strong>C</strong> Time to first visible BCC</td>
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<td><strong>D</strong> Visible BCC number</td>
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<td><strong>E</strong> Visible BCC volume</td>
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*Figure 5.* *In vivo* testing of therapeutically viable small molecule inhibitors of PI3K/AKT pathway for treating BCC. A and B, *in vivo* testing of therapeutically viable PI3K/AKT pathway inhibitors against microscopic and visible BCC assessment at age 21 and 28 weeks respectively, after 8 weeks of PI3K inhibitor treatment. For microscopic BCC assessment, dorsal skin biopsies of mice given oral formulations of PI3K inhibitors XL147, XL765, or GDC-0941 were analyzed to determine the microscopic BCC number (A) and size (B). Only XL765 demonstrated significantly chemopreventive efficacy against BCC number (−P < 0.001, Student t test) and size (P < 0.05, Student t test) compared with the vehicle control group. Mean and SEMs are shown. C, a Kaplan–Meier graph showed the time to the appearance of the first visible BCCs. Only XL765-treated mice had a delay in the first visible BCC appearance, although it was not statistically significant. XL147 and GDC-0941 did not seem to have any effect on the rate of BCC tumorigenesis. D and E, mice were assessed from age 28 weeks to determine the visible BCC number and size, respectively. Again, although different control vehicles were used, there were not significantly different from each other in terms of BCC numbers and size (data not shown), therefore the vehicle groups were combined. Only XL765 treatment reduced visible BCC number and volume by statistically significant amounts at the doses tested (P < 0.05, Student t test). Mean and SEMs are shown.
were treated with vehicle, tazarotene (5 mg/kg daily, 5 days per week), or XL765 (30 mg/kg twice daily, 5 days per week). Mice treated with XL765 had a reduction in tumor volume.

Overall, these data suggest that pharmacologic inhibition of PI3K/AKT/mTOR signaling can significantly inhibit the development of both microscopic and visible murine BCCs, that at the single dose of each tested, XL765 was considerably more effective than the other inhibitors tested, and that tumors resistant to tazarotene may retain susceptibility to PI3K inhibitors.

Discussion

We demonstrated previously the remarkable efficacy of tazarotene against murine BCC carcinogenesis, effects that likely are mediated via RARγ-mediated transcriptional activation of RA-target genes (8, 9). In this study we have identified downstream mechanisms of tazarotene’s antitumor effects by analyzing in vitro changes in global gene expression in a murine BCC cell line. Specifically, we found (i) gene expression changes in vitro suggesting that inhibition of the PI3K-AKT signaling pathway is a central node downstream of retinoid treatment; (ii) as in human BCCs, pathway activation in visible BCCs in our murine BCC model and in the ASZ001 murine BCC cell line; (iii) reduction of tazarotene’s antiproliferative effects by hyper-activation of the PI3K-AKT pathway in ASZ001 cells; and (iv) reduction of both ASZ001 cell proliferation in vitro and, crucially, BCC carcinogenesis in vivo by small molecule PI3K-AKT inhibitors. Thus, it is reasonable to expect that PI3K inhibitors could overcome resistance to retinoid therapy in BCCs whose resistance is because of loss of RAR expression.

Interactions between hedgehog and IGF-IR/PI3K/AKT signaling in cancers have been identified. One possible mechanistic explanation is that the hedgehog and IGF/PI3K/AKT pathways converge to coregulate downstream targets. Thus, for example, hedgehog signaling enhances N-myc expression, and PI3K/AKT signaling reduces N-myc phosphorylation and its proteolytic destruction by inhibiting GSK3 activity (28). N-myc is overexpressed not only in hedgehog-driven medulloblastomas but also in human BCCs (29). By contrast, some data indicate direct interactions between the 2 pathways. Thus, hedgehog signaling can enhance Igf2 transcription in some, albeit not all, contexts (30, 31) and PI3K/AKT/mTOR signaling can enhance Gli transcriptional activity by reducing GSK3-mediated phosphorylation and degradation and by phosphorylation-mediated release from binding to the suppressor SuFU (18, 32–34) and can reduce the development of resistance to the antitumor effects of hedgehog inhibitors (35, 36).

Contrary to our finding that the retinoid tazarotene inhibits IGF-IR/PI3K/AKT/mTOR signaling, in some contexts ATRA (which, like tazarotene, inhibits murine BCC carcinogenesis) activates PI3K/AKT signaling to induce a differentiation program (37–39). Therefore, the relationship between retinoid and PI3K/AKT signaling pathways seems to be complex and cell-type specific, but at least in murine BCCs PI3K/AKT signaling inhibits tumor development. Thus, our study not only points to enhanced PI3K/AKT signaling as a target for anti-BCC strategies but also suggests that results of previously published studies of the effects of topical tazarotene versus human BCCs may already have been a “proof of principle” of this idea. Of note, mTOR inhibition seems to be effective against cutaneous carcinogenesis in at least some contexts (40, 41). Of the PI3K inhibitors we assessed, the one with the greatest in vivo anti-BCC efficacy was XL765, the only one tested that inhibits not only PI3K but also mTOR and DNA-PK. Although we do not know whether the drugs used inhibited PI3K equivalently at the single doses tested in vivo and whether the effects of XL765 were mediated in part by mechanisms other than direct inhibition of PI3K, the finding that inhibition of multiple targets in the PI3K/AKT/mTOR pathway may have better antitumor efficacy than does inhibition of a single target is not a new one (42, 43).

In summary, our data suggest (i) that the PI3K/AKT/mTOR pathway is a positive effector of BCC carcinogenesis that is inhibited by tazarotene and (ii) that targeting this pathway directly may inhibit BCC carcinogenesis in tumors that are resistant to tazarotene. Also, our data suggest that short-term inhibition of this pathway can continue to inhibit BCC carcinogenesis even after treatment is completed. Therefore, it might be possible to treat and/or prevent BCCs using PI3K inhibitors for a relatively short...
duration, thereby avoiding potential toxic side effects (e.g., interference with glucose metabolism and the immune response) that occur with the chronic use of these agents.

Disclosure of Potential Conflicts of Interest

E. Epstein has commercial research grants from Genentech. Also, E. Epstein has been a consultant/advisory board member of Genentech and Novartis. No potential conflicts of interest were disclosed by the other authors.

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