The selective PI3K inhibitor XL147 (SAR245408) inhibits Tumor Growth and Survival and Potentiates the Activity of Chemotherapeutic Agents in Preclinical Tumor Models


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Abstract

Dysregulation of PI3K/PTEN pathway components, resulting in hyperactivated PI3K signaling, is frequently observed in various cancers and correlates with tumor growth and survival. Resistance to a variety of anticancer therapies, including receptor tyrosine kinase (RTK) inhibitors and chemotherapeutic agents, has been attributed to the absence or attenuation of downregulating signals along the PI3K/PTEN pathway. Thus, PI3K inhibitors have therapeutic potential as single agents and in combination with other therapies for a variety of cancer indications. XL147 (SAR245408) is a potent and highly-selective inhibitor of Class I PI3Ks (α, β, γ and δ). Moreover, broad kinase selectivity profiling of >130 protein kinases revealed that XL147 is highly selective for Class I PI3Ks over other kinases. In cellular assays, XL147 inhibits the formation of PIP₃ in the membrane, and inhibits phosphorylation of AKT, p70S6K and S6 phosphorylation in multiple tumor cell lines with diverse genetic alterations impacting the PI3K pathway. In a panel of tumor cell lines, XL147 inhibits proliferation with a wide range of potencies, with evidence of an impact of genotype on sensitivity. In mouse xenograft models, oral administration of XL147 results in dose-dependent inhibition of phosphorylation of AKT, p70S6K, and S6 with a duration of action of at least 24 h. Repeat-dose administration of XL147 results in significant tumor growth inhibition in multiple human xenograft models in nude mice. Administration of XL147 in combination with chemotherapeutic agents results in antitumor activity in xenograft models that is enhanced over that observed with the corresponding single agents.
Introduction

There are three classes of PI3Ks, among which the Class I kinases (subdivided into IA and IB subsets) convert phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$) in response to external cell stimuli (1, 2, 3). Activation of Class IA PI3Ks (PI3K\(\alpha\), -\(\beta\), and -\(\delta\)) is mediated by receptor tyrosine kinases (RTKs). G-protein–coupled hormone receptors are implicated in activation of PI3K\(\beta\) and Class IB PI3K (PI3K\(\gamma\)) (4). RAS can also directly bind to and activate p110\(\alpha\), the catalytic subunit of PI3K\(\alpha\), and PI3K\(\alpha\) can mediate cellular transformation by RAS (2,5). Similarly, p110\(\beta\), the catalytic subunit of PI3K\(\beta\), interacts with and serves as an effector of the GTPases RAC and cell division cycle 42 (CDC42) (2,6). Downstream effectors of PI3K signaling, such as phosphoinositide-dependent kinase-1 (PDK1) and AKT, bind to PIP$_3$ at the cell membrane and are subsequently activated by phosphorylation, resulting in activation of signaling cascades regulating tumor proliferation, survival, angiogenesis, invasion, and dissemination (1,2). TORC1, a multi-subunit complex including the mTOR catalytic subunit and Raptor, acts downstream of AKT and is itself a notable target for therapeutic intervention in cancer and other diseases (7,8).

Dysregulation of PI3K pathway components, resulting in upregulation of PI3K signaling, is observed in many cancers and is thought to promote tumor growth and survival (1,2). PIK3CA, the gene encoding p110\(\alpha\), is the most frequently mutated kinase in human tumors, with mutations evident in approximately 10% of human cancers (9). In addition, the tumor suppressor PTEN, which is a critical negative regulator of PI3K signaling that converts PIP$_3$ back to PIP$_2$, is frequently deleted or downregulated in human tumors (1,2,10). PTEN-deficient tumors are believed to be highly PI3K\(\beta\)-dependent, although it has recently been demonstrated that this dependence can be shifted to PI3K\(\alpha\) by concurrent mutations that activate p110\(\alpha\) (11). In addition to PTEN, other tumor suppressor genes negatively regulating the PI3K pathway have been identified, including liver kinase B1 (LKB1), type II inositol polyphosphate-4-phosphatase, and tuberous
sclerosis (TSC) – these are mutated/inactivated in a variety of familial and/or sporadic tumors (1,2).

Resistance to therapy, both primary and acquired, is a persistent problem in cancer treatment. Activation of the PI3K pathway has been implicated in resistance to a variety of agents including RTK inhibitors and chemotherapeutic agents (12). Consistent with this, blockade of the PI3K pathway has been shown to sensitize tumor cells to inhibitors of HER2, MET and EGFR as well as to taxanes and platinum drugs (12). Selective PI3K inhibitors therefore have potential both as single agents and in combination with other therapies and a number of these agents have entered clinical testing in recent years (1,2,13).

Here we describe the characterization of the preclinical pharmacology of the selective Class I PI3K inhibitor XL147 (SAR245408). Collectively, these data demonstrate that PI3K pathway inhibition by XL147 inhibits proliferation, angiogenesis, cellular invasion, and tumor cell growth and survival in preclinical models, and has the potential to enhance the efficacy of chemotherapeutic agents.
Materials and Methods

In vitro kinase inhibition assays and PIP₃ Mass Balance Assay

Kinase inhibition assays and the PIP₃ mass balance assay were performed as previously described (14).

pAKT and pS6 ELISA

ELISA for pAKT^{T308} and total AKT were performed on PC-3 cell lysates and analyzed as previously described (14). The pS6 ELISA was performed as previously described (15) with minor modifications.

Cell-based Assays

Cell lines were obtained from the American Type Culture Collection in 2001-2005 and maintained in culture conditions at 37°C under 5% CO₂ as previously described (14). For PI3K pathway status assessment following EGF treatment, the culture medium was replaced with test compounds dissolved in serum-free DMEM containing 0.3% DMSO. After incubation for 3 h, cells were stimulated with 100 ng/ml of EGF (R&D Systems, 236-EG) for 10 min and Western immunoblot analysis of cell lysates was performed as previously described (14). Assessment of mTOR pathway status in Ramos cells was performed as previously described (14). Cellular proliferation was assessed as previously described (16) using the Cell Proliferation ELISA, Bromo Deoxyuridine (BrdU) chemiluminescence kit (Roche, Applied Science). Cytotoxicity, apoptosis (Caspases 3/7), anchorage-independent growth, and PC-3 cell migration assays were performed as previously described (14). HGF-induced chemotaxis was assessed as previously described (16). The endothelial cell tube formation assay was performed as previously described (16), with minor modifications. Total tube length was quantified with Image Pro Plus software (Media Cybernetics).

For cell cycle analysis by FACS MCF7 cells were seeded at 1.2x10⁵ cells per well in 12-well plates (Nunc) in DMEM containing 10% FBS, 1% NEAA, and 1% penicillin
streptomycin (all from Cellgro). The cells were then incubated at 37°C, 5% CO₂ for 24 h. Serial dilutions of compounds in fresh growth medium at a final concentration of 0.3% DMSO (vehicle) were added to the cells and incubated for 48 and 72 h. After treatment, medium was removed and cells were washed with cold PBS and immediately harvested in ice-cold PBS. Cells were fixed by adding ice-cold ethanol drop-wise to the cell suspension to a final concentration of 75%. Cells were incubated in the fixative overnight at -20°C. The ethanol was washed off with PBS and cells were resuspended with propidium iodide (PI) staining solution to a final concentration of 5% PI (Molecular Probes), 10% RNase Cocktail (Ambion), and 85% PBS. Cells were stained for 1 h at 37°C. Samples were run through the FACS Calibur instrument (Becton Dickinson) and data acquired by the CellQuest program (Becton Dickinson). FACS data were analyzed using the ModFit LT program (Verity Software).

**In vivo pharmacodynamic assays, efficacy studies, and tumor histological assessment**

Female athymic nude mice and male nude mice were purchased from Taconic (Hudson, NY) and housed according to the Exelixis Institutional Animal Care and Use Committee guidelines. Tumor cells were cultured and established as xenografts in mice, and body and tumor weights assessed as previously described (14). Statistical significance was determined using the 2-tailed Student’s t-test (significance defined as p<0.05). XL147 was formulated in sterile water/10 mM HCl or water and administered at the indicated doses and regimens by oral gavage at a dose volume of 10 mL/kg. Paclitaxel was purchased from MP Biomedicals (Irvine, CA) and formulated for intravenous (IV) administration by dissolution of the dry powder into 1:1 EtOH/Cremophor mixture, with subsequent dilution (1:3) into normal saline. Carboplatin was purchased from PolyMed Therapeutics (Houston, TX) and formulated for IV administration by dissolution in normal saline containing 0.5% EtOH/0.5%CremophorEL.

For Western immunoblot assessment, tumors were collected at the indicated time-points and tumor lysates were prepared, processed, and analyzed as previously described (14). For histological assessment, after euthanasia, tumors from animals administered XL147
and/or other agents were excised and fixed in zinc fixative (BD Pharmingen) for 24-48 h before being processed into paraffin blocks. Five µm thin sections were cut serially to represent the largest possible surface for each tumor and Ki67 nuclear antigen, CD31 positive tumor vessels, or apoptotic cells (TUNEL) detected and analyzed as previously described (14).
Results

XL147 is a selective inhibitor of class I PI3Ks in biochemical assays

XL147 was identified following optimization of a quinoxaline scaffold for in vivo PI3K pathway inhibition and drug-like properties (XL147 structure is shown in Figure 1A). In assays performed using purified proteins in a luciferase-coupled chemiluminescence format, XL147 displayed potent inhibitory activity against Class I PI3K isoforms p110α, p110δ, and p120γ, with IC$_{50}$ values of 39, 36, and 23 nM, respectively (Table 1). XL147 was less potent against the remaining Class I isoform, p110β, with an IC$_{50}$ value of 383 nM. The IC$_{50}$ value for inhibition of PI3Kα by XL147 was determined at various concentrations of ATP, revealing XL147 to be an ATP-competitive inhibitor with an equilibrium inhibition constant (K$_{I}$) value of 42 nM.

XL147 had relatively weak inhibitory activity towards the Class III PI3K vacuolar sorting protein 34 (VPS34; IC$_{50}$ value of ~7.0 µM) and the PI3K-related DNA-dependent protein kinase (DNAPK; IC$_{50}$ value of 4.75 µM) [Table 1]. In an mTOR kinase immunoprecipitation assay using cell lysates, XL147 did not inhibit mTOR activity towards the physiologic substrate protein eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (IC$_{50}$ > 15 µM; Table 1). XL147 was also profiled against a panel of approximately 130 protein kinases with essentially no cross-reactivity observed (Supplemental Table S1). All assays were performed at ATP concentrations approximately equal to the Michaelis constant (K$_{M}$) values of the respective enzymes.

XL147 inhibits the PI3K pathway in multiple tumor cell models

MCF7 human mammary carcinoma cells and PC-3 human prostate adenocarcinoma cells were selected for the initial assessment of the effect of XL147 on PTEN/PI3K pathway signaling because they harbor, respectively, a heterozygous E545K activating mutation in the PIK3CA subunit of PI3Kα and a homozygous deletion of exons 3-9 of the PTEN tumor suppressor gene. PIP$_3$ is the product of a class IA or IB PI3K acting on its physiological substrate PIP$_2$. Hence PIP$_3$ levels serve as a direct assessment of PI3K activity. Consistent with its inhibitory activity against purified PI3K proteins, XL147 inhibited EGF-induced PIP$_3$ production in PC-3 and MCF7 cells in serum-free medium...
with IC$_{50}$ values of 220 and 347 nM, respectively (Table 2). The ability of XL147 to inhibit phosphorylation of key signaling proteins downstream of PI3K was examined by assessing its effects on EGF-stimulated phosphorylation of AKT and on non-stimulated phosphorylation of S6 in PC-3 cells in serum-free media by cell based ELISA. XL147 inhibited these activities with IC$_{50}$ values of 477 and 776 nM, respectively (Table 2).

The effects of XL147 on the PI3K signaling pathway were examined by Western immunoblot analysis in MCF7 and PC-3 cells (see Figure 1B and Supplemental Figure S1). XL147 inhibited AKT phosphorylation at both activation sites (T308 and S473) at concentrations consistent with the IC$_{50}$ values determined by ELISA in MCF7 and PC-3 cells stimulated with EGF following incubation for 3 hours in serum-free medium containing XL147. Inhibition of AKT substrate phosphorylation (PRAS40 and GSK3β) and inhibition of phosphorylation events downstream of mTOR (p70S6K, S6, and 4EBP1 phosphorylation) were also evident. XL147 induces a decrease in the levels of cyclin D1 protein, consistent with increased GSK3β activity as a result of inhibition of AKT leading to GSK3β-mediated phosphorylation and subsequent degradation of Cyclin D1 (Figure 1B and Supplemental Figure S1). In general, higher compound concentrations were required to inhibit phosphorylation events downstream of mTOR than more PI3K-proximal events such as AKT phosphorylation, consistent with selective inhibition at the PI3K level. XL147 does not inhibit nutrient-stimulated mTOR kinase activity in cells as assessed by S2481 autophosphorylation and p70S6K/4E BP1 phosphorylation, providing further evidence for lack of direct activity towards mTOR (Supplemental Figure S2).

The control compound ZSTK474 (an inhibitor of PI3K; Reference 17) at 10 µM robustly decreased the levels of all the phospho readouts assessed. The mTORC1 inhibitor rapamycin at 0.1 µM did not inhibit the phosphorylation of AKT or its direct substrates PRAS40 and GSK3β, but in fact appeared to stimulate phosphorylation of AKT. This is consistent with relief of p70S6K-dependent negative feedback of PI3K (2). As expected, rapamycin significantly decreased pp70S6K and pS6 levels, consistent with its well characterized ability to inhibit TORC1. None of the compounds had significant effects on ERK1/2 phosphorylation. The ability of XL147 to inhibit phosphorylation of key
signaling components of the PI3K pathway was also evident in the presence of 10% serum in PC 3 and MCF7 cells, although compound potency was reduced, presumably reflecting compound binding to serum proteins (Supplemental Table S2). The pharmacodynamic effects of XL147 were further profiled in additional cell lines bearing a variety of genetic lesions that activate/modulate PI3K signaling. These included OVCAR-3 (PIK3CA amplification), U87-MG (PTEN deletion), A549 (mutationally activated KRAS, loss-of-function mutation in gene encoding the mTOR-directed tumor suppressor gene LKB-1), MDA-MB-468 (PTEN deletion) and LS174T (PIK3CA and KRAS mutations) cells. XL147 demonstrated broad activity in these lines with no marked difference in sensitivity being evident. (Supplemental Figures S3 and S4).

**Effects of XL147 on proliferation in a panel of tumor cell lines**

In MCF7 and PC-3 cells, XL147 inhibits proliferation (monitored by BrdU incorporation) with IC$_{50}$ values of 9669 nM and 16492 nM, respectively. When tested in a broad panel of tumor cell lines with diverse origins and genetic backgrounds, XL147 was found to inhibit proliferation with IC$_{50}$ values ranging from approximately 1200 nM to >30000 nM (Figure 2 and Supplemental Table S3). In general, PIK3A mutant and, to a lesser extent, PTEN mutant cell lines tended to be relatively sensitive to XL147, whereas RAS or BRAF mutant cell lines tended to be less sensitive. Interestingly, several RAS mutant cell lines were relatively insensitive to XL147 in spite of their also harboring PI3CA mutations (Figure 2 and Supplemental Table S3). The micromolar IC$_{50}$ values demonstrated in these experiments may be attributable in part to the presence of 10% serum in these assays and the consistently high *in vitro* plasma protein binding exhibited by XL147, with 99.9% of this agent bound in mouse, rat, and human plasma. Similarly, *in vivo* plasma protein binding, quantified in plasma samples from mice and rats administered XL147, showed plasma protein binding of 99.9% and 99.8% at 100 mg/kg dosages. All values were determined by an equilibrium dialysis method. As discussed previously, the presence of serum also causes a significant increase in the IC$_{50}$ values for XL147 inhibition of AKT and S6 phosphorylation.
Anchorage-independent growth in soft agar is considered the most stringent assay for detecting malignant transformation of cells. To further characterize the effects of XL147 on tumor cell growth, an assay monitoring the anchorage-independent growth of PC-3 and MCF7 cells in soft agar over a 14-day period was employed. XL147 inhibits colony growth with an IC\textsubscript{50} of 3996 nM in PC-3 cells and 2730 nM in MCF7 cells. These IC\textsubscript{50} values are significantly lower than those required to inhibit growth of the cells in a monolayer, perhaps indicating an increased reliance on PI3K pathway signaling for growth in 3-dimensions.

To rule out direct cytotoxic effects of XL147 on tumor cells, its effects on cell viability were determined by bioluminescent measurement of cellular ATP. XL147 did not reduce ATP levels when incubated for 24 h, indicating a lack of cytotoxicity (Supplemental Table S3, footnote). Induction of cytoplasmic Caspases 3 and 7 was examined as an indication of apoptosis induction. XL147 did not affect the activity of these Caspases at the compound doses and time point tested (Supplemental Table S3, footnote). In MCF7 cells, the anti-proliferative effects of XL147 were associated with a specific block in the G1 phase of the cell cycle and an increase of sub G1 cell population (Supplemental Table S4). Therefore, at least in MCF7 cells, the anti-proliferative effects of XL147 in culture do not appear to be due to cytotoxic or pro-apoptotic effects, but rather reflect cytostasis.

**XL147 inhibits tumor cell migration, invasion, and angiogenesis**

One of the hallmarks of aggressive tumor cells is the ability to migrate in response to chemotactic stimuli and to invade surrounding tissue. HGF is one of the key stimulators of these behaviors, and cell lines expressing high levels of the HGF receptor MET, are highly invasive and metastatic in vivo. Since PI3K resides in the MET signaling pathway, the ability of XL147 to inhibit HGF-stimulated migration and invasion was tested using in vitro assays that measure these behaviors. Murine B16 melanoma cells express high levels of MET which becomes highly phosphorylated when the cells are treated with HGF. In 10% serum, B16 cells plated in the top well of a transwell chamber containing a barrier with 0.8 micron pores show very little ability to migrate to the lower chamber side. Addition of HGF to the lower transwell chamber greatly increases migration through the barrier over a 24 h period (Figure 3A). XL147 blocked this effect with an
IC$_{50}$ value of 899 nM. Inhibition of cell migration was observed at a five-fold lower concentration than that associated with cytotoxicity (IC$_{50}$ value of XL147 in B16 cells of 4494 nM). Therefore, the effects on inhibition of melanoma cell migration by XL147 are likely not due to cytotoxicity.

A second assay, the scratch assay, was utilized to test XL147 for activity against PC-3 tumor cell migration stimulated by EGF (Figure 3B). In this assay, a cell-free zone was scratched into a monolayer of tumor cells, and the ability of XL147 to block EGF-stimulated migration of the cells into the cell-free zone was determined. In the absence of growth factors, migration of cells bordering the scratch into the cell-free space is minimal during the 18 h time course of the experiment. The addition of EGF greatly stimulates migration, resulting in a nearly complete closure of the scratch over the same time course. XL147 inhibited cellular migration into the cell-free zone in response to EGF with an IC$_{50}$ value of 394 nM and exhibited no cytotoxicity (IC$_{50}$ > 3333 nM).

PI3K plays an important role in angiogenesis as a central mediator of signaling downstream of angiogenic RTKs (18,19). Endothelial tube formation was assayed to test the effect of XL147 in an in vitro model that reflects endothelial cell morphogenesis, a function thought to contribute to angiogenesis in vivo. When plated on a confluent layer of normal human diploid fibroblast cells, human microvascular endothelial cells (HMVECs) form extensive networks of tubules in response to VEGF over a 7 day period. Tubules are stained and quantitated using an antibody that recognizes the endothelial cell marker CD31, as illustrated in Figure 3C. XL147 inhibited VEGF-induced tubule formation with an IC$_{50}$ of 529 nM, similar to the IC$_{50}$ value of ZD6474 (646 nM), an inhibitor of VEGFR2, PDGFRβ, FGFR1, and FLT4 that is known to inhibit angiogenesis (20).

**XL147 strongly inhibits the PI3K pathway in tumor xenograft models and displays robust anti-tumor activity in tumor bearing mice**

Lysates of MCF7 xenograft tumors intradermally implanted in the hind flank of athymic nude mice contain high levels of constitutively phosphorylated AKT, p70S6K, and S6 proteins. The ability of XL147 to inhibit this endogenous phosphorylation of AKT,
p70S6K, and S6 was examined following a single oral dose of 10, 30, 100, or 300 mg/kg. The tumors were harvested 4 h, 24 h, or 48 h post-dose and homogenized in lysis buffer. Tumor lysates from each animal (n=4) were then pooled for each group and analyzed for levels of total and phosphorylated AKT, p70S6K, and S6 by Western immunoblotting (Figure 4A).

Administration of XL147 caused a dose-dependent decrease in phosphorylation of AKT, p70S6K, and S6 in the tumors, reaching a maximum of 81% inhibition of AKT phosphorylation at 300 mg/kg at 4 h. The dose response relationships derived from the 4 h time point predict 50% inhibition of AKT, p70S6K, and S6 phosphorylation at doses of ~100 mg/kg (pAKT\textsuperscript{T308}), 54 mg/kg (pAKT\textsuperscript{S473}), 71 mg/kg (p-p70S6K), and 103 mg/kg (pS6) [Supplemental Figure S5]. The inhibition of AKT, p70S6K, and S6 phosphorylation in MCF7 tumors following a 100 mg/kg dose of XL147 was maximal at 4 h, reaching 55-75%; however, the level of inhibition decreased to 8-45% by 24 h, and only minimal or no inhibition was evident by 48 h (Figure 3A). Following a 300 mg/kg dose of XL147, inhibition was also maximal at 4 h (65-81%). However, in contrast to the 100 mg/kg dose, inhibition at 24 h (51-78%) was almost comparable to that seen at 4 h, and partial inhibition (25-51%) persisted through 48 h (Figure 4A).

Similarly, administration of XL147 caused a dose-dependent decrease of phosphorylation of AKT, p70S6K, and S6 in PC-3 tumors in vivo, reaching a maximum of 74% inhibition of AKT phosphorylation at 300 mg/kg at 4 h post-dose (Figure 4B). The dose response relationships derived from the 4 h time point predict 50% inhibition of AKT, p70S6K, and S6 phosphorylation to occur at doses of 64 mg/kg (pAKT\textsuperscript{T308}), ~100 mg/kg (pAKT\textsuperscript{S473}), 95 mg/kg (p-p70S6K), and 99 mg/kg (pS6) [Supplemental Figure S5]. Blood was collected at the same time tumor tissue was harvested in both studies and plasma concentrations of XL147 were assessed (Supplemental Table S5). Based on these data, the plasma concentrations required to inhibit phosphorylation of AKT, p70S6K, and S6 by 50% in these tumor models ranged from approximately 90 to 180 µM. Hence XL147 exhibited comparable pharmacodynamic activity in PIK3CA-mutant MCF7 and PTEN-deficient PC-3 xenograft tumor models.
Multiple tumor models were utilized to explore the efficacy and potency of repeat-dose XL147 with regard to tumor growth inhibition in vivo. In addition to the previously described MCF7 and PC-3 models, the anti-tumor efficacy of XL147 was evaluated in xenograft models including OVCAR-3 (human ovarian xenograft tumor model exhibiting PIK3CA amplification), U-87 MG (human glioblastoma xenograft tumor model harboring a deletion at codon 54 in the gene encoding PTEN, resulting in a frameshift), A549 (human NSCLC xenograft tumor model harboring a homozygous activating mutation in KRAS and a homozygous loss-of-function mutation in LKB1), Calu-6 (human non-small cell lung carcinoma harboring mutationally activated KRAS Q61K), A2058 (PTEN-deficient human malignant melanoma harboring a homozygous V600E activating mutation in BRAF), and WM-266-4 (PTEN-deficient human malignant melanoma harboring a heterozygous V600D activating mutation in BRAF).

XL147 exhibited significant anti-tumor efficacy in vivo in all of these models (Figures 5 and 6, and Supplemental Table S6) at doses that proved well tolerated as assessed by daily monitoring of mouse weights (Supplemental Figure S6). The most efficacious once daily dose assessed was 100 mg/kg, suggesting that sustained pathway inhibition is required for maximal efficacy. This dose generally resulted in stasis or near-stasis of tumor growth, except in the case of the tumors harboring KRAS or BRAF mutations where tumors generally continued to growth although at a reduced rate (Figure 6 and Supplemental Table S6). Immunohistochemical analysis of MCF7 tumors collected at the end of the dosing period revealed significant, dose-dependent decreases in staining for Ki67, a marker of cell proliferation (Table 3). Similarly, administration of XL147 at 100 mg/kg once daily for 2 weeks resulted in a 51% decrease in Ki67 staining in PC-3 tumors. Moreover, decreased tumorvascularization was also observed in MCF7, PC-3, and Calu-6 tumors (Table 3 and Figure 6). Thus, inhibition of PI3K by XL147 results in an anti-proliferative effect, as well as a modest anti-vascular effect in xenograft tumors. In PC-3 and Calu-6 tumors there was also evidence for increased apoptosis as judged by TUNEL staining (Figure 6) although the absolute percentage of TUNEL positive tumor cells remained small, suggesting that increased apoptosis was not a significant contributor to anti-tumor efficacy. Plasma concentrations at the end of these efficacy studies were higher than those evident following single-dose administration, presumably reflecting
accumulation. For example, in the MCF7 efficacy study, average plasma concentrations for XL147 administered at the 100 mg dose were 233, 329 and 112 μM at the 1 hour, 4 hour and 24 h time-points, respectively (n=3 per time-point), compared with 179 and 86 μM for the 4 and 24 hour time-points for the same dose in the single dose MCF7 pharmacodynamic study shown in Figure 4A (Supplemental Table S5).

In addition to its anti-tumor efficacy as monotherapy, XL147 potentiated the anti-tumor efficacy of the cytotoxic agents paclitaxel and carboplatin (Figure 6). When XL147 was combined with paclitaxel or carboplatin apoptosis was induced to an extent greater than that seen for either agent alone while there was also an enhanced reduction in tumor angiogenesis evident (Figure 6). It is noteworthy that XL147 administered in these combinations proved generally well-tolerated as assessed by daily monitoring of mouse body weights (Supplemental Figure S6).
Discussion

To date three PI3K pathway inhibitors have been approved by FDA for the treatment of cancer. These include the rapamycin analogs TORISEL™ (temsirolimus; Pfizer) for patients with advanced renal cell carcinoma (RCC) and Afinitor™ (everolimus; Novartis) for the treatment of patients with advanced RCC after failure of treatment with sunitinib or sorafenib, and for advanced estrogen receptor-positive breast cancer after failure of treatment with a nonsteroidal aromatase inhibitor. However, the efficacy of these agents may be limited by the fact that mTORC1 inhibition enhances tumor cell survival by upregulating PI3K/AKT signaling via inhibition of a mTORC1-dependent negative feedback loop acting through PI3K (2). In contrast, inhibition of PI3K offers the potential for concerted inhibition of both PI3K and mTORC1, while obviating this feedback loop. The PI3Kδ specific inhibitor idelalisib has been approved by FDA for administration in combination with rituximab for patients with relapsed chronic lymphocytic leukemia (CLL) for whom rituximab alone would be considered appropriate therapy, and as monotherapy for patients with relapsed follicular B-cell non-Hodgkin lymphoma (FL) and small lymphocytic lymphoma (SLL) who have received at least two prior systemic therapies, but selective inhibition of PI3Kδ has limited impact in solid tumor types.

The results detailed above demonstrate that XL147 (SAR245408) is a potent and selective inhibitor of Class I PI3Ks. In cellular assays, treatment with XL147 inhibits phosphorylation of proteins downstream of PI3K, including AKT and ribosomal protein S6, in multiple tumor cell lines with diverse molecular alterations impacting the PI3K pathway. In a broad panel of tumor cell lines XL147 inhibits proliferation with a wide range of potencies, which appeared to be influenced by genetic background. In human xenograft tumor models in athymic nude mice, oral (PO) administration of XL147 results in dose-dependent inhibition of PI3K pathway components with a duration of action of approximately 24 hours. On repeat dosing, XL147 shows significant tumor growth inhibition in multiple human xenograft models at well-tolerated doses. When administered in combination with cytotoxic chemotherapy, XL147 showed anti-tumor efficacy which was enhanced over that seen with the corresponding monotherapies.
A major cause of PI3Kα activation in human tumors is gain of function mutations in PIK3CA, particularly in Exons 9 and 20 which encode the helical and kinase domains of the p110α catalytic subunit of PI3K (3). Molecular alterations directly affecting PI3KB are rare in human cancer (1), but PI3Kβ is considered to be the major driver of dysregulated PI3K pathway activity associated with PTEN deficiency, although PI3Kα may be the more important isoform in PTEN-deficient tumors where p110α is concurrently activated by mutated RAS (11). Hence, concerted inhibition of PI3Kα and PI3Kβ is likely desirable in terms of broad potential utility in treating solid tumors. In biochemical assays, XL147 appears to be more potent against PI3Kα than against PI3Kβ (Table 1). However, in cellular assays XL147 shows comparable activity versus PI3K pathway signaling in MCF7 breast (PIK3CA mutant) and PC-3 prostate (PTEN-deleted) tumor cells. Moreover, XL147 showed comparably pharmacodynamic activity against these cell lines when they were grown as xenograft tumors in mice. These data demonstrate that XL147 exhibits functionally equivalent activity against PI3Kα and PI3Kβ in cultured cells and preclinical tumor models.

XL147 exhibited a wide range of anti-proliferative activity against tumor cells grown as monolayers. In MCF7 cells, these effects were associated with a G1 arrest, but not with acute cytotoxicity or induction of apoptosis. There was a trend suggesting enhanced sensitivity of cells exhibiting PIK3CA mutations to XL147, consistent with similar observations previously reported for the PI3K inhibitors GDC-0941 and CH5132799 (21,22). Likewise, the relative insensitivity of RAS mutant cell lines, regardless of PIK3CA status, to inhibition of proliferation by XL147 is consistent with preclinical observations with other PI3K pathway targeting agents (23).

When administered as monotherapy, XL147 showed efficacy in models with diverse genetic lesions activating the PI3K pathway, specifically a PIK3CA E545K mutation (MCF7), PIK3CA amplification (OVCAR-3), PTEN deletion/deficiency (PC-3, U-87 MG, A2058, WM-266-4), KRAS mutation (A549 and Calu-6), and LKB1 mutation (A549). The fact that efficacy was observed in all these models suggests that XL147 may
have broad utility in tumors with activation of the PI3K pathway. It is intriguing that, of these models, XL147 appeared to be least efficacious against Calu-6, a KRAS-mutant tumor lacking detectable genetic alterations in PIK3A, PTEN, or LKB-1. Based on IHC/IF analyses efficacy across models was likely mediated by a combination of anti-proliferative and anti-angiogenic effects (Table 3 and Figure 6). The plasma concentrations associated with pharmacodynamic activity and anti-tumor efficacy in mice are comparable to plasma concentrations associated with PI3K pathway inhibition in a single-agent Phase 1 clinical study (compare Supplemental Table S5 with clinical pharmacokinetics summarized in reference 24). Hence XL147 pharmacokinetics and pharmacodynamic activity appear to translate well between mice and humans.

Based on the xenograft data presented here, and the clinical experience gained thus far with XL147 and other PI3K inhibitors in clinical development, it is not yet clear whether the presence of PIK3CA mutations or PTEN deficiency will be predictive of greater clinical responsiveness. In a single-agent Phase 1 study, robust pharmacodynamic activity across diverse tumors was evident regardless of mutational status. For example, PI3K pathway inhibition was roughly comparable in a tongue squamous cell carcinoma harboring a PIK3CA E545K mutation to that seen in tumors lacking detectable PI3K pathway alterations (24). Moreover, in that study a partial response was evident in a non-small cell lung cancer patient, although no mutations affecting the PI3K pathway were detected in archival tumor tissue from the patient (24). It is also not yet clear whether the presence of PIK3CA mutations or PTEN deficiency will be predictive of greater clinical responsiveness to PI3K pathway inhibitors in general, although an analysis based on combining the results of multiple early stage trials suggested that PIK3CA H1047R mutations are associated with response (25).

The ability of XL147 to be combined with cytotoxic agents in preclinical tumor models at well-tolerated doses is promising and consistent with encouraging early results in XL147-003, a single arm, open label, dose escalation study of XL147 in combination with paclitaxel and carboplatin in subjects with refractory solid tumors (26; NCT00756847). Moreover, the enhanced efficacy evident when XL147 is combined with
trastuzumab or lapatinib in mouse models (27,28) provides strong support for exploring combinations of XL147 with RTK inhibitors. These and similar data provide a rationale supporting clinical studies where XL147 is combined with other targeted or cytotoxic agents (eg, NCT01042925, NCT01082068, NCT00692640).
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References


### Table 1

**Kinase Inhibition Profile of XL147**

<table>
<thead>
<tr>
<th>Family</th>
<th>Kinase</th>
<th>IC₅₀ ± SEM (nM)</th>
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<tr>
<td>PI3K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class IA</td>
<td>PI3Kα</td>
<td>39 ± 10</td>
</tr>
<tr>
<td></td>
<td>PI3Kβ</td>
<td>383 ± 78</td>
</tr>
<tr>
<td></td>
<td>PI3Kδ</td>
<td>36 ± 8</td>
</tr>
<tr>
<td>Class IB</td>
<td>PI3Kγ</td>
<td>23 ± 8</td>
</tr>
<tr>
<td>Class III</td>
<td>VPS34</td>
<td>6974</td>
</tr>
<tr>
<td>PI3K-related</td>
<td>DNA-PK</td>
<td>4750 ± 2000</td>
</tr>
<tr>
<td></td>
<td>mTOR</td>
<td>&gt; 15,000ᵇ</td>
</tr>
</tbody>
</table>

DNAPK, DNA protein kinase; IC₅₀, concentration required for 50% target inhibition; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; VPS34, vacuolar sorting protein 34.

ᵃ Immunoprecipitation kinase assay using cell lysates.
ᵇ Highest concentration tested.
Table 2

Effects of XL147 on PIP₃ Production and AKT and S6 Phosphorylation

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>PIP₃ IC₅₀ (nM)</th>
<th>pAKT IC₅₀ (nM)ᵃ</th>
<th>pS6 IC₅₀ (nM)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3</td>
<td>220</td>
<td>477</td>
<td>776</td>
</tr>
<tr>
<td>MCF7</td>
<td>347</td>
<td>ndᵇ</td>
<td>ndᵇ</td>
</tr>
</tbody>
</table>

ᵃIC₅₀ values determined using ELISA assay.
ᵇnd, not determined

Table 3

Immunohistochemical Analyses of Proliferation and Vascularity in MCF7 Xenograft Tumors

<table>
<thead>
<tr>
<th>Group</th>
<th>Ki67 Analysis</th>
<th>CD31 Analysis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Positive Cellsᵃ</td>
<td>% Reductionᵇ</td>
<td>MVDᵃ,c</td>
</tr>
<tr>
<td>Vehicle 10 ml/kg PO qd</td>
<td>37 ± 4</td>
<td>na</td>
<td>78 ± 7</td>
</tr>
<tr>
<td>XL147 30 mg/kg PO qd</td>
<td>30 ± 5</td>
<td>20</td>
<td>59 ± 9</td>
</tr>
<tr>
<td>XL147 100 mg/kg PO qd</td>
<td>25 ± 5</td>
<td>32</td>
<td>49 ± 7</td>
</tr>
<tr>
<td>XL147 300 mg/kg PO twice weekly</td>
<td>21 ± 5</td>
<td>43</td>
<td>39 ± 7</td>
</tr>
</tbody>
</table>

ᵃValues are the mean ± standard deviation;ᵇValues are relative to vehicle control (in all cases p < 0.05).
ᶜMean Vessel Density (mean vessel count/mm²).
na, not applicable.
Figure Legends

Figure 1
(A) Chemical structure of XL147 (SAR245408). (B) XL147 inhibits PI3K pathway signaling in EGF-stimulated MCF7 cells. After incubation with XL147 at the indicated concentrations, ZSTK474 (10 µM), or rapamycin (0.1 µM), MCF7 cells were stimulated with 100 ng/ml of EGF for 10 min. The cells were then lysed and effects of compound on PI3K pathway signaling assessed by Western blotting.

Figure 2
Relative sensitivity of tumor cells to XL147 as a function of genetic status. a Cell proliferation IC\textsubscript{50} values are presented normalized to that for BT474 (most sensitive cell line). See Materials and Methods and Supplemental Table S3 for details.

Figure 3
XL147 inhibits (A) B16 melanoma cell migration, (B) PC-3 cell migration, and (C) VEGF-induced HMVEC tubule formation. Assays were performed as previously described (14, 16).

Figure 4
Administration of XL147 inhibits PI3K pathway signaling in MCF7 and PC-3 tumors. A single dose of XL147 or vehicle was administered by oral gavage to MCF7 (A) or PC-3 (B). Tumors were resected at the indicated times post dose and the effects of XL147 on phosphorylation of AKT, p70S6K, and S6 were assessed by Western blotting.

Figure 5
XL147 administration results in tumor growth inhibition and/or regression of established xenograft tumors. MCF7 or OVCAR-3 xenograft tumors were implanted. When tumors reached ~100 mg in size administration of vehicle or XL147 was initiated at the indicated doses and regimens. Data points represent the mean ± standard error for each treatment group (N = 9–10).
Figure 6

XL147 administration potentiates the anti-tumor efficacy of paclitaxel or carboplatin. PC-3 or Calu-6 xenograft tumors were implanted. When tumors reached ~100 mg in size administration of vehicle or the indicated agents was initiated at the indicated doses and regimens. Data points represent the mean ± standard error for each efficacy treatment group (N = 10). At the end of each study tumors were resected from each group and effects on tumor apoptosis and microvessel density were assessed (values represented as mean ± standard deviation). Key: a, p < 1 x 10^{-6} cf vehicle; b, p < 2 x 10^{-6} cf either monotherapy; c, non-significantly inhibited cf vehicle; d, p < 5 x 10^{-5} cf vehicle; e, p < 0.04 cf either monotherapy; f, p < 1 x 10^{-8} cf vehicle; g, p < 1 x 10^{-7} cf either monotherapy; h, p < 0.003 vs vehicle; i, p < 0.01 vs either monotherapy.
Figure 1. (A) Chemical structure of XL147 (SAR245408). (B) Effects of XL147 on PI3K Signaling Pathway in EGF-Stimulated MCF7 Cells.
**Figure 2**
Sensitivity to proliferation inhibition by genotype
Figure 3
Anti-migratory and anti-angiogenic effects of XL147

A

HGF alone
HGF+ 370 nM
HGF+ 1111 nM
HGF+ 3333 nM
No HGF

B

EGF alone
EGF+ 13 nM
EGF+ 1111 nM
EGF+ 3333 nM
No EGF

C

VEGF alone
VEGF+ 370 nM
VEGF+ 3333 nM
VEGF+ 10000 nM
No VEGF
Figure 4
Inhibition of AKT, p70S6K, and S6 Phosphorylation in MCF7 and PC-3 Xenograft Tumors after a Single Oral Dose
Figure 5
Anti-tumor efficacy of XL147 administered as a single agent
Figure 6
Potentiation of anti-tumor efficacy of chemotherapy by XL147
The selective PI3K inhibitor XL147 (SAR245408) inhibits Tumor Growth and Survival and Potentiates the Activity of Chemotherapeutic Agents in Preclinical Tumor Models

Paul Foster, Kyoko Yamaguchi, Pin Pin Hsu, et al.

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http://mct.aacrjournals.org/content/suppl/2015/03/26/1535-7163.MCT-14-0833.DC2

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