DATA SUPPLEMENT

SUPPLEMENTARY METHODS

Phase I clinical trial

Patients ≥ 18 years old with metastatic or unresectable solid tumors for which standard curative or palliative measures do not exist, are not tolerable, or are no longer effective were eligible to enroll in this study. Patients were required to have an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 1, adequate liver, renal, and bone marrow function. Patients were excluded if they had total cholesterol >300 mg/dL and fasting triglycerides > 2.5x normal institutional limits, and uncontrolled diabetes mellitus with fasting serum glucose > 1.5x normal institutional limits. In addition, patients were excluded if they had recent major surgery, chemotherapy, radiotherapy, or an investigational agent; recent or current active bleeding or bleeding diathesis; recent hemoptysis or known endobronchial lesions or involvement of large pulmonary vessels by tumor; uncontrolled infection; HIV disease, or major cardiovascular and cerebrovascular events within the prior 6 months. Blood pressure was required to be controlled to ≤ 140/90. Women of child-bearing age were required to use contraception, and pregnant and breastfeeding women were excluded. Prior therapy with everolimus and/or pazopanib was excluded. This study was conducted with institutional review board approval in accordance with the Declaration of Helsinki and Good Clinical Practice and was registered with the National Institutes of Health (NCT01184326). Each patient provided written informed consent.

Study design

The primary endpoint of the study was to determine the maximum tolerated dose (MTD) and dose limiting toxicities (DLT) of combination therapy with everolimus and pazopanib. A standard 3 + 3 design was used starting with pazopanib 600 mg and everolimus 5 mg given orally once daily on a continuous basis during a 28-day cycle. The dose escalation was planned to alternate sequential escalations up to pazopanib 800 mg daily and everolimus 10 mg daily, or de-escalation to pazopanib 400 mg daily and everolimus 5 mg daily.

Safety

Safety evaluations included physical examination, ECOG performance status, vital signs, concomitant medication assessment, electrocardiogram, and laboratory evaluations (serum chemistry, blood counts, lipids, hepatitis B testing, and urinalysis). Patients were evaluated every two weeks for the first two cycles of therapy, and then monthly starting with cycle 3. Adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0. DLTs were defined as grade 3 or 4 treatment-related non-hematologic adverse events, grade 3 thrombocytopenia with bleeding or grade 4 thrombocytopenia, grade 4 neutropenia lasting longer than 7 days, and grade 4 neutropenia with fever, if they occurred during the first 28 days of combination therapy. Missing more than 5 days of study therapy due to drug-related intolerable grade 2 toxicity or toxicity severe enough to require a dose reduction were considered DLTs. Nausea and vomiting controlled with antiemetics, grade 3 hypertension controlled to ≤ 150/90 within 7 days of adjustment or addition of antihypertensives, and grade 3 clinically insignificant laboratory abnormalities were excluded from the definition of DLTs.

Pharmacokinetics

Pharmacokinetic sampling was performed on day 15 of the first cycle of therapy to insure that steady-state conditions for the repeated dosing schedule for both drugs had been achieved. Blood specimens were obtained before initiating treatment, immediately before dosing on day 15, at 1, 2, 4 and 6 hours after dosing, and before dosing on the following day. At each time point, two blood samples (2 mL) were drawn from a peripheral arm vein into evacuated tubes containing potassium EDTA. One tube of whole blood was not processed and the other tube was centrifuged (1,100-1,300 *g*, 4°C, 10 min) to afford plasma. The samples were maintained at -20ºC until assayed.

The concentrations of everolimus in whole blood and pazopanib in plasma were measured by two different analytical laboratories. The sample preparation procedure, chromatographic conditions and detection by electrospray ionization mass spectrometry in the negative ion mode that comprised the analytical method for everolimus were adapted from previously reported methods(1). Pazopanib was determined by high performance liquid chromatography-tandem mass spectrometry after protein precipitation, as previously reported(2, 3). Concentration ranges of the calibration curves were 0.50-50 ng/mL for everolimus and 0.10-50 µg/mL for pazopanib. During the analysis, calibration curves for both drugs exhibited correlation coefficients >0.995 and the interday accuracy was within ±15% of the known concentration of all calibration standards and quality control samples with a precision ≤15%.

The steady-state minimum concentration (C_{min}^{ss}) of drug in whole blood or plasma for individual patients was calculated as the geometric mean of the assayed concentration of drug in the two samples collected before dosing on days 15 and 16. The steady-state maximum concentration (C_{max}^{ss}) of drug in whole blood or plasma for individual patients was the sample with the highest assayed concentration. Plasma concentration-time

curves were analyzed by standard noncompartmental methods using WinNonlin Professional 5.0. The log-linear trapezoidal algorithm was used to estimate the area under the plasma concentration-time curve for the 24 h dosing interval (AUC_{24}^{ss}). Values of the pharmacokinetic parameters are reported as the geometric mean ± SD.

Efficacy

Objective tumor response, progression-free survival, and duration of response were evaluated by Response Evaluation Criteria in Solid Tumors (RECIST) Committee v. 1.1(4).

Whole Exome Sequencing

Sequencing studies were approved by the Dana-Farber/Harvard Cancer Center Institutional Review Board, and written informed consent was obtained from the patient. We used standard techniques to extract genomic DNA from the tumor and blood specimens. Whole exome sequencing was performed as previously described(5). Briefly, genomic DNA libraries were generated from tumor and normal DNA and were subjected to solution-phase hybrid capture using whole-exome RNA baits (Agilent), followed by massively parallel sequencing. We sequenced 76 bases from both ends of library DNA fragments using an Illumina HiSeq instrument, achieving 244,965,864 and 86,179,420 reads in the normal and tumor sample, respectively. This yielded target gene haploid coverage of 90- and 76-fold from the blood and tumor biopsy, respectively. The sequencing data were analyzed for SNVs and indels using established algorithms (http://www.broadinstitute.org/cancer/cga/Home and (6))

Reagents and point mutations

The wild-type human mTOR expression vector "pcDNA3-Flag mTOR wt" was acquired from Addgene (plasmid #26603). All mTOR point mutations were generated using the Stratagene QuikChange II XL kit (200521) according to the manual. The primers used were designed with the Stratagene QuikChange Primer Design tool and generated by IDT. To generate the double E2014K/E2419K mTOR mutant, the E2014K mutation was generated first, followed by mutation with the E2419K primers. All point mutations were verified with DNA sequencing through Eton Biosciences. Rapamycin was from Tocris. Pazopanib was from Selleck Chemicals. GAPDH (GTX627408) antibody was from GeneTex, Flag-epitope antibody was from Sigma (M2), pS6K1 (9234) and S6K1 (9202) antibodies were from Cell Signaling.

mTOR activity assay

To measure activity of wildtype or mutant mTOR constructs, $2x10^6$ 293T cells were seeded onto 10-cm dishes in DMEM with 10% IFS. The following day, cells were transfected with 2 ng of pRK5-HA-GST-S6K1 and 5 μg of pCDNA3-Flag-mTOR using Roche Xtreme-GENE 9 DNA transfection reagent (06365787001). 24 hours after transfection, media was changed to serum-free DMEM. 48 hours after transfection, cells were washed once in PBS and lysed in 1ml of NP-40 lysis buffer (50mM Tris HCl ph7.4, 150mM NaCl, 1% NP-40, 1mM EDTA, 100mM NaF, 10mM NaPyrophosphate, 1.5mM Na_3VO_4, 10mM Na-β-glycerophosphate, and protease inhibitor tablets). Following lysis, 20 μg of total lysate was loaded into each lane of an SDS-PAGE gel from Invitrogen (NP0336). Gels were transferred onto a PVDF membrane and blotted with indicated antibodies.

Supplementary Table 1. Number of patients with non-hematologic toxicities

Toxicity	*Maximum Grade*

	G1	G2	G3	G4
Abdominal pain	1	-	-	-
Alanine aminotransferase increased	3	-	1	-
Alkaline phosphatase increased	1	-	-	-
Anorexia	-	1	-	-
Aspartate aminotransferase increased	2	2	-	-
Bone pain	1	-	-	-
Cholesterol high	2	-	-	-
Creatinine increased	-	1	-	-
Diarrhea	1	-	1	-
Fatigue	1	4	-	-
Headache	-	1	-	-
Hyperglycemia	1	1	-	-
Hypertension	1	1	-	-
Hypertriglyceridemia	1	-	-	-
Hyperuricemia	-	-	-	1
Hypocalcemia	2	-	-	-
Hypokalemia	2	-	-	-
Hypomagnesemia	1	-	-	-
Hypophosphatemia	-	2	1	-
Lipase increased	-	-	2	-
Mucositis oral	2	1	-	-
Nausea	4	-	-	-
Palmar-plantar erythrodysesthesia syndrome	1	1	-	-
Pneumothorax	-	-	1	-
Pruritus	-	-	1	-
Rash acneiform	1	-	-	-
Rash maculo-papular	-	1	1	-
Serum amylase increased	1	-	-	-
Vomiting	3	-	-	-
Weight loss	1	-	-	-

G1, grade 1. G2, grade 2. G3, grade 3. G4, grade 4.

Supplementary Table 2. Number of patients with hematologic toxicities

Toxicity	***Maximum Grade Heme***		
	G1	***G2***	***G3***
Anemia	2	1	-
Neutropenia	1	-	1
Thrombocytopenia	3	1	1
Leukopenia	-	1	-
Maximum Grade	1	2	2

G1, grade 1. G2, grade 2. G3, grade 3.

Supplementary Table 3. Steady-state pharmacokinetic parameters for everolimus in whole blood and pazopanib in plasma when given concurrently.

	Everolimus	**Pazopanib**
Daily dose	5 mg	400 mg
No. of patients	7	6
$\mathbf{C_{min}^{ss}}$	6.1 ± 4.9 ng/mL (5.4 ± 1.8, n=4)(7)	13.1 ± 7.4 µg/mL (10.4 ± 9.0)(3)
$\mathbf{C_{max}^{ss}}$	37.9 ± 26.1 ng/mL (32 ± 9, n=4)	26.8 ± 9.5 µg/mL (22 ± 12)
$\mathbf{AUC_{24}^{ss}}$	335 ± 189 ng·h/mL (238 ± 77, n=4)	452 ± 186 µg·h/mL (447 ± 186)

Mean ± SD of pharmacokinetic variables determined in the present study are shown above historical data for the same dose and schedule of each drug given alone in parentheses with the citation number of the reference.

Supplementary Table 4. Somatic mutations identified by whole exome sequencing

(see accompanying spreadsheet)

Supplementary Figure 1. Effect of pazopanib on S6K1 phosphorylation in cells expressing activating *MTOR* mutations. Constructs expressing $mTOR^{E2014K}$, $mTOR^{E2419K}$, $mTOR^{E2014K/E2419K}$, or wildtype mTOR were coexpressed in 293T cells along with HA-GST-tagged S6K1, the downstream target of mTOR. The levels of both endogenous and exogenously expressed phosphorylated/total S6K1 as well as Flag-tagged-mTOR and GAPDH are shown for HEK-293T cells expressing the *MTOR* mutations after a 6-hour incubation at 0 μM, 1.0 μM, or 10.0 μM pazopanib as indicated.

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