Material and Methods

Study Design and Subjects

This phase 1 trial was a prospective, single-center, dose-escalating study to examine the pharmacodynamics, pharmacokinetics, safety and tolerability of intravenous PZ-128 administered to subjects with stable coronary artery disease or multiple risk factors for coronary artery disease. The protocol was approved by the Institutional Review Board of Sinai Hospital of Baltimore (IRB445 and IRB2996) and Tufts Medical Center (IRB577 and IRB1236) as well as by the US Food and Drug Administration (FDA, Center for Drug Evaluation and Research). An independent, advisory Data and Safety Monitoring Board (DSMB) consisting of clinicians from University of Maryland, Johns Hopkins, Duke University, and UCSD monitored the overall conduct of the trial. Written informed consent was obtained from each subject before any study-related activities were performed.

The study enrolled male and non-pregnant female subjects between 18 and 75 years with documented coronary artery disease (CAD) or ≥2 risk factors for CAD. Key exclusion criteria included: 1) presence or history of conditions or concomitant medications with the potential to affect drug disposition, platelet function or coagulation. 2) any clinically significant physical exam, electrocardiogram (ECG), laboratory, coagulation, or pulmonary function abnormalities; and 3) history of alcohol or substance abuse. Aspirin therapy was allowed. Complete inclusion/exclusion criteria are detailed in the online-only Data Supplement.

After a screening period of up to 28 days, subjects were enrolled in one of 8 cohorts and received a single, weight-adjusted dose of intravenous PZ-128 at 0.01, 0.03, 0.1, 0.3, 1 or 2 mg/kg over a 1 h continuous infusion, or 0.5 or 1 mg/kg over a 2 h infusion (Figure 1). The dose range was selected based on safety and tolerability in monkeys and guinea pigs, and efficacy in baboons.[1] The maximum recommended starting dose (MRSD) for this First-in-

Human (FIH) clinical trial was based on the FDA Guidance *Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers*.[2] The human equivalent dose (HED) was determined from the no observed adverse effect level (NOAELs) in the tested non-human primates using allometric scaling based on body surface area. A safety factor of 16 was applied to the lowest HED obtained in monkeys, resulting in the final MRSD of 0.01 mg/kg (0.003-0.01 mg/mL).

PZ-128 was supplied in vials (AmbioPharm, Inc. North August, SC) as a dry lyophilized powder and was stored at -20° C. PZ-128 was prepared by a research pharmacist and delivered in sterile 5% Dextrose USP (150 or 250 mL). A pre-treatment regimen of anti-histamines +/- corticosteroid was instituted for allergy prophylaxis at the 2 mg/kg dose (1 h infusion), and 0.5 and 1 mg/kg doses using a 2 h infusion. Within 28 days of the screening visit, fasted subjects (no food for 8 h overnight) received a single dose of PZ-128 and remained in the clinical research unit for at least 24 h for monitoring (without food for 8 h post dosing). Peripheral intravenous catheters (18 to 20-gauge) were placed for drug delivery and phlebotomy during the inpatient stay, otherwise blood was drawn by venipuncture. Subjects returned for a follow-up visit 7-10 d thereafter and completed the study protocol with a 30-d telephone call.

Pharmacokinetic Measurements

Serial blood samples for pharmacokinetic (PK) evaluation were obtained at time 0 and 0.25, 0.5, 1, 2, 4, 6, 8, 24 h and 7-10 d following the start of the PZ-128 infusion. Whole blood samples were collected into K_2 EDTA-containing tubes, and centrifuged (850*g* for 15 min at 4 °C) within 30 min. The resultant plasma samples were frozen and stored at -70 °C until analysis. PZ-128 drug levels were determined with an API 4000 system equipped with a Zorbax 300SB-C8 column using an UHPLC-MS/MS method (WIL Research, Ashland, OH). PK evaluation was

carried out by using a non-compartmental approach with the aid of Phoenix WinNonlin. PK parameters included peak plasma concentration (C_{max}), time to C_{max} (t_{max}), area under the plasma concentration-time curve from dosing to last measurable time point (AUC_{last}) and to infinity (AUC_{inf}), terminal-phase half-life ($t_{1/2}$), total body clearance (Cl), and volume of distribution at steady state (V_{ss}). Urine collections were performed at predose, 4 and 24 h and the samples were immediately mixed with urine stabilizer stock solution and then centrifuged (4000*g* for 10 min at 4 °C). The resultant aliquots of supernatant samples were frozen and stored at -70 °C until analysis. PZ-128 drug levels in urine were determined with a TSQ Vantage system equipped with a Kinetex C8 column using a LC-SRM/MS method (NovaBioAssays, Woburn, MA) and Xcalibur software (Thermo Scientific).

Pharmacodynamic Measurements

The primary efficacy objective was inhibition of 8, 12 and 20 μM SFLLRN-induced aggregation 0.5 to 24 hours after the start of PZ-128. The secondary efficacy variables (160 μM AYPGKF, 5 and 20 μM ADP, 4 μg/mL collagen-induced aggregation) were used to assess specificity of the inhibitory effect of PZ-128. Pharmacodynamic effects of PZ-128 on platelet function were assessed by light transmission aggregometry (LTA) as previously described.[1] Blood samples were obtained at time 0 and 0.5, 1, 2, 6, 24 h and 7-10 d following the start of the PZ-128 infusion. Whole blood was drawn into pre-filled conical tubes containing 200 μM D-phenylalanine-proline-arginine-chloromethyl ketone (PPACK) and 4% sodium citrate (0.4% final) anti-coagulants (Sigma). Anticoagulated blood was centrifuged at 120*g* for 5 min to harvest platelet-rich plasma (PRP). The remaining blood sample was subjected to further centrifugation at 850*g* for 10 min to recover platelet-poor plasma (PPP). Platelets were stimulated with multiple agonists to induce aggregation: 1) 8, 12 and 20 μM SFLLRN (PAR1

agonist peptide, (synthesized by the Tufts Peptide Core Facility)); 2) 5 and 20 μM ADP (Sigma); 3) 160 μM AYPGKF (PAR4 agonist peptide (Tufts)); and 4) 4 and 20 μg/mL collagen (fibrillar equine type 1 (Chrono-Log)). Aggregation was measured using a Chrono-Log Lumi-Aggregometer, Model 490-4D, with Aggro/Link software. Maximum and final aggregation were recorded using PPP as a reference and reported as % inhibition of platelet aggregation (IPA) relative to baseline.

Bleeding Time

Bleeding times were assessed with a Surgicutt® device (ITC, Piscataway, NJ) at predose and at 1-2 hours post start of infusion.

Safety and Tolerability

Safety and tolerability were monitored up to 30 d post-dose and were evaluated based on the incidence of adverse events (AEs) including clinically significant changes in physical examinations, vital signs, standard clinical laboratory tests (CBC, chemistries, coagulation [including prothrombin time (PT), international normalized ratio (INR), activated partial thromboplastin time (aPTT), and activated coagulation time (ACT)] and urinalysis), and 12-lead ECGs. Injection-site evaluations and pulmonary function tests (peak expiratory flow, oxygen saturation) were also conducted. The National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE) v4.0 grading system was used to classify the nature and severity of the AEs.[3]

Sample Size Calculation

The primary efficacy objective was to evaluate inhibition of 8, 12 and 20 μM SFLLRN-induced

aggregation 0.5 to 24 hours after the start of PZ-128. Based on a standard deviation (SD) of 22%, and choosing a power of 88% and with an alpha 0.05, it was calculated that the study would require at least 6 subjects in order to obtain a significant result if the true differences between baseline and the treatment groups were as small as 28% with a standard deviation of 22%.[1] A $p<0.05$ was considered statistically significant. Statistical calculations were carried out using SigmaStat Software (Point Richmond, CA).

Statistical Analyses

Statistical analyses were performed with the use of SAS, Statistica, and KaleidaGraph. Descriptive statistics (mean ± SD) were used to summarize demographics, pharmacodynamic pharmacokinetic safety data by dose level. Summary statistical analyses for each dose cohort (ANOVA) and time dependence of effects versus baseline (Dunnett's multiple comparisons tests) were conducted with the normalized maximal and final LTA data. A *post-hoc* analysis of the pharmacodynamic effects in the subgroup of subjects who received concomitant aspirin (ASA) was examined in the 0.5, 1 and 2 mg/kg dose cohorts. Clinical laboratory evaluations, bleeding time, ECG parameters and pulmonary function tests were analyzed with each dose as a categorical variable to determine a global P value by ANOVA and using the baseline value (at predose) as a covariate. Individual dose cohorts were also compared versus the lowest dose group (0.01 mg/kg) serving as a surrogate 'placebo' group. In addition, laboratory parameters and bleeding time were analyzed for the effect of PZ-128 dose by repeated measures and mixed effects models. Steroid premedication and outcome at baseline (predose) were included as covariates and PZ-128 dose was a continuous variable. A P-value <0.0016 was considered significant based on Bonferroni post-hoc test correction.

ECG parameters (Heart rate, RR, PR, QRS, QT, QTc and QTd intervals) were analyzed by repeated measures and mixed effects models. Outcome at baseline (predose) was included as a covariate and PZ-128 dose was a continuous variable. P-value <0.007 was considered significant based on Bonferroni post-hoc test correction. The change in QTc at each time point from baseline QTc for each subject was also analyzed by repeated measures and mixed effects models with PZ-128 as a continuous variable. Injection site evaluations at various time points after initiation of PZ-128 infusion were analyzed by linear regression analysis using PZ-128 as a continuous variable.

1. Zhang P, Gruber A, Kasuda S, Kimmelstiel C, O'Callaghan K, Cox DH, Bohm A, Baleja JD, Covic L, Kuliopulos A. Suppression of arterial thrombosis without affecting hemostatic parameters with a cell-penetrating PAR1 pepducin. *Circulation.* 2012;126:83-91.
2. Guidance for Industry Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers. In: CDER, ed.: U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research; 2005.
3. Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0. In: NIH-NCI, ed.: U.S.DEPARTMENT OF HEALTH AND HUMAN SERVICES; 2010.