Supplementary Materials

Patients and Methods

Pharmacokinetic assessments

Blood samples for pharmacokinetic (PK) analyses were collected pre-morning dose on days 1, 2, 8, 15, 27 of cycle 1; days 1, 15, 22 and 23 of cycle 2; day 1 of cycles 3 and 4; and every 4 cycles thereafter. Post-morning dosing blood samples were collected at 0.25, 0.5, 1, 2, 3, 4, 6, 8 and approximately 10–12 hours on days 1 and 27 of cycle 1, and on day 22 of cycle 2. To investigate drug washout, no doses were given following the morning dose on day 27 of cycle 1 and PK samples were taken at 24 and 48 hours post-dose prior to cycle 2. In addition, 2-hour post-morning dose samples were obtained on day 8 of cycle 1, day 1 of cycles 2–4, and day 1 of every fourth cycle thereafter.

Bioanalytical and pharmacokinetic analyses

Plasma was separated from PK blood samples by centrifugation within 30 minutes of collection and stored frozen (<–20 °C) until analysis. Urine samples were collected within 2 hours pre-dose on cycle 1 day 1, with a 12-hour urine collection (twice-daily dosing) and 24-hour urine collection (once-daily dosing) after the morning dose on cycle 1 day 27. Amount of SAR245409 excreted unchanged in urine was calculated as concentration in urine x total urine volume. The percentage of SAR245409 excreted unchanged in urine was calculated as (amount excreted unchanged in urine/total dose given) x 100. Concentrations of SAR245409 were initially

measured in a small subset of patients. Based on the low amount of unchanged SAR245409 found in urine at steady-state, no further analysis of urine samples was performed. Not all PK samples were obtained in all patients as the PK blood and urine sampling schedules were adjusted by amendment during the study. Bioanalysis of human plasma and urine samples was performed using a validated liquid chromatography/tandem mass spectrometry (LC-MS/MS) method. The internal standard was D_5-SAR245409. Clean-up of K_2EDTA anticoagulant-treated plasma samples or urine samples, 100 µL, was prepared by protein precipitation using 400 µL acetonitrile/methanol (95/5, v/v). A portion of supernatant (300 µL) was transferred to a 96-well plate and evaporated to dryness with nitrogen gas at 45°C. The sample was then reconstituted with 200 µL of water/acetonitrile (75/25, v/v) containing 0.1% of formic acid and subsequently 20 µL of solution was injected into the LC-MS/MS system. The standard curve covered a linear range of 1–1000 ng/mL in human plasma and urine. The lowest detection limit of the method was 1 ng/mL.

The extracted samples were analyzed by LC-MS/MS consisting of a Shimadzu LC-20AD integrated HPLC system and an Applied Biosystems/MDS Sciex API 4000 mass spectrometer with an APCI interface. An isocratic mobile phase containing acetonitrile/water (75/25, v/v) with 1% formic acid at a flow rate of 0.25 mL/min and an HPLC analytical column (MAC-MOD Analytical Inc., ACE C18, 2.1 x 50 mm, 5 µm) were employed for the LC-MS/MS analysis. A positive ion multiple reaction monitoring (MRM)

mode was used to detect analyte (m/z 271.1 → 243) and internal standard (D_5-AR245409, m/z 276.0 → 244.2).

PK samples collected after a prior dose hold, dose reduction, or missed dose were excluded from the PK analysis. To enter the analysis, pre-dose samples were confirmed to have been taken prior to dosing by comparing the actual dosing and PK collection times. PK parameters for a given day were only calculated if at least 6 PK samples were taken with at least one sample collected between 2 and 4 hours inclusive.

PK data analysis based on actual sample times and measured plasma concentrations was performed using non-compartmental methods implemented within WinNonlin® Professional Version 5.2 software (Pharsight Corp., Mountain View, CA ,USA). The first-order rate constant associated with the terminal (log-linear) portion of the curve (λ_z) was estimated by linear regression of log concentration versus time, and the terminal half-life was estimated as $\ln(2)/\lambda_z$. The area under the plasma concentration–time curve from 0 to t hours post-dose (AUC_{0-t}) was calculated using linear up/log-linear down trapezoidal integration. The maximum plasma concentration (C_{max}), time to reach C_{max} (t_{max}) and minimum plasma concentration (C_{min}) were estimated directly from the observed plasma concentration–time data. Results tabulation and formatting was performed in Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA, USA). Concentration–time profiles and exposure figures were generated using S-Plus Version 8.0 (Tibco Software Inc., Palo Alto, CA, USA).

Pharmacodynamic assessments

The pharmacodynamic effects of SAR245409 on multiple components of the phosphatidylinositol-3-kinase (PI3K) pathway were assessed using circulating candidate biomarkers, peripheral blood mononuclear cells (PBMCs), fixed hair sheaths, frozen skin biopsies and frozen tumor biopsies.

Blood-based biomarker analysis

Plasma biomarkers were evaluated using ELISA analysis of plasma samples collected from patients fasting overnight through the 2-hour time point on days 1, 8 and 27/28 and administered SAR245409 on the twice-daily schedule at 15 mg (n=3), 30 mg (n=6), 50 mg (n=8), 60 mg (n=10) and 120mg (n=7), as well as the SAR245409 once-daily schedule at 70 mg (n=4) and 100 mg (n=7). PBMCs were collected and lysed in RIPA buffer on site at protocol-defined time-points. Frozen lysates were transported to Exelixis and analyzed by western blotting to document impact on $pAKT^{S473}$, $pPRAS40^{T246}$ (AKT-dependent phosphoepitope), and phosphorylated eIF4E-binding protein-1 $(p4EBP1)^{T37/T46}$ (mTORC1-dependent phosphoepitope), using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or ezrin as a loading control.

Pharmacodynamic analysis of tissue samples

The pharmacodynamic effects of SAR245409 on multiple PI3K pathway components were assessed in fixed hair sheaths, frozen skin biopsies and frozen tumor biopsies by tissue immunofluorescence staining analysis. Hairs were collected optionally pre- and 4 hours post-dosing on days 1, 8, 15, 27/28 of cycle 1 and pre-dose at the start of selected additional cycles. Paired skin biopsies were collected optionally at baseline and 4–8 hours post-dosing on days 1, 27, 28, of cycle 1 and day 1 of cycle 2 pre-dosing. Paired tumor biopsies were collected during the screening period prior to commencement of therapy and ~4 hours following dosing on day 27 of cycle 1. The following epitopes were evaluated using immunofluorescence staining methods: $pAKT^{T308}$ (3-phosphoinositide-dependent protein kinase-1-dependent phosphoepitope and readout for PI3K activity), $pAKT^{S473}$ (mTORC2-dependent phosphoepitope), $pPRAS40^{T246}$, $p4EBP1^{T70}$/$pS6^{S240/S244}$ (mTORC1-dependent phosphoepitopes) and the proliferation marker Ki67. For some sets of paired tumor biopsies, the MAPK pathway components phosphorylated mitogen-activated protein kinase kinase $(pMEK)^{S217/S221}$, phosphorylated extracellular-signal-regulated kinase $(pERK)^{T202/Y204}$ and total MEK and ERK were also assessed.

Hairs were collected either from the scalp (head hair) or eyebrows using blunt-nosed forceps with analysis done either with all hair samples collected from the head or eyebrows for each patient. Sample sets from four patients were analyzed. Technical failure rate of hair sample due to loss of attached sheath cells was substantial. Samples containing hair sheaths were trimmed to approximately 1–2 cm in length containing the sheath and bulb region, placed

in 1.5 ml cryotubes with screw-top closure and immersed completely in zinc fixative for 48 h (BD Pharmingen, # 550523) at room temperature followed by transfer into 70% ethanol and storage at –80°C. For immunofluorescence analysis of hair, frozen samples were thawed on ice, and 2–4 hairs per patient embedded together in Histogel support matrix and processed into a paraffin block; 5 µm thick serial sections were prepared onto positively charged Shandon Colormark Plus microscopy slides (# B9992010CS). To mitigate any variation in marker expression along the length of hair sheaths, every 3rd, 4th, or 5th section was placed on each microscopy slide, to support sequential analysis for 3, 4, or 5 readouts across the total length of a hair sheath. Sections were stained to detect expression of phosphorylated AKT^{T308} (CST #4056, dilution 1:200), AKT^{S473} (CST #3787, 1:25), $4EBP1^{T70}$ (CST #9455, 1:50), $PRAS40^{T246}$ (Biosource #44-1100G, 1:200) and $S6^{S240/S244}$ (CST #2215, 1:100). Sections were then incubated with Alexa-594 goat anti-rabbit secondary antibody (Molecular probes, #A11037, dilution 1:200). Stained sections were mounted in fluorescent mounting medium (DAKO #S3023) containing 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, #D3571) as a nuclear counterstain.

For immunofluorescence analysis of skin, cryo-preserved skin biopsy samples were collected frozen in O.C.T™ (#4583, Sakura) on dry ice and stored at -80°C prior to sectioning. Skin sample sets collected from 12 patients from the dose-escalation part were analyzed. Technical failure rate of skin samples was negligible. Samples were serially sectioned at 10 µm thickness. Twenty serial sections were obtained from each sample, with slide 1 stained with

hematoxylin and eosin (H&E) to assess tissue quality and subsequent slides stained to detect expression of phosphorylated AKT^{T308} (CST #4056, dilution 1:200), $AKTS^{473}$ (CST #3787, 1:25), $4EBP1^{T70}$ (CST #9455, 1:50), $PRAS40^{T246}$ (Biosource #44-1100G, 1:200) and $S6^{S240/S244}$ (CST #2215, 1:100) overnight at 4°C. Skin sections were then incubated in goat anti-rabbit Alexa 594-conjugated secondary antibody (Molecular Probes #A11037, dilution 1:200), with DAPI (Invitrogen #D3571) as nuclear counterstain.

For immunofluorescence analysis of tumor tissue, cryo-preserved tumor biopsy samples were collected frozen in O.C.T™ (#4583, Sakura) on dry ice and stored at −80°C prior to sectioning. Samples were serially sectioned at 10 µm thickness. Twenty serial sections were obtained from each sample, with slide 1 stained with H&E to assess tissue quality and subsequent slides stained to detect expression of phosphorylated AKT^{T308} (CST #4056, dilution 1:200), AKT^{S473} (CST #3787, 1:25), $4EBP1^{T70}$ (CST #9455, 1:50), $PRAS40^{T246}$ (Biosource #44-1100G, 1:200), $S6^{S240/S244}$ (CST #2215, 1:100) and $MEK^{S217/S221}$ (CST #9121, 1:200). All protocols were executed in dual-probe fashion to detect phosphorylated marker and Ki67 nuclear antigen (Vector, #VP-K452, clone MM1, dilution 1:100) to delineate tumor cell ROI vs normal tissue compartments. For detection of pERK and total ERK, 20 serial sections were obtained from each sample, with slide 1 stained with H&E to assess tissue quality and subsequent slides dual-probe stained to detect expression of phosphorylated $ERK^{T202/Y204}$ (CST #4376, dilution 1:50) and total ERK (CST #9107, 1:100) in the same section. After overnight incubation with primary antibody at 4°C, sections were incubated in goat anti-rabbit or goat anti-

mouse Alexa 488-conjugated secondary antibody (Molecular Probes #A11037 and #A11032; 1:200 dilution) with DAPI (Invitrogen #D3571) as nuclear counterstain. TUNEL was carried out using an *in situ* cell death detection kit according to the manufacturer's instruction (Roche #11 684 817 910).

Digital immunofluorescent images were quantified and a normalized intensity score calculated. Mean and SD values for baseline, pre-dose and post-dose samples were calculated, and then the percentage modulation in biomarker expression compared with baseline was calculated. Statistical analysis employed two-tailed student t-test analysis with $P<0.05$ considered statistically significant. Bonferroni adjustment was applied if multiple comparisons against a single baseline sample were performed.

Gene sequencing was performed on archival and/or fresh frozen tumor tissue for a core panel of genes deemed relevant to SAR245409: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), phosphatase and tensin homolog (*PTEN*), *KRAS* and *BRAF*, and some other genes as warranted by indication such as epidermal growth factor receptor (*EGFR*), *KIT* and *TP53*. For some patients, additional or supportive molecular profiling data were available as part of the pathology/molecular profiling reports shared by the sites with the sponsor. Genomic DNA was isolated from formalin-fixed paraffin-embedded biopsies using the modified QIAquick DNA extraction protocol or the PicoPure DNA extraction kit (Molecular Devices, Sunnyvale, CA). For fresh-frozen tumor tissue, similar

methods were used to isolate DNA from OCT sections. DNA was amplified by polymerase chain reaction (PCR) using primers flanking known mutational hotspots. The resulting PCR products were sequenced bidirectionally and analyzed on automated ABI 3730XL sequencers (Applied Biosystems, Foster City, CA), and traces were analyzed using the Mutation Surveyor software package (SoftGenetics, State College, PA) and manual inspection of the traces. When direct Sanger sequencing was not feasible, 454 sequencing was utilized and analyzed using AVA software. Identified mutations were confirmed by independent PCR amplification and Sanger sequencing. Quantitative PCR was used to evaluate PIK3CA, EGFR, MET and KIT gene amplification in some samples. The relative target copy number was derived by standardizing the input DNA to two reference genes (ALB, chromosome 4, and RNase P, chromosome 14). Dilution of cancer cells with non-cancer cells could have led to an underestimate of the degree of amplification in some samples.

PTEN protein expression was evaluated by immunohistochemistry in formalin-fixed, paraffin-embedded tissue sections (5 μm) with anti-PTEN antibody (NCL-PTEN, clone 28H6, diluted 1:300, Novocastra Laboratories Ltd) utilizing standardized procedures. Predominantly nuclear over cytoplasmic PTEN staining in tumor and non-neoplastic tissues was observed and quantified. PTEN staining in the non-neoplastic normal vascular endothelium and extratumoral stromal cells served as the internal positive control for each patient. PTEN-stained tumor samples were scanned onto an Aperio XT digital slide scanner and quantified using an H-Score.