Concomitant Inhibition of PI3Kβ and BRAF or MEK in PTEN-Deficient/BRAF-Mutant Melanoma Treatment: Preclinical Assessment of SAR260301 Oral PI3Kβ-Selective Inhibitor

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Abstract

Class IA PI3K pathway activation resulting from PTEN deficiency has been associated with lack of sensitivity of melanoma to BRAF kinase inhibitors. Although previous studies have shown synergistic activity when pan-PI3K inhibitors were combined with MAPK inhibitors in the treatment of melanoma exhibiting concurrent genetic abnormalities, overlapping adverse events in patients limit optimal dosing and clinical application. With the aim of specifically targeting PTEN-deficient cancers and minimizing the potential for on-target toxicity when inhibiting multiple PI3K isoforms, we developed a program to discover PI3Kβ-selective kinase inhibitors and identified SAR260301 as a potent PI3Kβ-selective, orally available compound, which is now in clinical development. Herein, we provide a detailed biological characterization of SAR260301, and show that this compound has outstanding biochemical and cellular selectivity for the PI3Kβ isoform versus the α, δ, and γ isoforms and a large panel of protein and lipid kinases. We demonstrate that SAR260301 blocks PI3K pathway signaling preferentially in PTEN-deficient human tumor models, and has synergistic antitumor activity when combined with vemurafenib (BRAF inhibitor) or selumetinib (MEK inhibitor) in PTEN-deficient/BRAF-mutated human melanoma tumor models. Combination treatments were very well tolerated, suggesting the potential for a superior safety profile at optimal dosing using selective compounds to inhibit multiple signaling pathways. Together, these experiments provide a preclinical proof-of-concept for safely combining inhibitors of PI3Kβ and BRAF or MEK kinase modulators to improve antitumor activity in PTEN-deficient/BRAF-mutant melanoma, and support the evaluation of SAR260301-based combinations in clinical studies. Mol Cancer Ther; 15(7); 1460–71. ©2016 AACR.

Introduction

The PI3K pathway mediates cell signaling in response to stimulation by growth factors and promotes key cellular functions, including survival, proliferation, growth, and metabolism. Downstream effectors of PI3K include AKT/PKB and mTOR. Class I PI3K lipid kinases comprise isoforms (PI3Kα, PI3Kβ, PI3Kγ, PI3Kδ) that have distinct roles within PI3K signaling and normal physiology. They are subdivided into: Class IA, comprising α, β, δ isoforms activated by receptor tyrosine kinases, G protein–coupled receptors (GPCR), and Ral sarcoma protein (RAS); and Class IB, whose single member, PI3Kγ, is activated by GPCRs. Class IA PI3Ks are heterodimeric enzymes composed of p85 regulatory and p110 catalytic subunits. All four isoforms generate phosphatidylinositol 3,4,5-triphosphate (PIP3), a secondary messenger involved in AKT activation. Cellular PIP3 levels are tightly regulated by PTEN, a lipid phosphatase that converts PIP3 into phosphatidylinositol 4,5-biphosphate (PIP2; refs. 1, 2).

Several findings suggest that Class IA PI3K signaling is involved in the etiology of human cancers, making these proteins ideal candidates for anticancer targeted therapy. In human tumors, activating mutations have been identified in PIK3CA, which encodes the p110α catalytic subunit of PI3Kα. In addition, tumor cells frequently lose expression of negative PI3K regulatory proteins (e.g., PTEN), resulting in constitutive activation and downstream signaling (3–5). While PI3Kα blockade inhibits growth of tumors harboring PIK3CA mutations, PTEN-deficient tumors are dependent on PI3Kβ activity. Indeed, conditional knockout of PI3KCB, which encodes the p110β catalytic subunit, but not PIK3CA, has been reported to inhibit tumor growth and prevent formation of cancers induced by PTEN deficiency (6–8). Thus, PI3Kβ-selective kinase inhibitors are being developed for the treatment of PI3Kβ-deficient malignancies, including SAR260301, an agent discovered and characterized in our laboratory (9).
The RAS/MAPK pathway is another major pathway mediating signaling from growth factor receptors and activated in many tumors. Several genetic changes can hyperactivate this pathway, as activating mutations in *RAS* or *BRAF* are found in approximately 20%–30% of solid tumors (10, 11). In melanoma, both MAPK and PI3K pathways are major mediators of tumorigenic signaling, and associated genetic abnormalities are frequent, particularly *NRAS* mutations (in ~20% of tumors), *BRAF* mutations (in ~45%), and *PTEN* loss (in 10%–30%). Although *PTEN* abnormalities and *NRAS* mutations are often mutually exclusive, concurrent *PTEN* deficiency and activating *BRAF* mutations are common in melanomas, resulting in activation of both PI3K and MAPK pathways (12–14). In *BRAF*-mutant melanoma, BRAF inhibitors provide clinical benefits but responses are transient; hence, understanding mechanisms of resistance could enable rational therapeutic strategies. For example, preclinical studies suggest that PTEN deficiency contributes to intrinsic BRAF inhibitor resistance (15, 16), and in a phase I study of dabrafenib (BRAF inhibitor), *PTEN* deficiency was associated with shorter progression-free survival (17). These findings support the rationale for combined targeting of PI3K and BRAF pathways in *PTEN*-deficient/BRAF-mutant melanoma.

This article describes the biological characterization of SAR260301, including its mechanism of inhibition, biochemical/ cellular selectivity, determinants of sensitivity based on pathway modulation, and anticancer effects in *BRAF*-mutant/ *PTEN*-deficient melanoma models, both as a single agent and in combination with MAPK pathway inhibitors. Two classes of MAPK pathway inhibitors were investigated: vemurafenib (PLX4032) BRAF inhibitor and selumetinib (AZD6244) MEK inhibitor. Together, these experiments provide the preclinical proof-of-concept for combining inhibitors of PI3K and BRAF or MEK to improve antitumor activity in BRAF-mutant/ *PTEN*-deficient melanoma.

Materials and Methods

Compounds

SAR260301 was synthesized at Sanofi (9). For cell-based studies, 10 or 30 nmol/L stock solutions of SAR260301, vemurafenib, selumetinib (Selleck Chemicals) were prepared in 100% DMSO. For mice studies, formulations were: 12.5% ethanol, 12.5% polysorbate80 (Solutol HS15), 5% polyethylene glycol 400 (PEG400), 2% DMSO, 0.1% polysorbate80 (selsulfonate), 10 or 30 mmol/L stock solutions of SAR260301, vemurafenib, selumetinib (Selleck Chemicals) were prepared in 100% DMSO.

PI3K lipid kinase assays

Enzymatic assays were performed using the PI3K homogeneus-time-resolved-fluorescence (HTRF) kit (Millipore) in 384-well format, as described previously (9). IC_{50} values and 95% confidence intervals (CI) were calculated using a four-parameter logistic model (18) in Biost@t Speed internal software.

PI3K kinase activity was also measured using LC-MS to characterize inhibition mechanism. ATP Km app was determined by preparing reaction mixtures containing 2 nmol/L PI3K (p110β,107α-His/phosphorytin, 0.224 heterodimer, produced in-house), 10 μmol/L Pip2 (Echelon), and different ATP concentrations in (50 mmol/L HEPES-Na, 5 mmol/L MgCl₂, 0.05% CHAPS, 5 mmol/L DTT, pH 7.4), incubated at room temperature. At specified time points, 5-μL aliquots were added to 45-μL stop solution [20 mmol/L EDTA, pH 8.0, 100 mmol/L 1-[2R-phosphohydroxyl]-inositol-3,4-bisphosphate, trisodium salt (S-PiP2, Cayman Chemicals; used as internal standard)]. Mixtures were analyzed using LC-MS (HPLC mobile-phase-gradient: water with 0.2% N, N-DMIPA/acetonitrile, 0.2% DMIPA; XBridge C18 3.5 μm, 2.1 × 30 mm-column; AB Qtrap5500-instrument). PI3P (Echelon) was used to calibrate MS signal. The k_{on} values were calculated as ratios of reaction rates over PI3K concentration (2 nmol/L). For IC_{50} measurement, methods were similar except that 62 μL of mixtures containing PI3K, ATP, and SAR260301 were preincubated for 15 minutes, and reactions started by adding 38-μL PiP2 solution. Final reaction mixtures containing 2 nmol/L PI3K, 10 μmol/L Pip2, various ATP concentrations, SAR260301 in reaction-buffer containing 2% DMSO were incubated for 25 minutes.

Kinase profiling studies

mTOR, VPS34, and DNA-PK assays were evaluated using time-resolved-fluorescence-resonance-energy-transfer (TR-FRET) technology.

mTOR. GFP-4EBP1-Thr46 phosphorylation was assayed using Terbium chelate–labeled antibody (Tb-anti-pThr46-4EBP1; Invitrogen). Reaction mixtures, containing mTOR (Invitrogen) 0.15 nmol/L in assay buffer (50 mmol/L HEPES, pH 7.5; 1 mmol/L EGTA; 0.5% glycerol; 0.01% BSA; 10 mmol/L MnCl₂; 0.1% NV10; 1 mmol/L DTT), were incubated for 60 minutes at room temperature with 0.4 μmol/L GFP-4EBP1 (Invitrogen). 10 μmol/L ATP, SAR260301 (in 3% DMSO). Reactions were stopped by adding 19 mmol/L EDTA containing 0.2 nmol/L Tb-anti-pThr46-4EBP1. After ≥2 hours at room temperature, fluorescence was measured with UltraEvolution reader (TECAN), excitation 340 nm. Phosphorylation was quantified using the fluorescence ratio emitted at 520 nm (pThr46-4EBP1) and 495 nm (Tb-anti-pThr46-4EBP1).

VPS34. Phosphatidylinositol (PI) phosphorylation was assayed by quantifying ADP generated from ATP using Transscreen ADP-kit (Cisbio). ATPs (2 μL) were combined with 8-μL PI (Sigma)/VPS34 (Invitrogen) in reaction buffer containing 50 mmol/L HEPES, 5 mmol/L MgCl₂, 0.1% (v/v) CHAPS, and 2 mmol/L TCEP at pH 7.1. Reactions were started by adding 10-μL ATP (Sigma) in reaction buffer, followed by incubation for 60 minutes at room temperature. Reactions contained 3% DMSO, 10 μmol/L ATP, 5 nmol/L VPS34, 0.51 nmol/L to 10 μmol/L SAR260301 (adapted to generate ~2 μmol/L ADP). ADP/ATP standards were prepared and reactions stopped per manufacturer’s instructions. Fluorescence emission at 620/665 nm was detected using a RubyStar reader (BMG Labtech).

DNA-PK. Ser15 phosphorylation in a fluorescein-labeled p53-derived peptide was quantified using Tb-anti-pSer15-p53 antibody. DNA-PK (Promega; 1.2 U/μL) and DNA (20 μg/mL) from calf thymus were mixed in buffer (HEPES 50 mmol/L, pH 7.5; EGTA 1 mmol/L; MgCl₂; 10 mmol/L; Brij35 0.01%; DTT 1 mmol/L). In 5 μL 1.6 μmol/L fluorescein-p53-peptide, 10 μmol/L ATP, SAR260301 were added under 1-μL DMSO 10% in assay buffer. Incubations were started by adding 5-μL DNA-PK (0.6 μU/μL). After 60 minutes at room temperature, reactions were stopped with 10-μL Tb-anti-pSer15-p53 and EDTA in buffer [20 mmol/L Tris-HCl, pH 7.5; sodium azide...
0.02% NP-40 0.01% (v/v)]. Final concentrations of Tb-anti-pSer15-p53 and EDTA were 5 and 10 mmol/L, respectively. Fluorescence transfer was measured using a PHERAstar (BMG Labtech) with Lanthascreen filter.

SAR260301 was profiled against (i) a 192 kinase panel using Micro-fluidic Mobility Shift Assay technology (PerkinElmer); (ii) a 442 kinase panel at KINOMEscan (DiscoverX) (19); (iii) a 190 kinase panel at ActivX-Biosciences Inc. (20); and (iv) two lipid kinases at Millipore (Kinase Profiler).

Cell lines and cell culture

The following cell lines were purchased from ATCC (date): A2058 (12/2006), A575 (04/1997), AN3CA (03/2007), BT474 (09/2000), BT549 (12/2003), HCC1954 (08/2013), HCT116 (2005), LnCaP (04/2001), MDA-MB-468 (2003), MiaPaCa-2 (06/2010), NCI-H1155 (08/1996), NCI-H460 (1997), NCI-H596 (12/2003), NCI-N87 (12/2006), PC3 (04/2002), RPMI-7951 (07/2004), SK-BR-3 (03/2003), T47D (06/2000), U-87MG (2003), and WM-266.4 (05/2003) and RAW 264.7 (05/2006). UACC-62 (12/1988) and UCSD-242L (09/1988) were from (2003), and WM-266.4 (05/2003) and RAW 264.7 (05/2006). UACC-62 (12/1988) and UCSD-242L (09/1988) were from NCI; MCF7 (2003) from European Collection of Cell Cultures; and Colo-849 (05/2005) from DSMZ. They have not been authenticated since receipt. They were stored within a centralized bank, used up to passage 20, and routinely screened for mycoplasma contamination using MycoAlert mycoplasma detection kit (Lonza). Cells were cultured in DMEM high-glucose medium (Invitrogen), except for: BT549, Colo-849, NCI-H596, NCI-N87, RPMI-7951, UACC-62, UCSD-242L, WM-266.4 (RPMI1640); AN3CA, MCF7 (MEM α); SK-BR-3 (McCoa 5A). Media were supplemented with 10% FCS, 2 mmol/L L-glutamine, and growth factors (Invitrogen) recommended by cell suppliers.

Mechanistic models. MEF-3T3-myr-p110β and MEF-3T3-myr-p110δ conditionally overexpressing a myristoylated-p110β or p110δ protein, were established in-house by transfecting MEF-3T3-Tet-Off cells (Clontech, 05/2005) with pTRE2Hygro-myc vector containing human myristoylated wild-type PI3KCB or PI3KCD sequences. These clones overexpress activated p110β or p110δ protein only in absence of doxycycline, resulting in constitutive AKT phosphorylation. Cells were cultured in DMEMα containing 100 mg/mL hygromycin-B, 2 mmol/L l-glutamine, 10% Tet-approved-FCS (Clontech 631106). SAR260301 cellular selectivity against VPS34 was evaluated in GFP-2xFYVE- and in GFP-LC3-HeLa-derived cells as described previously (21). FYVE binds the PI3K complexes VPS34 was evaluated in GFP-2xFYVE- and in GFP-LC3-HeLa-derived cells as described previously (21). FYVE binds the PI3K complexes (VPS34), a 442 kinase panel at ActivX-Biosciences Inc. (20); and (iv) two lipid kinases at Millipore (Kinase Profiler).

AKT phosphorylation assays

Cells were seeded in 96-well plates in complete medium and treated with increasing SAR260301 concentrations for 1.5 hours. For MCF7, after SAR260301 treatment, cells were incubated for 10 minutes with IGF1 (R & D Systems) at 125 ng/mL final concentration in serum-free culture medium.

AKT phosphorylation on Ser473 or Thr380 (pAKT-Ser473 or pAKT-Thr380) was measured using a sandwich immunoassay kit (Meso Scale Discovery), as recommended by the manufacturer and described previously (22, 23). IC₅₀ values were calculated using the Biost@t SPEED internal software and a four-parameter logistic model.

Western blot analyses

Cell lines were treated with SAR260301, vemurafenib, or both drugs at given concentrations (0.1% DMSO final concentration). Cell lysates were prepared using standard procedures. Protein (20 µg) were separated on NU-PAGE 4–12% gels (Invitrogen). All primary antibodies, from rabbit sources, were purchased from Cell Signaling Technology. PARP cleavage was detected using anti-cleaved PARP (Asp214) polyclonal antibody (#9541). Other antibodies used were monoclonal anti-GAPDH (14C10; #2118), polyclonal anti-phosphorylated S6 ribosomal protein (Ser240/244; #2215), monoclonal anti-phosphorylated AKT (Thr380; #4056), or (Ser473; #4060), polyclonal anti-phosphorylated PRAS40 (Thr246; #2640). Antibody binding was revealed using anti-rabbit IgG-HRP conjugate (W4018; Promega). Luminescence was detected using FujiFilm (Ray Test) apparatus.

Proliferation studies

Cell proliferation was measured by quantifying intracellular ATP using CellTiter-Glo kit (Promega). Cells seeded into 96-well black microplates in complete medium, were treated with single or combined agents 0.3 nmol/L–30 µmol/L (0.1% DMSO final concentration) for 96 hours, according to the Ray Design methodology (24). Briefly, a matrix of concentrations is prepared to allow the interaction between two compounds at different proportions/ratios, each corresponding to a “ray”. The design includes one ray for each single agent (Rays 1 and 5) and 4 for combinations. The effective fractions, corresponding to the proportional effect of each compound alone according to their IC₄₀ value, were estimated between 0.05 and 0.95. The combination rays correspond to SAR260301/vemurafenib or SAR260301/selumetinib ratio of 1:3, 1:1, 3:1 and 9:1, estimated based on effective fractions. These fractions were based on IC₅₀ calculations because SAR260301 single agent being poorly active, IC₅₀ values were better estimated than IC₅₀. The combination index (Ki), based on the Loewe additivity equation, was determined for each ray. Each Ki and 95% CI were estimated using a global nonlinear model fitted with the NL MIXED procedure in SAS V9.2 software. Conclusions were: additivity when the CI included 1, significant synergy when CI upper bound<1, significant antagonism when CI lower bound >1.

In vivo studies

Animal procedures were conducted in accordance with conditions established by the European Community (2010/63/EU Directive) and approved by the Sanofi Animal Care and Use Committee. Female SCID mice (Charles River, France), 8–10 weeks, weighing at least 18 g, with free access to food and sterile water, were implanted subcutaneously with UACC-62 or WM-266.4 cells (3 × 10⁶ cells/animal). Drugs were administered orally to seven mice (median tumor size at start of treatment: 125–126 mm³ for SAR260301/vemurafenib and 126–144 mm³ for SAR260301/selumetinib). SAR260301 (150 mg/kg twice daily)
and vemurafenib (50 or 100 mg/kg once daily) or selumetinib (10 or 25 mg/kg once daily) were administered from days 8–15 (vemurafenib/UACC-62), 21–31 (vemurafenib or selumetinib/WM-2664), or 11–22 (selumetinib/UACC-62), after tumor implantation, and antitumor activity was measured on day 15 (vemurafenib studies) or 22 (selumetinib studies). Tumor volumes [in mm$^3$ based on the following formula, volume = length (mm) × width$^2$ (mm$^2$)/2] were measured twice weekly and body weight recorded daily. Endpoints collected were tumor volumes and the percentage of tumor regression (% of volume decrease posttreatment compared with pretreatment). Statistical significance was determined by a Dunnett test after a two-way ANOVA on rank-transformed tumor volume changes from baseline with $P < 0.05$ considered significant.

**Results**

**SAR260301 is a potent ATP-competitive PI3Kβ inhibitor**

Using a TR-FRET assay, SAR260301 inhibited PI3Kβ lipid kinase activity with an IC$_{50}$ of 52 nmol/L (95% CI, 29–95 nmol/L) at 100 µmol/L ATP. PI3Kβ kinetics and SAR260301 inhibition were further investigated using LC-MS to quantify the reaction product PIP3. The reaction rate in this assay was constant for more than 30 minutes and its dependence on ATP concentration could be described by the Michaelis– Menten equation with the following parameters: $K_m$ app = 108 ± 8 µmol/L, $K_{obs}$ = 12 ± 3/min (mean ± range values for two independent experiments; Fig 1A). IC$_{50}$ values for PI3Kβ inhibition by SAR260301 were measured at varying ATP concentrations (Fig 1B). The linear dependence of obtained IC$_{50}$ values on ATP concentration conformed to the ATP-competitive inhibitory mechanism assumed from the X-ray crystal structure of PI3Kβ ligand–bound SAR260301 (Fig 1C, ref. 9). Fitting this dependence to the equation for tight binding competitive inhibitors \[ IC_{50} = K_i \times ([S]/K_m \text{ app} + 1) + [E]/2 \] (25), where [E] is the concentration of PI3Kβ), produced a $K_i$ value for SAR260301 of 3.5 nmol/L.

**SAR260301 is a selective PI3Kβ inhibitor in biochemical assays**

The PI3K$\beta$ selectivity of SAR260301 was evaluated by determining its biochemical potency against other recombinant PI3K isoforms (PI3Kz, PI3Kβ, PI3Kγ) in vitro using TR-FRET technology (Table 1). Other than PI3Kβ, PI3Kγ was the only isoform inhibited at submicromolar concentrations (IC$_{50}$ 403 nmol/L vs. 1,869 nmol/L for PI3Kz and >10,000 nmol/L for PI3Kγ). SAR260301 did not inhibit mTOR (IC$_{50}$ >10 µmol/L). Furthermore, in a large panel of lipid and protein kinases (>400), the only other kinase inhibited by SAR260301 was VPS34 lipid kinase (IC$_{50}$ 180 nmol/L). Table 1 shows lack of activity against several other phylogenetically related kinases. Thus, SAR260301 is a potent and selective PI3Kβ kinase inhibitor, showing moderate inhibitory activity against PI3Kβ (7.8-fold selectivity) and VPS34 (3.5-fold selectivity).

**SAR260301 is a selective PI3Kβ inhibitor in cellular assays**

The PI3K$\beta$-isoform selectivity of SAR260301 in biochemical settings was confirmed in cellular assays (Table 1). SAR260301 inhibition of AKT-Ser473 phosphorylation was measured in cellular systems relevant for PI3Kz, PI3Kβ, PI3Kγ, and PI3Kδ (PI3KCA-mutated H460 lung tumor cells, MEF-3T3-myr p110β, C5a-stimulated RAW 264.7 mouse macrophages, and MEF-3T3-myr p110β, respectively; ref. 23). Selectivity against VPS34 was investigated in GFP-2xFYVE and GFP-1C3 HeLa–expressing cells. SAR260301 at 10 µmol/L did not induce GFP-FYVE relocalization to cytoplasm and did not prevent starvation-induced autophagy (Supplementary Fig. S1). In these studies, SAR260301 inhibited PI3Kβ with a potency 26-fold higher than PI3Kδ (IC$_{50}$ 32 vs. 823 nmol/L), showed lower potency against PI3Kz (IC$_{50}$ 2,825 nmol/L) and no inhibitory activity against PI3Kγ or VPS34 (<30% inhibition at 3 or 10 µmol/L). In conclusion, SAR260301 is a PI3Kβ-selective inhibitor in cellular setting, inhibiting PI3Kβ 26-fold, 88-fold, >94-fold, and >313-fold more potently than PI3Kβ, PI3Kz, PI3Kγ, and VPS34, respectively.

**SAR260301 inhibits the PI3K pathway preferentially in PTEN-deficient cells**

To investigate genetic determinants of sensitivity, modulation of AKT phosphorylation (pAktSer473 and/or pAktThr308) induced by SAR260301 was determined in a panel of 24 tumor cell lines exhibiting different genetic abnormalities. SAR260301 was preferentially active in PTEN-deficient compared with PTEN-expressing cell lines (Fig. 2). IC$_{50}$ values for pAKT inhibition in PTEN-deficient PC3 and LNCaP prostate carcinoma, U-87MG glioblastoma, and MDA-MB-468 and BT-549 breast carcinoma cells ranged from 39 to 310 nmol/L for pAKT-Ser473 and 20–209 nmol/L for pAKT-Thr308. Interestingly, SAR260301 inhibited pAKT in tumor cell lines exhibiting additional genetic abnormalities besides PTEN deficiency as BRAF, KRAS, PIK3R1, and FGFR2 mutations. In contrast, SAR260301 was not active (IC$_{50}$ > 1,000 nmol/L) in PTEN wild-type cells, including PIK3CA-mutated and/or HER2-amplified tumor cells (Supplementary Table S1; Fig. 2). In conclusion, SAR260301 modulates PI3K pathway preferentially in PTEN-deficient human tumor cells, and concurrent BRAF, KRAS, or PIK3R1 and FGFR2 mutations do not negatively impact PI3K pathway modulation by SAR260301.

SAR260301 was also shown to inhibit, in a dose- and time-dependent manner, phosphorylation of other PI3K/AKT pathway downstream effectors, including PRAS40 and S6RP (Supplementary Fig. S2).

SAR260301 has moderate antitumor activity as a single agent

Antitumor activity of SAR260301 was first evaluated in vitro. In MEF-3T3-myr-p110β mechanistic model, SAR260301 inhibited PI3Kβ-dependent proliferation/viability in low serum conditions with an IC$_{50}$ of 196 nmol/L (95% CI, 117–327 nmol/L; Supplementary Fig. S3A). In PTEN-deficient human prostate tumor cells, SAR260301 inhibited LNCaP cell proliferation in low and high serum conditions with an IC$_{50}$ values of 2.9 and 5.0 µmol/L, respectively, after 4-day treatment, whereas it was inactive in these conditions in PC3 cells at concentrations up to 10 µmol/L, despite target engagement. After prolonged treatment, SAR260301 at 3 or 10 µmol/L inhibited PC3 cell proliferation in low serum conditions, with a cytostatic effect achieved for 14 days, despite some cell death induction observed at 10 µmol/L (Supplementary Fig. S3B and S3C). In Lncap model, SAR260301 induced moderate cell apoptosis at 10 µmol/L with 3.5-fold increase in PARP cleavage vs. control (Supplementary Fig. S3D). SAR260301 also led to antitumor activities in PTEN-deficient/BRAF-mutant human melanoma cells, inhibiting cell proliferation with IC$_{50}$ values of 6.5 and 3.3 µmol/L for UACC-62 and WM-266.4, respectively, after 4-day treatment. (IC$_{50}$ values providing better estimates than IC$_{50}$ based on the moderate potency).
Figure 1. SAR260301 is a potent ATP-competitive PI3Kβ inhibitor. A, time course of PIP3 generation catalyzed by PI3Kβ at varying concentrations of ATP and the dependence of $k_{obs}$ for the reaction on ATP concentration (LC-MS assay); representative of two independent experiments performed. B, inhibition by SAR260301 of PI3Kβ-catalyzed generation of PIP3 (mean ± range) at varying concentrations of ATP (LC-MS assay), and dependence of the observed IC50 values on ATP concentration (see Results). C, chemical structure of SAR260301 and X-ray cocrystal structure of SAR260301 bound to the ATP-binding site of p110β subunit of PI3Kβ.
SAR260301 PI3Kβ-Selective Inhibitor in PTEN-Mutated Melanoma

Table 1. Potency of SAR260301 in biochemical and cell-based assays

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| NOTE: IC_{50} values and 95% confidence intervals (CI) were derived from the mean of at least two independent experiments. Biochemical and cellular IC_{50} of SAR260301 for kinases of interest are shown, illustrating that SAR260301 is a potent and selective inhibitor of PI3Kβ.

Abbreviations: CI, confidence interval; nd, not defined.

aBiological selectivity profile against a panel of >400 kinases; IC_{50} = concentration required for 50% target inhibition.

bDetermined using the Millipore panel.

cDetermined using the ActivX Biosciences panel.

In vitro antitumor activity of SAR260301, administered orally at 150 mg/kg twice daily in mice xenographed subcutaneously with PTEN-deficient tumors, was reported previously, showing significant tumor growth inhibition against UACC-62 tumors with growth delay but nonsignificant effect against PC3 (9).

SAR260301 synergizes with MAPK pathway inhibitors to inhibit growth of PTEN-deficient and BRAF-mutant melanoma cells in vitro and in vivo

PTEN deficiency is associated with lack of sensitivity to targeted therapies, particularly resistance of BRAF-mutant melanoma to BRAF or MEK inhibitors (15–17, 26). Concomitant genetic abnormalities in P13K and MAPK signaling pathways in melanoma may require combination treatment to inhibit both pathways concomitantly (Supplementary Fig. S4). Thus, the activity of SAR260301 combined with a MAPK inhibitor against PTEN-deficient/BRAF^{V600E} melanoma was investigated in vitro and in vivo using vemurafenib (BRAF inhibitor) and selumetinib (MEK1/2 inhibitor). The in vitro antitumor interaction between SAR260301 and either agent was characterized using a ray design approach in UACC-62 and WM-266.4 models. Compounds were first evaluated as single agents. In these studies, vemurafenib and selumetinib inhibited UACC-62 and WM-266.4 cell proliferation/viability with IC_{50} values of 26 and 57 nmol/L (vemurafenib), and 19 and 46 nmol/L (selumetinib). In contrast, single-agent SAR260301 had modest antiproliferative activity, with IC_{40} values of 6.5 (UACC-62) and 3.3 nmol/L (WM-266.4). Combining SAR260301 with vemurafenib or selumetinib resulted in synergistic activity or additivity with tendency to synergy (Ki<1) against both UACC-62 and WM-266.4 cells and for all drug ratios tested (Fig. 3A–D). These studies show that SAR260301 synergizes with both a BRAF inhibitor and a MEK1/2 inhibitor, increasing their antitumor activity against PTEN-deficient/BRAF-mutant melanoma cells. Further studies showed that the synergy observed with SAR260301 plus vemurafenib correlated with increased apoptosis induction (based on PARP cleavage) and more sustained inhibition of S6RP phosphorylation in UACC-62 and WM-
266.4 cells compared with either agent alone (Fig. 4), indicating that combined treatment also impacts the mTORC1 pathway.

Combined treatments were also investigated in vivo in SCID mice bearing UACC-62 and WM-266.4 subcutaneous melanomas. Drugs administered orally, included SAR260301 (150 mg/kg twice daily) and vemurafenib (50 or 100 mg/kg once daily) or selumetinib (10 or 25 mg/kg once daily). In UACC-62 model, combined SAR260301 plus vemurafenib treatment resulted in enhanced antitumor effects as compared with single agents, with tumor stasis achieved at both vemurafenib dose levels (Fig. 5A). In this experiment, SAR260301 induced similar pAKT inhibition when administered as a single agent (58%) or combined with vemurafenib 50 (60%) or 100 mg/kg (68%), 4 hours after last treatment. Combination with selumetinib in this model also resulted in significantly greater antitumor activity than single-agent treatments, with tumor regression, whereas selumetinib alone induced only tumor growth delay (10 mg/kg) or stasis (25 mg/kg; Fig. 5B). Synergy was also demonstrated in WM-266.4 model for combination with both agents with tumor growth delay or tumor stasis achieved (Fig. 5C and D). In WM-266.4 experiments, single-agent MAPK inhibitors induced similar level of pERK inhibition as compared with combined treatments (54% for vemurafenib single agent vs. 60% in combination and 85% for selumetinib single agent vs. 81%–87% in combination), 4 hours after last treatment. Importantly, all treatments were well tolerated with no significant loss of body weight for single or combined treatment. Overall, these findings showed that the benefit of combined treatments observed in cellular studies was also present in animal studies. Taken together, these studies show that selective PI3K inhibition with SAR260301 blocks PI3K/AKT pathway signaling of PTEN-deficient/mutant melanoma, and has synergistic antitumor activity when combined with BRAF or MEK inhibitors in relevant human tumor models.

Discussion

The high prevalence of PTEN deficiency in several malignancies with high unmet medical need has motivated the search for drugs targeting this oncogenic event. Various PI3K pathway inhibitors have been investigated in PTEN-deficient cancers, including pan-PI3K inhibitors that inhibit all four PI3K isoforms and/or mTORC1. However, the application of pan-PI3K inhibitors has been hampered by dose-limiting toxicities (DLT) resulting from on-target adverse events (e.g., hyperglycemia, fatigue, rash, and diarrhea). Furthermore,
MTDs of pan-P13K inhibitors may not potently inhibit the PI3Kβ isoform. Aiming to specifically target PTEN-deficient cancers and reduce adverse events, we undertook a PI3Kβ isoform-specific discovery program and identified SAR260301 as a potent and highly selective PI3Kβ inhibitor (9).

Similar to studies of genetic suppression of PIK3CB (8), our data describe the preferential activity of SAR260301 against PTEN-deficient tumor cells, demonstrating that PI3Kβ kinase activity is involved in PI3K pathway modulation in this selective genetic context. This has been reported with other p110β small-molecule inhibitors (27, 28). Interestingly, our data show that concurrent BRAF or KRAS mutations do not impact sensitivity to SAR260301, which may be advantageous for treating human tumors exhibiting genetic abnormalities on MAPK and PI3K signaling pathways. Clinical trials with pan-P13K inhibitors have shown no clear correlation between molecular alterations in PI3K pathway and antitumor effect; clinical responses were observed in patients with KRAS-mutant cancers and BRAF-mutant melanoma, which was unexpected because tumors with activating mutations in MAPK pathway proteins may be resistant to PI3K inhibitors (29). Nor did KRAS mutation confer resistance to the PI3Kβ inhibitor BYL719 in PIK3CA-mutated cell lines (30). These observations suggest that the impact of KRAS or BRAF mutations on sensitivity to PI3K pathway inhibitors depends on the cellular context and the tumorigenic signaling pathway.

Figure 3.
SAR260301 synergizes with vemurafenib and selumetinib to inhibit PTEN-deficient/BRAF-mutant melanoma cell lines in vitro. The panels contain isobolograms from representative experiments showing the synergy evaluation for SAR260301 with vemurafenib (A and B) and selumetinib (C and D) in two different models: UACC-62 (A and C) and WM-266.4 (B and D). Cells were treated with SAR260301/vemurafenib or SAR260301/selumetinib, either as single agents or in combination, at concentrations ranging from 0.3 nmol/L to 30 pmol/L, for 96 hours, according to Ray Design methodology and cell proliferation was measured using a commercially available kit. Three to four experiments were performed for each cell line. For each ray, the combination index $K_i$ based on the Loewe additivity equation and 95% CIs were determined. Additivity was concluded when the CI of the $K_i$ included 1; significant synergy was concluded when the upper bound of the CI of $K_i$ was <1 and significant antagonism was concluded when the lower bound of the CI of $K_i$ was >1.
We demonstrate that although SAR260301 has potent single-agent antiproliferative activity in vitro in a p110β-dependent cell model, it has moderate in vitro and in vivo antitumor activity as a single agent in PTEN-deficient human tumor models, despite potently inhibiting the PI3K pathway. Our studies were performed in prostate cancer and melanoma models, indications in which PTEN-deficiency is highly prevalent and in which PTEN has been shown to play a key role in the cell transformation process. SAR260301 inhibited prostate cancer cell proliferation in vitro with moderate activity and higher potency in LnCaP compared with PC3 cells after 4-day treatment in low or high serum conditions. The higher sensitivity of LnCaP versus PC3 has previously been observed with the PI3K/Δ inhibitor AZD8186, which inhibits growth of PTEN-deficient prostate carcinomas except in androgen receptor (AR)-negative PC3 cells; some antitumor response to AZD8186 was observed in PC3 model in vitro, indicating borderline sensitivity likely dependent on the experimental assay conditions (28, 31). Similarly, SAR260301 inhibited PC3 cell proliferation in vitro with higher potency after 14-day treatment in low serum conditions, inducing a cytostatic effect. Some cell death induction was observed at 10 μmol/L, but it is difficult to ensure PI3Kβ selectivity at this high concentration and we cannot exclude a potential effect of another target. In melanoma cells, single-agent SAR260301 inhibited UACC-62 and WM-266.4 cell proliferation in vitro with moderate potency after 4-day treatment in low and high serum conditions. In vitro, SAR260301 monotherapy had limited antitumor activity both against PC3 prostate tumors and UACC-62 and WM-266.4 melanoma, leading only to tumor growth delay, despite potent pathway modulation (9). This is consistent with another study showing that genetic suppression of PIK3CB but not PIK3CA in PC3 tumors, while inhibiting PI3K pathway signaling, had modest antitumor effects (AT/AC of 49%; ref. 8). Given the multiple genetic abnormalities found in human cancers, it is conceivable that PTEN-deficient tumors are not exclusively addicted to PI3Kβ-mediated signaling, as first anticipated, suggesting that PI3Kβ inhibition is necessary but not sufficient to cause tumor regression. The PI3K pathway is regulated at several levels with positive and negative feedback loops that can cause pathway reactivation, dependent on cell lineage and genetic context. This has also been reported previously with selective PI3Kα or PI3Kβ inhibition of PIK3CA-mutant breast cancer or PTEN-deficient prostate cancer, respectively (32, 33). In PTEN-deficient prostate cancer, combined treatment with hormonal therapy improved AZD8186 efficacy, certainly due to upregulation of AR-target genes following PI3K/Δ inhibition and compensatory crosstalk between the PI3K and AR pathways (31). Overall, these reports suggest that PI3Kβ inhibition alone may not be a viable therapeutic strategy and may need to be combined with other targeted therapies to achieve tumor regressions in a complex genetic context encountered in most human cancers.

We investigated SAR260301 in melanoma because in this tumor, PTEN has a well-established tumor suppressor role and loss of expression is associated with carcinogenesis (34–37), which may be potentiated by BRAF mutation (16, 38). In patients with BRAF-mutant tumors treated with the BRAF inhibitor dabrafenib, PTEN deficiency has been associated with worse outcomes (17). The high frequency of genetic abnormalities in both PI3K and MAPK pathways in melanoma has prompted studies of concomitant inhibition of targets within both pathways, and combining pan-PI3K with MAPK inhibitors has consistently shown synergistic activity against melanoma in vitro and in vivo (39–44). We found that SAR260301 had synergistic in vitro activity when combined with BRAF.
SAR260301 combined with BRAF or MEK inhibitors results in improved antitumor activity in a PTEN-deficient/BRAF-mutant melanoma model. Mice bearing established subcutaneous tumors derived from UACC-62 (A and B) or WM-266.4 (C and D) melanoma cell lines were treated daily from day 8 to 15 (UACC-62) with vemurafenib, from day 11 to 22 (UACC-62) with selumetinib, or from day 21 to 31 (WM-266.4) with vemurafenib or selumetinib and/or with either SAR260301 or vehicle twice daily. Each data point represents the median + MAD of calculated tumor volumes in experimental groups of seven mice, the MAD representing the median absolute deviation. Statistical differences of test versus control, or combination versus single agents, was determined using Dunnett tests after two-way ANOVA on rank-transformed tumor volume changes from baseline, with \(P < 0.05\) considered significant. Respective symbols indicate statistically significant differences: \(\dagger\), between treated and control experimental groups; \(\dagger\dagger\), between combination and best single agent; \(\dagger\dagger\dagger\dagger\), \(P < 0.0001\); \(\dagger\dagger\dagger\), \(P < 0.001\); \(\dagger\dagger\), \(P < 0.01\); \(\dagger\), \(P < 0.05\); \(\dagger\dagger\dagger\dagger\dagger\), \(P < 0.0001\); \(\dagger\dagger\dagger\), \(P < 0.001\); \(\dagger\dagger\), \(P < 0.01\); \(\dagger\), \(P < 0.05\). A and C, combination with vemurafenib. B and D, combination with selumetinib. B, combined treatment in UACC-62 tumor-bearing mice of SAR260301 with selumetinib 10 and 25 mg/kg resulted in 8.2% and 29.8% tumor regression, respectively. bid, twice daily; qd, once daily.

(vemurafenib) or MEK (selumetinib) inhibitors in two different PTEN-deficient/BRAF-mutant melanoma models. This synergy was observed with different ratios of effective drug doses, which is an advantage for clinical application. Our studies demonstrate that using equipotent concentrations, combination treatment can achieve the same antiproliferative effects as single-agent treatment but with a 5- to 10-fold lower drug concentration. Interestingly, the synergy observed with SAR260301 plus vemurafenib correlated with increased apoptosis induction and more sustained inhibition of S6RP phosphorylation compared with either agent alone, indicating that combined treatment impacts the mTORC1 pathway. Notably, enhanced in vivo antitumor activity was achieved using SAR260301 with suboptimal single-agent doses of vemurafenib and selumetinib. Strong PI3K and MAPK pathway modulation were maintained in combined treatments. Importantly, combination treatments were well tolerated in mice at two different dosages, suggesting that combinations of selective compounds may have limited toxicity. This is an important finding because pan-PI3K and MAPK inhibitors have overlapping adverse event profiles in patients and optimal dosing is limited by toxicity. Nevertheless, clinical studies are needed to evaluate the potential for DLTs that cannot be predicted by preclinical models, for example, fatigue.
Several studies have shown that resistance to BRAF or MEK inhibitors in melanoma can be overcome by concurrent PI3K pathway blockade, including inhibitors of PI3K, AKT, mTOR, and/or IGF-1R (45–47). Studies have also revealed that PI3K influences the interplay between PI3K and MAPK pathways. In melanoma samples and cells, BRAF-mutant tumors had significantly higher levels of pAKT than BRAF-wild-type tumors, and almost all tumors with elevated pAKT had low PTEN levels (48). Furthermore, in BRAF-mutant melanoma cells, sensitivity to MEK inhibition was associated with loss of PTEN and PI3K pathway activation (high pAKT level; refs. 26, 42, 45, 46). PTEN expression therefore represents a therapeutically relevant parameter that could inform treatment choices in melanoma.

Our findings confirm that PI3Kβ inhibitors modulate PI3K pathway preferentially in PTEN-deficient tumors and are the first to demonstrate that combining PI3Kβ inhibition with BRAF or MEK inhibition has synergistic activity in PTEN-deficient/BRAF-mutant melanoma. In addition to SAR260301, other selective PI3Kβ inhibitors in clinical development include GSK2636771, which is being examined in phase I/IIa trials as monotherapy for PTEN-deficient solid tumors (NCT01458067) and combined with enzalutamide for castration-resistant prostate cancer (CRPC; NCT02215096; ref. 49), and AZD8186 PI3Kβ/δ inhibitor in phase I (NCT01884285), which has shown inhibitory activity against PTEN-deficient tumor cells and preclinical antitumor potential in combination with signaling pathway inhibitors (49, 50). Interestingly, phase I data showed that GSK2636771 is well tolerated with manageable DLTs (hypophosphatemia and hypomagnesemia), and has shown inhibitory activity against PTEN-deficient tumors and is the first to demonstrate that combining PI3Kβ inhibition with BRAF or MEK inhibition has synergistic activity in PTEN-deficient/BRAF-mutant melanoma. In addition to SAR260301, other selective PI3Kβ inhibitors in clinical development include GSK2636771, which is being examined in phase I/IIa trials as monotherapy for PTEN-deficient solid tumors (NCT01458067) and combined with enzalutamide for castration-resistant prostate cancer (CRPC; NCT02215096; ref. 49), and AZD8186 PI3Kβ/δ inhibitor in phase I (NCT01884285), which has shown inhibitory activity against PTEN-deficient tumor cells and preclinical antitumor potential in combination with signaling pathway inhibitors (49, 50). Interestingly, phase I data showed that GSK2636771 is well tolerated with manageable DLTs (hypophosphatemia and hypomagnesemia), and has preliminary single-agent activity in a PTEN-deficient tumor (PR observed in a patient with PTEN-deficient CRPC).

Supported by the preclinical studies summarized here, a phase I/IIb trial has been initiated that examines SAR260301 monotherapy in patients with advanced cancer and combination therapy with vemurafenib in patients with melanoma (NCT01673737). This trial will provide an initial assessment of whether combining inhibitors of PI3Kβ and MAPK pathway has the potential to provide clinical benefits for patients with melanoma.

References


Concomitant Inhibition of PI3Kβ and BRAF or MEK in PTEN-Deficient/ BRAF-Mutant Melanoma Treatment: Preclinical Assessment of SAR260301 Oral PI3K β-Selective Inhibitor

Hélène Bonnevaux, Olivier Lemaitre, Loic Vincent, et al.

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