Durable Pharmacological Responses from the Peptide ShK-186, a Specific Kv1.3 Channel Inhibitor That Suppresses T Cell Mediators of Autoimmune Disease^[S]

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ABSTRACT

The Kv1.3 channel is a recognized target for pharmaceutical development to treat autoimmune diseases and organ rejection. ShK-186, a specific peptide inhibitor of Kv1.3, has shown promise in animal models of multiple sclerosis and rheumatoid arthritis. Here, we describe the pharmacokinetic-pharmacodynamic relationship for ShK-186 in rats and monkeys. The pharmacokinetic profile of ShK-186 was evaluated with a validated high-performance liquid chromatography-tandem mass spectrometry method to measure the peptide's concentration in plasma. These results were compared with single-photon emission computed tomography/computed tomography data collected with an ¹¹¹In-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid-conjugate of ShK-186 to assess whole-blood pharmacokinetic parameters as well as the peptide's absorption, distribution, and excretion. Analysis of these data support

a model wherein ShK-186 is absorbed slowly from the injection site, resulting in blood concentrations above the Kv1.3 channel-blocking IC₅₀ value for up to 7 days in monkeys. Pharmacodynamic studies on human peripheral blood mononuclear cells showed that brief exposure to ShK-186 resulted in sustained suppression of cytokine responses and may contribute to prolonged drug effects. In delayed-type hypersensitivity, chronic relapsing-remitting experimental autoimmune encephalomyelitis, and pristane-induced arthritis rat models, a single dose of ShK-186 every 2 to 5 days was as effective as daily administration. ShK-186's slow distribution from the injection site and its long residence time on the Kv1.3 channel contribute to the prolonged therapeutic effect of ShK-186 in animal models of autoimmune disease.

Introduction

The Kv1.3 channel has been an active target of pharmaceutical development for more than 15 years (Chi et al., 2012). The interest in this channel derives from its important function in activated effector-memory T (T_{EM}) cells, which are major mediators of autoimmune disease (Wulff et al., 2003; Beeton et al., 2006). Engagement of the T cell receptor by antigen-presenting cells results in an influx of calcium into the cytoplasm, initially from the endoplasmic reticulum

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ABBREVIATIONS: T_{EM}, effector-memory T; ACN, acetonitrile; ANOVA, analysis of variance; AUC, area under the concentration-time curve; CFA, complete Freund's adjuvant; CS, clinical score; CT, computed tomography; SPECT, single-photon emission CT; DA, dark agouti; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; CR-EAE, chronic-relapsing EAE; HPLC, high-pressure liquid chromatography; Gd, gadolinium; IACUC, institutional animal care and use committee; ID, injected dose; IDRI, Infectious Disease Research Institute; IL, interleukin; In, indium; MS, mass spectrometry; MS/MS, tandem MS; OVA, ovalbumin; PBMC, peripheral blood mononuclear cell; PD, pharmacodynamics; PIA, pristane-induced arthritis; PK, pharmacokinetics; SD, Sprague-Dawley; TFA, trifluoroacetic acid.

but subsequently from the extracellular space via the Ca^{2+} release-activated Ca^{2+} channel (Cahalan and Chandy, 2009). The opening of voltage-gated Kv1.3 and calcium-activated KCa3.1 potassium channels in the T cell membrane and the resulting efflux of potassium ions promotes calcium entry and sustains intracellular calcium at concentrations necessary for T cell activation (Cahalan and Chandy, 2009). Resting T cells express a mixture of both K^+ channels. However, upon activation, naive and central-memory T cells increase expression of the KCa3.1 channel, whereas T_{EM} cells up-regulate Kv1.3 channel expression (Wulff et al., 2003). In the latter cell population, the degree of Kv1.3 expression is a measure of cell activation, and the channel is required for the maintenance of the T_{EM} cell phenotype (Hu et al., 2007, 2012).

We and others have shown previously that Kv1.3^{HIGH} CCR7⁻-activated T_{EM} cells are present at sites of inflammation in autoimmune disease, and auto-reactive T cells from subjects with multiple sclerosis, type 1 diabetes, and rheumatoid arthritis express the Kv1.3^{HIGH} phenotype of activated T_{EM} cells (Rus et al., 2005; Beeton et al., 2006). In addition, specific Kv1.3 inhibitors have been found to be effective in numerous animal models of inflammation including chronic relapse-remitting experimental autoimmune encephalomyelitis (CR-EAE) and adoptive EAE (Beeton et al., 2005, 2006), pristane-induced arthritis (PIA) (Beeton et al., 2006), the delayed-type hypersensitivity (DTH) reaction (Koo et al., 1997; Beeton et al., 2005; Matheu et al., 2008), allergic contact dermatitis (Azam et al., 2007), allogeneic kidney transplant (Grgic et al., 2009), spontaneous autoimmune diabetes (Beeton et al., 2006), vascular neointima hyperplasia (Cheong et al., 2011), antglomerular basement membrane glomerulonephritis (Hyodo et al., 2010), and psoriasis (Gilhar et al., 2011). For these reasons, numerous groups have focused on developing specific and potent inhibitors of the Kv1.3 channel for the treatment of inflammation and autoimmune disease (Cahalan and Chandy 2009, Rangaraju et al., 2009). Much of this effort has focused on developing small-molecule inhibitors of Kv1.3, but identifying compounds that are adequately specific has proved challenging, and to date, no drug specifically targeting Kv1.3 has entered clinical trials.

Our group is developing ShK-186, a 37-amino acid selective peptide inhibitor of Kv1.3, as a therapeutic for autoimmune diseases. To date, the Food and Drug Administration has approved more than 55 peptide drugs in approximately 33 mechanistic classes, making peptides one of the most active areas of biologics drug research. However, the peptide field has suffered from a perception that frequent dosing is required for sustained pharmacodynamic (PD) activity and patients have a poor acceptance of parenteral therapies. Therefore, careful attention to dose frequency and dose presentation are important to the development of a commercially viable peptide drug. Here, we report on the optimization of ShK-186 dose and dose frequency in animal models of autoimmune disease as part of our recently completed non-clinical program for the peptide.

Previous studies from our group have characterized the level of Kv1.3 channel-blocking activity in the serum of Lewis rats after single subcutaneous injections of three related analogs, ShK(L5), ShK-186, and ShK-192 (Beeton et al., 2005; Matheu et al., 2009; Pennington et al., 2009). Peak

serum drug activity occurred 30 min after injection and returned to baseline by 7 h postdose, but ~200 pM concentration of functionally active peptide was detected in the blood 24 and 48 h after injection, and approximately 100 pM was detectable at 72 h (Beeton et al., 2005; Matheu et al., 2009; Pennington et al., 2009). However, previous animal studies that investigated ShK peptides were performed by using a once-daily or more than once-daily dosing frequency (Beeton et al., 2001, 2005, 2006; Matheu et al., 2008; Pennington et al., 2009). Here, we demonstrate that ShK-186 has a long-lasting therapeutic effect in three different rat models of autoimmune disease because of its slow release from the site of subcutaneous injection and its tight binding to and slow release from the Kv1.3 channel on T cells.

Materials and Methods

Animals. DA and Lewis rats (8–10 weeks old) were purchased from Harlan (Indianapolis, IN) and housed under pathogen-free conditions with food and water ad libitum. DTH trials were approved by the Baylor College of Medicine and Infectious Disease Research Institute (IDRI; Seattle, WA) institutional animal use and care committees (IACUCs). EAE trials were approved by the IACUCs of the University of California at Irvine and the IDRI. PIA trials were approved by the Baylor College of Medicine IACUC.

Sprague-Dawley (Crl:CDSD) rats (6–9 weeks old) were purchased from Charles River Laboratories, Inc. (Wilmington, MA) and housed in a temperature (64°C–79°C)- and humidity (30–70%)-controlled facility. Food and water were ad libitum. PK studies in SD rats were approved by either the MPI Research or IDRI IACUCs.

Non-naive cynomolgus (*Macaca fascicularis*) and squirrel monkeys (*Saimiri boliviensis*) were between 2 and 5 years of age and transferred from the MPI Research stock colony to the study site (Mattawan, MI). All cynomolgus monkeys were of Chinese origin. The squirrel monkey was of Bolivian origin and obtained originally from the University of Texas MD Anderson Cancer Center (Houston, TX). Animals were housed individually in stainless-steel cages in an environmentally controlled room. The monkeys were provided environmental enrichment; fluorescent lighting was provided 12 h/day. Temperature was maintained between 64 and 84°C; humidity was 30 to 70%. Animals were provided Certified Primate Diet (PMI Nutrition International, Inc., St. Louis, MO) twice daily. Primate treats and other enrichment foods were provided on a regular basis. Water was available ad libitum. Primate studies were approved by the MPI Research IACUC.

Kv1.3 Peptide Inhibitors. ShK-186 and ShK-198 (derivatives of ShK; accession no. P29187) were manufactured by using an fluorenylmethoxycarbonyl-tertiary butyl solid-phase strategy on an amide resin. All of the coupling steps were mediated with 6-Cl-*N*-hydroxybenzotriazole in the presence of diisopropyl carbodiimide. Fluorenylmethoxycarbonyl removal was facilitated with 20% piperidine in dimethylformamide containing 0.1 M *N*-hydroxybenzotriazole to buffer the piperidine and minimize potential racemization at the six Cys residues. After assembly, the peptide was cleaved from the resin and simultaneously deprotected by using a TFA cleavage cocktail (reagent K) containing aromatic cationic scavengers for 2 h at room temperature. The crude peptide was filtered from the spent resin and subsequently isolated by precipitation into ice-cold diethyl ether. The crude peptide was dissolved in 50% acetic acid and subsequently diluted into 3 liters of H_2O . The pH of this peptide solution was adjusted to 8.0 with NH_4OH and allowed to slowly stir overnight. Disulfide bond formation was mediated by air oxidation or through the addition of a glutathione exchange system. ShK and its derivatives spontaneously fold to a major thermodynamically favored isomer, which is the biologically active form of the peptides. The folded peptide was loaded onto a preparative RP-HPLC column

and purified by using a gradient of ACN versus H₂O containing 0.05% TFA. The fractions containing the desired peptide purity were pooled together and lyophilized to produce an acetate salt. Drug was formulated at 0.5 to 25 mg/ml in 10 mM sodium phosphate, 0.8% NaCl, and 0.05% polysorbate 20, pH 6.0.

Serum and Plasma Stability Studies. Shk-186 peptide was solubilized at a concentration of 10 mg/ml in formulation buffer. Twenty five microliters of the stock peptide solution was spiked into 475 μ l of a 1:1 dilution of serum, plasma, or whole blood with RPMI to a final concentration of 0.5 mg/ml and incubated at 37°C for the indicated period. Fifty microliters of the sample was then mixed with 50 μ l of 4% trichloroacetic acid, and the sample was vortexed for 30 s and placed at 4°C for 15 min. The trichloroacetic acid mixture was centrifuged at 12,000 rpm for 5 min to pellet the precipitated proteins, and the supernatant was analyzed on an Agilent Technologies (Santa Clara, CA) 1100 HPLC system fitted with a Grace (Deerfield, IL) Vydac C18, 5.0- μ m, 300-Å, 4.6 $\times$ 250-mm column. Mobile phase A was composed of 0.1% TFA in 95% water/5% ACN, and mobile phase B was composed of 0.1% TFA in 5% water/95% ACN. The system flow rate was 1 ml/min.

HPLC-MS/MS Method for Measuring ShK-186 and Metabolites in Plasma. Whole-blood samples were collected into K₂EDTA-containing tubes and processed by centrifugation to plasma. Plasma was supplemented 20:1 (v/v) with the HALT Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA) and stored frozen at -70°C until analysis. Fifty microliters of an internal standard [2 μ g/ml ShK (parent peptide; accession number P29187)] in 90:10 H₂O/ACN (v/v) (Bachem, Bubendorf, Switzerland) were added to 100 μ l of plasma. The combined sample was diluted with 300 μ l of H₂O and purified by using a Waters (Milford, MA) Sep-Pak tC18, 25-mg, 96-well SPE plate. Samples were eluted in 500 μ l of TFA/H₂O/ACN (1:70:30, v/v) and evaporated under N₂. The residue was reconstituted in 200 μ l of TFA/H₂O/ACN (0.02:90:10, v/v/v) and analyzed by HPLC-MS/MS. HPLC was performed on an Agilent 1200 series instrument fitted with an ACE 5 C18-PFP 50 $\times$ 2.1-mm, 5- μ m column (Advanced Chromatography Technologies, Aberdeen, UK). Mobile phase A was formic acid/H₂O (2:1000, v/v), and mobile phase B was formic acid/H₂O/ACN (2:500:500, v/v/v). The flow rate was 300 μ l/min. Mass spectrometry was performed on a SCIEX API 5000 instrument (AB Sciex, Foster City, CA) using turbo ion spray in the positive mode. Mass detection was performed by using multiple reaction monitoring (ShK-186, m/z 741.5 $\rightarrow$ 841.0; ShK-198, m/z 728 $\rightarrow$ 840.6; internal standard, m/z 676.8 $\rightarrow$ 769.0). The dwell times for the internal standard, ShK-186, and ShK-198 were each 500 ms.

SPECT/CT Scanning of Radiolabeled ShK-221. ShK-221 (100 μ g) was radiolabeled with 2 mCi ¹¹¹indium chloride (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) in a 300- μ l reaction containing 50 mM sodium acetate, pH 5.0 for 30 min at 95°C. The reaction was quenched by the addition of EDTA to a final concentration of 50 mM, and the radiolabeling efficiency was assessed by reverse-phase HPLC [Luna 5 μ C18(2) 100A 250 $\times$ 4.6-mm column; Phenomenex, Torrance, CA] on an Agilent 1100 system using an IN/US Systems Gamma RAM model 4 radio-HPLC detector (LabLogic Systems, Brandon, FL). The labeling efficiency varied from 89 to 98% by this method. SPECT/CT scanning (NanoSPECT/CT Preclinical Imager; Mediso, Budapest, Hungary) was carried out on anesthetized animals in four 15-min scans during the first hour and one scan each at 4, 8, 24, 48, 72, 120, and 160 h postdose. The individual projection frame time for each helical SPECT was set such that the duration of each scan would last for approximately 15 to 45 min (varying by time point to account for isotope decay) and allow for significant collection of statistics within each frame. The characteristic peaks detected from the spectra for ¹¹¹In were 245 and 171 keV (primary and secondary, respectively). The resulting projection data were reconstructed after each scan by using an iterative model that takes advantage of the pinhole geometry to achieve a resolution of approximately 2 mm.

Approximately 10 μ l of blood samples were collected after each scan, and the amount of radioactivity in the sample was measured by

using a Wallac Wizard 1470 scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). Drug concentrations were computed by taking into account the specific activity of the administered dose, the half-life of ¹¹¹In (67.3 h), and the counting efficiency of the instrument.

Suppression of Cytokine Responses in Peripheral Blood Mononuclear Cells. PBMCs were isolated from human whole blood by using CPT Vacutainers (BD Biosciences, San Jose, CA) and dispersed into RPMI media. One hundred microliters of media containing 2 $\times$ 10⁵ PBMCs were added to each well of a 96-well dish and treated with the addition of 50 μ l of ShK-186 in media at varying concentrations for 1 h at 37°C, 5% CO₂. Cells were washed twice with RPMI, resuspended in 200 μ l of fresh media supplemented with 40 μ M thapsigargin, and stimulated for 48 h at 37°C, 5% CO₂. Cytokine production was measured in the overlying media by using a Luminex Assay (Millipore Corporation, Billerica, MA) specific for IL-2.

DTH Model in Rat. Active DTH was induced and monitored as described previously (Beeton and Chandy, 2007). In brief, Lewis rats were immunized in the flanks with ovalbumin (OVA) (200 μ g/rat) emulsified in complete Freund's adjuvant (CFA) (Sigma, St. Louis, MO). Seven to nine days later, animals were challenged under isoflurane anesthesia in the pinna of one ear with 20 μ g of OVA dissolved in saline and in the other ear with saline. Animals received one or two subcutaneous injections of either ShK-186 or vehicle at the time of challenge or on the 4 days before challenge. Thickness of both ears was measured 24 h after challenge with a spring-loaded micrometer (Mitutoyo America Corporation, Aurora, IL).

CR-EAE Model in Rat. The CR-EAE model in DA rats (Lorentzen et al., 1995) was used with minor modifications. In brief, animals were immunized by subcutaneous injection at the base of the tail with 0.2 ml of a 1:1 emulsion of homogenized SD rat spinal cord (Bioreclamation LLC, Westbury, NY) in CFA supplemented to 4 mg/ml *Mycobacterium tuberculosis* H37Ra (Sigma) under isoflurane anesthesia. Each rat received ~80 mg of spinal cord and 400 μ g of H37Ra. Either ShK-186 or placebo was administered subcutaneously on alternate flanks as appropriate. Animals were observed daily by measuring their body weight and assessing clinical signs of disease. Animals were included in the study and randomized into experimental groups sequentially as they reached a clinical score of 1. Scores were assigned as follows: 0, no illness; 0.5, no tail coil; 1, no tail coil and flaccid tail (tail dropped straight down five consecutive times); 2, mild paraparesis, wobbling; 3, moderate to severe paraparesis, falling on its side, unable to stand on hind legs; 3.5, one-limb paralysis; 4, two-limb paralysis; 5, two-limb paralysis with incontinence; 6, death. A cumulative clinical score (CS) was calculated for each rat by adding the daily scores from the day of disease onset (CS = >1) until the end of treatment and averaged to obtain a mean cumulative clinical score. Disease prevalence was calculated as the number of animals with CS >0.5 divided by the number of living animals per day and expressed as a percentage. During the chronic phase, an animal was considered to have a relapse if its clinical score was more than 1.

Pristane-Induced Arthritis in Rats. Female DA rats received 150 μ l of pristane (2,6,10,14-tetramethylpentadecane; CosmoBio, Carlsbad, CA) by subcutaneous injection in two sites at the base of the tail under isoflurane anesthesia. ShK-186 or vehicle was administered subcutaneously in the scruff of the neck daily or every other day for the duration of the trial. All four limbs were monitored for arthritis as described previously (Rintisch et al., 2009). In brief, a score of one point was given for each swollen and red toe, and each midfoot, digit, or knuckle, and five points for each swollen ankle or wrist (maximum score per limb, 15; maximum score per animal, 60). All of these studies were done under an IACUC-approved protocol at the Baylor College of Medicine.

Statistical and Computational Analysis. Statistical analysis was carried out by using one-way ANOVA (EAE model and cytokine expression studies), the two-tailed, Mann-Whitney *U* test (DTH model), or the paired *t* test (pharmacokinetic studies). Goodness of

model fit was determined by using the R^2 statistic. Pharmacokinetic calculations were as follows: $C_{\max}$ and $T_{\max}$ were as observed in the dataset. AUC was computed by using a linear trapezoidal method. The terminal elimination half-life was computed from the slope of the regression with the best adjusted R^2 value. $AUC_{t \rightarrow \infty}$ was calculated by dividing the last observed drug concentration by the terminal elimination slope.

Results

Pharmacokinetic Properties of ShK-186 in Representative Species. As a precursor to PK studies of ShK-186 in nonclinical species, we evaluated the stability of the peptide in serum, plasma, and whole blood from humans, SD rats, and cynomolgus monkeys (*M. fascicularis*). Spiking-in studies showed the formation of a single metabolite in all samples analyzed from all three species. The metabolite was characterized by mass spectrometry and shown to be the dephosphorylated form of ShK-186 (referred to as ShK-198; Supplemental Fig. 1, A–C). ShK-198 is identical to the previously described analog ShK(L4) with the exception of containing a C-terminal amide in place of a carboxyl (Beeton et al., 2005). ShK(L4) blocks Kv1.3 with an IC_{50} of 48 pM (Beeton et al., 2005), which is similar to the potency of ShK-198 (Fig. 1, A and C; $IC_{50} = 41.4 \pm 7.25$ pM; $n = 5$). Conversion of ShK-186 to ShK-198 occurred most readily in serum and in plasma samples treated with citrate or heparin as the anticoagulant. ShK-186 is most stable in plasma containing K_2 EDTA or specific phosphatase inhibitors (sodium fluoride, sodium orthovanadate, sodium pyrophosphate, and β -glycerophosphate), suggesting that endogenous phosphatases are responsible for its conversion.

We developed and validated an HPLC-MS/MS method to measure concentrations of ShK-186 and ShK-198 in K_2 EDTA plasma of rats and cynomolgus monkeys. The method had a lower limit of quantitation of 2 ng/ml (~ 450 pM) for each analyte and showed equivalent recovery of ShK-186 from both spiked plasma and spiked buffer QC samples. Using this method, we measured the in vivo pharmacokinetic properties of ShK-186 after a single subcutaneous administration (the intended clinical route of administration) to rats and monkeys. Both rats and monkeys have T_{EM} cells that express large numbers of the Kv1.3 channel after activation and are therefore relevant nonclinical species for testing ShK-186 (Chi et al., 2012).

The PK profile of ShK-186 is characterized by a short residence time in the central compartment of rat and monkey species. In rats, after a single administration, ShK-186 reaches a maximum plasma concentration between 1 and 5 min, whereas its metabolite reaches its $C_{\max}$ between 1 and 15 min (Fig. 2, A and B; Tables 1 and 2). Although the $C_{\max}$ and AUC for both the parent and metabolite increase with dose in rats, the relationship between $C_{\max}$ and dose was largely nonlinear ($R^2 = 0.8$), whereas the relationship between AUC and dose was approximately linear through the 500 μ g/kg dose ($R^2 = 0.97$; Fig. 2E). The half-life in rat ranged from 4.4 to 9.2 min for ShK-186 and from 6.1 to 16.4 min for ShK-198 (Tables 1 and 2). Clearance values for ShK-186 and ShK-198 in rats were not significantly different (paired t test; $t_4 = 2.2$; $p = 0.1$) and ranged from 441.4 to 1453.3 ml/kg $\cdot$ min.

The PK of ShK-186 (Fig. 2C) and ShK-198 (Fig. 2D) after a single subcutaneous administration to monkeys demon-

strated a linear relationship between dose and both AUC and $C_{\max}$ (slope = 1.02–1.27; $R^2 = 0.97$; Fig. 2F) through the entire range of doses from 35 to 1000 μ g/kg. The monkey half-life ranged from 8.8 to 23.8 min for ShK-186 and from 16.7 to 73.5 min for ShK-198. Clearance rates in the monkey were generally lower for ShK-198 than ShK-186 and ranged from 18.7 to 141.3 ml/kg $\cdot$ min (Tables 1 and 2).

These data indicate that ShK-186 and its metabolite ShK-198 reach a maximum concentration in plasma 1 to 15 min after a single subcutaneous injection of drug in both species, and both are rapidly cleared from the central compartment. The limit of quantitation of the HPLC-MS method is approximately 10-fold higher than the IC_{50} of ShK-186 and ShK-198 on the Kv1.3 channel and thus lacks the sensitivity to estimate the terminal elimination phase at low, but potentially therapeutic, concentrations.

Absorption, Distribution, Metabolism, and Excretion Studies with a Radiolabeled Analog of ShK-186.

We developed a radiolabeled analog of ShK-186 to measure the biodistribution of the peptide and evaluate its total concentration in whole blood. ShK-186 contains a single iodinateable tyrosine at position 23. However, iodine incorporation into the ring, which is predicted to interact within the pore region of the Kv1.3 channel (Pennington et al., 1996), results in disruption of the channel binding properties of the peptide. We therefore modified the amino terminus of ShK-198 with a six-carbon linker attached via a peptide bond to one of the carboxylic acids of a 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid chelate (Supplemental Fig. 2A). The 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid conjugate, designated ShK-221, was readily coordinated with indium or gadolinium (Supplemental Fig. 2, B and C). In patch-clamp experiments, the gadolinium- and indium-labeled ShK-221 peptides blocked Kv1.3 with IC_{50} values similar to ShK-186 and ShK-198: Gd-ShK-221, 58.23 ± 1.38 pM ($n = 5$); In-ShK-221, 63.8 ± 2.25 pM; ($n = 3$); ShK-186, 68.99 ± 4.01 pM ($n = 5$); and ShK-198, 41.4 ± 7.25 pM ($n = 5$) (Fig. 1C). We prepared and administered ^{111}In -labeled ShK-221 by subcutaneous injection to SD rats (1.0 mCi; 100 μ g/kg) and squirrel monkeys (0.83 mCi; 35 μ g/kg). The radiolabeling efficiency ranged from 89 to 98% over the series of experiments as determined by HPLC. Biodistribution of radiolabeled ShK-221 was evaluated by SPECT imaging continuously for the first hour postdose, and then at 4, 8, 24, 48, 72, 120, and 160 h (Supplemental Videos 1–12). The background level in the detection system was approximately 0.1 μ Ci/ml (~ 5 ng/ml of ShK-221 at the initial time point and 26 ng/ml at the last time point). Blood samples were collected after each scan, and total radioactivity in whole blood was measured by gamma counting. Computed tomography was performed at each time point to enable colocalization of the radiolabel with key anatomical structures.

Studies in Squirrel Monkeys. Biodistribution of ^{111}In -ShK-221 in squirrel monkeys was characterized principally by slow absorption from the injection site over the entire 160-h period (Fig. 3, A and E). The quantity of ^{111}In -ShK-221 present at the injection site followed a biphasic exponential decay ($R^2 = 0.95$) with an initial half-life of approximately 1 to 1.5 h and a terminal half-life of >48 h (Fig. 3E). During the first hour, significant radioactivity could be observed in the kidney, increasing in intensity through 1 h [$\sim 1\%$ injected dose (ID)/g; Supplemental Table 1] and slowly declining to approximately baseline by 48 h. Radioactivity in the monkey

Fig. 1. Biophysical properties of ShK-186, ShK-186 dephosphorylated form (ShK-198), and ShK-186 analogs. A, representative whole-cell Kv1.3 currents in the absence and presence of dephosphorylated ShK-186 (ShK-198). B, representative whole-cell Kv1.3 currents in the absence and presence of In-ShK-221 (a ShK-186 analog). In-ShK-221 was in 100% serum. C, comparison of dose-response curves for ShK-186, ShK-198, Gd-ShK-221, and In-ShK-221 on Kv1.3. The IC_{50} values were: ShK-186, 68.99 ± 4.01 pM ($n = 5$); ShK-198, 41.4 ± 7.25 pM ($n = 5$); Gd-ShK-221, 58.23 ± 1.38 pM ($n = 5$), and In-ShK-221, 63.8 ± 2.25 pM ($n = 3$). D, dose-response curves of ShK-186 on Kv1.3 in the presence of varying concentrations of serum. E, dose-response curves of In-ShK-221 in the presence or absence of serum. Electrophysiological recordings were carried out in the whole-cell configuration of the patch-clamp technique as described previously (Beeton et al., 2005; Wulff et al., 2000). The external solution was sodium Ringer's, and the pipette solution was potassium fluoride (300 mOsm). Kv1.3 currents were elucidated by 200-ms depolarizing pulses from a holding potential of -80 to 40 mV. All analogs were each tested at several concentrations. The reduction in peak current at 40 mV for each concentration was used to generate a dose-response curve by using Origin software (OriginLab Corp., Northampton, MA).

TABLE 1 ShK-186 pharmacokinetic parameters in Sprague Dawley rats and cynomolgus monkeys							
Species	Dose	<i>n</i>	<i>T</i> _{max}	<i>t</i> _{1/2}	<i>C</i> _{max}	AUC _{0-inf,obs}	Clearance
	μg/kg		min		ng/ml	ng/ml · min	ml/kg · min
Monkey	35	10	5	8.8	12.8	247.6	141.3
	100	14	5	14.5	33.6	823.1	121.8
	150	16	5	23.8	69.8	1935.9	78.5
	250	2	5	11.3	106.6	2220.9	112.2
	300	5	5	14.0	173.8	3496.4	86.0
	500	2	5	15.8	192.5	4734.0	105.6
	1000	2	5	13.0	358.0	9552.7	104.6
Rat	50	6	1	4.4	26.2	115.9	441.4
	150	6	1	9.2	27.0	249.5	640.9
	250	3	5	4.7	29.7	282.9	886.3
	500	9	1	5.2	42.7	587.5	847.2
	1000	3	5	7.9	48.0	690.6	1453.3

TABLE 2 ShK-198 pharmacokinetic parameters in Sprague Dawley rats and cynomolgus monkeys							
Species	Dose	<i>n</i>	<i>T</i> _{max}	<i>t</i> _{1/2}	<i>C</i> _{max}	AUC _{0-inf,obs}	Clearance
	μg/kg		min		ng/ml	ng/ml · min	ml/kg · min
Monkey	35	10	5	22.7	23.0	771.9	46.0
	100	14	5	24.5	55.6	2298.4	43.6
	150	16	5	16.7	94.2	2555.9	58.7
	250	2	15	22.8	117.0	5943.2	42.1
	300	5	5	73.5	168.0	8588.5	35.1
	500	2	15	30.8	321.0	17,750.6	28.2
	1000	2	15	25.4	852.5	53,389.9	18.7
Rat	50	6	1	6.1	11.8	102.5	486.6
	150	6	5	10.4	11.6	243.9	617.9
	250	3	5	7.5	36.4	480.5	522.1
	500	9	15	16.4	33.9	1039.6	488.5
	1000	3	5	8.7	53.9	1219.2	819.9

kidney was observed primarily in the cortical and medullary regions during all time points and was comparatively absent in the renal pelvis except for the first hour (Fig. 3B). Significant bladder associated radioactivity ($T_{max} = 0.75$ – 1 h; 0.34% ID/g) was observed only during the first 4 h, after

which relatively little radiolabel was detected in bladder. No other organ showed significant levels of radioactivity except for liver, which peaked at 0.75 to 1 h after dose administration (0.166% ID/g). Muscle, heart, and brain all had $<0.1\%$ ID/g at all time points measured (Supplemental Table 1).

Fig. 2. Pharmacokinetic profiles of ShK-186 and ShK-188 in plasma after a single dose to rats and monkeys. A to D, ShK-186 was administered by subcutaneous injection to SD rats (A and B; $n = 3$ –9 animals/dose/time point) and cynomolgus monkeys (C and D; $n = 2$ –16 animals/dose/time point), and plasma samples were collected at time intervals ranging from 1 min to 24 h. Samples were analyzed by using a validated HPLC-MS/MS method that can resolve both the parent compound (ShK-186; A and C) and its metabolite (ShK-198; B and D). E and F, C_{max} and AUC_{0-inf} were computed for rat (E) and monkey (F) at each dose level by using noncompartmental analysis and the linear trapezoidal method.

Evaluation of blood-associated radioactivity in monkeys at each time point also demonstrated a biphasic exponential decay ($R^2 = 0.99$) with an initial half-life of approximately 1 h and a terminal half-life of >64 h (Fig. 3F). In monkeys much of the terminal elimination phase was reflected by blood concentrations of ^{111}In -ShK-221 (>200 pM) well above the IC_{50} for ShK-186 (69 pM), ShK-198 (41 pM), and In-ShK-221 (64 pM) for 6 days postdose. Even though ultrafiltration studies demonstrated significant binding of ShK-186 to plasma proteins, the channel-blocking activity of ShK-186 and In-ShK-221 were unaffected by the presence of serum (Fig. 1, B, D, and E), suggesting that the bulk of the peptide detected in blood is functionally active.

Studies in Rats. Biodistribution of ^{111}In -ShK-221 in the rat was similar to that in monkeys and characterized by slightly faster absorption from the injection site and excretion through the urine over the first 24 h (Fig. 3, C and E). Significant radioactive label was observed in rat bladder (9.4% ID/g), kidney (2.9% ID/g), and liver (0.4% ID/g) during the first hour (Fig. 3C; Supplemental Table 2). Although little label was identified in the bladder at later time points, the amount of drug in liver and kidney was relatively constant through the first 24 h. Cross-sectional views of the rat kidney showed that, with the exception of the first hour,

radioactivity was concentrated primarily in the cortical regions similar to the monkey (Fig. 3D).

The whole blood-associated radioactivity in the rat also showed a biphasic exponential decay ($R^2 = 0.99$) with an initial half-life of approximately 1.7 h and a terminal half-life of >72 h (Fig. 3F). The ShK-221 concentrations in the rat (200 pM) were well above the IC_{50} for ShK-186 and ShK-198 until 5 days postdose. In summary, the biodistribution of radiolabeled ShK-221 in squirrel monkeys and rats is characterized by a slow absorption from the injection site, significant concentrations of drug peripherally in the injection site, kidney, and liver, and a long terminal elimination phase in whole blood.

Suppression of Cytokine Responses in Human PBMCs. Another way that ShK-186 could exert a durable PD effect is through a sustained interaction with the Kv1.3 channel on lymphocytes. We therefore assessed the duration of lymphocyte suppression after exposure to ShK-186. We have previously shown that ShK-186 suppresses thapsigargin-induced expression of IL-2, IL-17, interferon- γ , and IL-4 in freshly isolated human PBMCs (Chi et al., 2012). Here, we exposed PBMCs to the drug either continuously during a 48-h period of thapsigargin stimulation, for 1 h before 48 h of stimulation, or for 1 h up to 16 h before stimulation and measured the IL-2

Fig. 3. Biodistribution studies with radiolabeled ShK in rat and squirrel monkey. ^{111}In -labeled ShK-221 was administered to SD rats (100 $\mu\text{g/kg}$; 1.0mCi) and squirrel monkeys (35 $\mu\text{g/kg}$; 0.84mCi) as a single subcutaneous injection to the scapular region of each animal. SPECT and CT scans were collected continuously during the first hour ($4 \times 15\text{-m}$ intervals) and at 4, 8, 24, 48, 72, 120, and 160 h postdose. A and C, flattened 2D images of the 3D reconstructions are shown for the 4-, 24-, 72-, and 160-h time points for monkeys (A) and the 1-, 8-, and 24-h time points for rats (C). Both animals showed slow absorption of drug from the injection site and significant early and sustained distribution to kidney and to a lesser extent liver. B and D, kidney-associated radioactivity in monkeys (B) and rats (D) was principally identified in the cortex. E, quantification of ^{111}In -ShK-221 at the injection site in monkeys (top) and rats (bottom) revealed a biphasic decay with an initial half-life of approximately 0.5 to 1.5 h and a terminal half-life of >48 h. F, drug concentrations in monkey (top) and rat (bottom) whole blood followed a similar biphasic decay with an initial half-life of approximately 1.5 h and a terminal half-life of >64 h. Blood concentrations remained above the IC_{50} for Kv1.3 throughout the entire study period and above the 80% saturation concentration (233 pM) through the first 120 h, which is consistent with a slow, continuous distribution from the injection site throughout the study period.

Fig. 4. Durable effect of drug treatment on human PBMCs. Time of treatment studies were conducted in human PBMCs in which the effect of drug treatment timing and duration on thapsigargin-stimulated IL-2 responses was measured. Continuous drug treatment during the 48-h stimulation period (A; black bars) was compared with drug pretreatment for 1 h followed by a brief washout and stimulation for 48 h (B; open bars) or drug pretreatment (1 h) followed by washout, media incubation (16 h), and thapsigargin stimulation (48 h) (C; hatched bars). Drug treatment for a 1-h period up to 16 h before stimulation was statistically indistinguishable from continuous drug treatment. None of the treatment times and durations were significantly different (one-way ANOVA; $F_{3,12} = 0.40$; $p = 0.76$).

responses by enzyme-linked immunosorbent assay (Fig. 4). In cases of transient exposure, the cells were thoroughly washed with media before thapsigargin treatment. There was no statistically significant difference (one-way ANOVA; $F_{3,12} = 0.40$; $p = 0.76$) between continuous drug treatment versus transient drug exposure up to 16 h before thapsigargin stimulation. These data are consistent with a model where ShK-186 rapidly associates with, but slowly dissociates from, the Kv1.3 channel of lymphocytes.

Suppression of the DTH Response In Vivo: Dose Frequency and Dose-Response Studies.

Our absorption, distribution, metabolism, and excretion studies suggested that a low, but therapeutically, relevant drug concentration persists in the blood for several days after a single administration of ShK-186. We therefore evaluated the relationship between dose frequency and therapeutic efficacy in animal models of effector-memory T cell-mediated disease. The simplest model for assessing drug effects is the DTH reaction, an immune response largely mediated by skin-homing effector-memory CD4⁺ T cells (Soler et al., 2003; Matheu et al., 2008). We immunized groups of five to eight Lewis rats with OVA in CFA followed 7 to 9 days later by elicitation of the DTH response in the ears of immunized animals. Ear swelling was measured 24 h postelicitation. The durability of drug effect was assessed by treating animals with ShK-186 at varying times before or coincident with the elicitation phase. The day of ear challenge is referred to as day 0 for reference.

Animals that were treated with two doses of 10 or 100 $\mu\text{g/kg}$ ShK-186 on days 0 and 1 (the standard regimen) were compared with single 100 $\mu\text{g/kg}$ doses administered on days 0, -1, -2, -3, and -4. Two doses administered on days 0 and 1 and single doses administered on days -1 to -4 yielded statistically significant reductions in ear swelling relative to placebo-treated animals (two-tailed Mann-Whitney test; Fig. 5A). Single doses administered before day -4 did not result in a significant reduction in ear swelling relative to the control group (data not shown). We conclude that a single subcutaneous injection of ShK-186 is sufficient to suppress the DTH response in rat for 5 days, consistent with the long terminal elimination half-life of radiolabeled ShK-221.

To establish a relationship between dose level and the time of dosing, we evaluated the suppressive effect of a single ascending dose (0.1–100 $\mu\text{g/kg}$) of ShK-186 administered 2 days before ear challenge. A dose-dependent reduction in ear swelling was observed over the three-log dose range, with doses ≥ 1 $\mu\text{g/kg}$ achieving statistical significance relative to vehicle-treated animals (Fig. 5B).

Treatment Strategies in the CR-EAE and PIA Models. CR-EAE is widely used as a model of multiple sclerosis. This model has been extensively characterized in guinea pigs (Raine et al., 1977; Keith et al., 1979) and DA rats (Feurer et

Fig. 5. Time of administration and dose-dependent suppression of the DTH response. Lewis rats were immunized with ovalbumin in CFA and challenged 7 to 9 days later by injection of ovalbumin to the ear pinna. The day of challenge is represented as day 0. The DTH reaction was measured 24 h postchallenge (day 1). A, ShK-186 administered at 10 or 100 $\mu\text{g/kg}$ in two doses on days 0 and 1 or as a single 100 $\mu\text{g/kg}$ dose on days -1, -2, -3, and -4 resulted in a significant reduction in ear swelling relative to placebo (PL)-treated animals when measured on day 1 postchallenge (two-tailed Mann-Whitney). These data indicate that a single ShK-186 dose can suppress the DTH response for up to 5 days. B, using the DTH model, a dose-response study was conducted with a single dose of ShK-186 administered on day -2. Doses between 0.1 and 100 $\mu\text{g/kg}$ showed a clear dose-response relationship, with all but 0.1 $\mu\text{g/kg}$ achieving statistical significance relative to placebo. *, $p < 0.05$; **, $p < 0.01$.

Fig. 6. Alternate dosing schedules are effective in the chronic EAE model. A, an initial study evaluated lead-in dosing followed by less than daily drug administration. Male DA rats were immunized with spinal cord homogenate in CFA and administered ShK-186 (100 $\mu\text{g/kg/day}$; $n = 25$) or vehicle ($n = 12$) during the prodromal period and for 7 days after the onset of EAE, after which drug-treated animals were randomized to receive a maintenance dose of 100 $\mu\text{g/kg}$ ShK-186 every 2 days ($n = 12$)

al., 1985; Lorentzen et al., 1995), and unlike the acute EAE models includes relapses and some degree of demyelination (Lassman and Wisniewski, 1979; Lorentzen et al., 1995; Matheu et al., 2008). Moreover, because of its protracted nature, it is an adequate model for testing dosing schedules. The initial wave of disease is largely adjuvant mediated and composed primarily of central memory T cells, whereas subsequent waves of disease are principally T_{EM} cell-mediated and therefore Kv1.3-dependent (Matheu et al., 2008). We have previously shown ShK-186 to be effective in the CR-EAE model by using daily dosing at 100 $\mu\text{g/kg}$ (Matheu et al., 2008). In the present study, we explored the effect of less frequent dose administration on drug efficacy in this model.

Two study designs were explored. In the first, a lead-in period with daily drug administration was initiated at the time of disease induction and continued throughout the first wave of disease. Seven days after disease onset, animals were randomized to receive 100 $\mu\text{g/kg}$ ShK-186 every 2 or 3 days through the remainder of the study. Daily drug treatment caused a statistically significant reduction in mean clinical score compared with placebo-treated animals during the first wave of disease (Fig. 6A). After lead-in, drug administration every 2 or 3 days resulted in continued significantly lower mean daily clinical score in treated relative to control animals. The average cumulative clinical score was 161.1 ± 162.3 , 72.5 ± 98.1 , and 71.3 ± 103.3 for the placebo, every-2-days, and every-3-days dosing groups, respectively. Animals were scored twice daily.

In a second series of experiments, we evaluated the effect of reduced dose frequency on EAE without the drug lead-in period. In this study, groups of DA rats were randomized to receive placebo, daily ShK-186, or ShK-186 every 3 days beginning at a clinical score ≥ 1 . Both drug-treated groups exhibited a significantly lower mean daily clinical score than placebo-treated animals (Fig. 6B). However, there was no statistically significant difference between daily and every-third-day dose administration. Mean cumulative clinical scores were 49.7 ± 26.7 , 36.9 ± 20.6 , and 30 ± 19.3 for the placebo, daily, and every-third-day dosing groups, respectively. The percentage of rats with one or more relapses was 70% (9/13), 21% (3/14), and 21% (3/14), and the total number of relapses observed during the chronic phase were 15, 6, and

or 3 days ($n = 13$). ShK-186 significantly reduced ($p < 0.001$) the severity of the initial episode of disease. Maintenance therapy administered at 48- or 72-h intervals significantly ($p < 0.001$; one-way repeated-measures ANOVA) reduced the severity of CR-EAE. B, a second study measured the effect of infrequent drug administration beginning at disease onset. Female DA rats ($n = 13/\text{group}$) were immunized with spinal cord homogenate in CFA to elicit a chronic, relapsing EAE. After the onset of symptoms (clinical score > 1), animals were randomized to receive daily placebo, daily ShK-186, or ShK-186 every 2 or 3 days. Drug treatment resulted in significantly lower clinical scores and a lower frequency of disease incidence (inset) during the chronic phase in the groups treated daily and groups treated every third day compared with placebo (repeated-measures ANOVA; $F_{2,60} = 39.4$; $p < 0.0001$). Mean clinical scores were not lower relative to placebo in the group treated on alternate days in this study (data not shown). C, a final study measured the durability of response of a period of drug therapy. Female DA rats ($n = 23$) were immunized and allowed to proceed to CR-EAE. Ten days after the onset of symptoms, animals were randomized to receive daily placebo ($n = 10$) or 100 $\mu\text{g/kg}$ ShK-186 ($n = 13$) for 14 days. Therapy was discontinued on day 24, and the time required for treated animals to return to baseline disease was evaluated. The previously treated group had mean clinical scores significantly lower than placebo controls on days 24, 25, 26, and 28 (Supplemental Table 2), which is consistent with a 5-day window for disease recurrence.

5 for the placebo, ShK186 daily, and every-third day-treated groups, respectively. Animals were scored once daily.

Finally, we wanted to evaluate the durability of disease suppression in the CR-EAE model after cessation of drug administration. Disease was elicited in groups of DA rats, and the animals were allowed to develop a relapsing disease. On day 10 after disease onset (clinical score ≥ 1), animals were randomized to receive daily placebo or 100 $\mu\text{g/kg}$ ShK-186 for 14 days. Drug treatment was discontinued on day 24, and the animals were monitored for an additional 15 days. There was no difference between the placebo- and drug-treated groups before drug treatment (Fig. 6C). During the 14 days of daily drug administration, there was a statistically significant reduction in mean clinical score in drug-treated versus control animals. Once treatment was withdrawn, the mean clinical score in the drug-treated group slowly returned to the level of placebo over approximately 5 days (Supplemental Table 3). However, there was no rebound effect observed in the previously treated animals.

As a final test of the effectiveness of less than daily dosing, we evaluated ShK-186 administration every other day in the PIA model of rheumatoid arthritis (Beeton et al., 2006). ShK-186 administered once daily (100 $\mu\text{g/kg}$) has been previously reported to reduce the number of joints affected and the severity of joint swelling in the PIA model in DA rats (Beeton et al., 2006). Here, we demonstrate that ShK-186 administered on alternate days (100 $\mu\text{g/kg}$) was as effective in ameliorating disease (Fig. 7) as our previously reported effect with once-daily administration (Beeton et al., 2006).

The data from these four studies are consistent with a model of durable drug effects after a single subcutaneous dose of ShK-186 and observations made using the DTH model.

Discussion

Here, we present five complementary types of data that relate the PK properties of ShK-186 to its therapeutic efficacy in rat models of autoimmune diseases. Through serum/plasma stability studies, we identify the sole metabolite of ShK-186 in rat, monkey, and human samples as the dephosphorylated peptide ShK-198. ShK-198 blocks Kv1.3 (IC_{50} 40 pM) with approximately the same potency as ShK-186 (69

Fig. 7. Pristane-induced arthritis treated with alternate day administration of ShK-186. Arthritis was induced in DA rats by injection of 75 μl of pristane at the base of the tail. Disease was allowed to evolve to a clinical score of 1 when animals were randomized to receive placebo or 100 $\mu\text{g/kg}$ ShK-186 every other day (EOD). Clinical score is computed as according to Gillett et al. (2010). Overall disease severity in the study was high. Animals receiving ShK-186 every other day had a statistically significant reduction in mean score (placebo, 20.4 ± 5.9 ; drug, 14.6 ± 4.1 ; paired t test; $p < 0.0001$).

pM). Second, we demonstrate that the C_{max} of both ShK-186 and ShK-198 is rapidly reached after subcutaneous injection to rats and monkeys. Both parent and metabolite have a short apparent half-life in the central compartment with $>90\%$ of the C_{max} eliminated by 2 h in both species at all tested doses. The half-life is shorter and the apparent clearance greater for ShK-186 than ShK-198, probably reflecting the independent action of endogenous phosphatases on the conversion of ShK-186 to its metabolite. Third, SPECT imaging of rats and monkeys administered an ^{111}In -labeled ShK-186 analog (ShK-221; IC_{50} 65 pM) by subcutaneous injection revealed that the peptide is released slowly from the injection site and has an extended terminal half-life, with whole-blood levels above the IC_{50} value for Kv1.3 block for 5 days in rats and 7 days in monkeys. The Kv1.3 channel-blocking affinity of ShK-186 and ShK-221 is not affected by the presence of serum, indicating that plasma protein binding, if any, does not affect the functional activity of these peptides. Fourth, in vitro proliferation assays demonstrate that brief 1-h exposure of PBMCs to ShK-186 is sufficient to suppress interleukin-2 production 64 h later. This suggests that the peptide, once bound to the Kv1.3 channel, dissociates very slowly. Finally, in three different rat models of T_{EM} cell-mediated inflammatory diseases (DTH, CR-EAE, and PIA) ShK-186 is as effective in ameliorating disease when administered every 2 to 5 days as when it is administered once daily. The durable PD effect of ShK-186 is contrary to conventional wisdom that frequent administration of peptide therapeutics is required to sustain PD activity.

Our findings highlight a potential advantage of developing peptides from venoms as drug candidates. SPECT imaging studies with ^{111}In -labeled ShK-221 revealed a biphasic (fast then slow) release from the subcutaneous injection site over 7 days in rats and monkeys. This type of rate-limiting absorption resulting in prolonged plasma exposure has been described for whole animal venoms (Barral-Netto et al., 1990; Audebert et al., 1994). The long absorption phase has been used to explain the frequent relapse in envenomation patients long after exposure and/or treatment with antivenom (Dart et al., 2001; Seifert and Boyer, 2001; Gutiérrez et al., 2003), and venoms are often described as having a depot effect. The parent peptide of ShK-186 was originally isolated from the venom of *Stichodactyla helianthus* (Castañeda et al., 1995), and ShK-186 may share some of the in vivo characteristics of the complex peptide mixtures found in animal venom. Venom and toxin PK parameters are also characterized by large apparent volumes of distribution and slow elimination from deep and shallow peripheral compartments (Ismail et al., 1996). The apparent volume of distribution for ShK-186 is large in both rats (2743–16,458 ml/kg) and monkeys (1729–2664 ml/kg), consistent with that reported for other animal venoms. The blood concentration of ^{111}In -ShK-221 mimicked the absorption of the peptide from the injection site and was characterized by a rapid initial phase and a very long terminal phase. The terminal half-life computed by using ^{111}In -ShK-221 was >64 h in monkeys with sustained blood levels for 7 days above the IC_{50} values for ShK-186 (69 pM) and ShK-198 (40 pM). The presence of serum does not affect channel-blocking affinity of these peptides, indicating that plasma protein binding, if any, does not affect functional activity.

Glomerular filtration is the principal elimination pathway for the peptide shortly after subcutaneous injection. Significant amounts of radioactivity were observed in the bladder of both rats (~17% injected dose) and monkeys (~1% injected dose) at the earliest time points after administration of ^{111}In -ShK-221. The large amount of drug excreted by the rat in the first hour is most likely a reflection of the increased metabolism of the rat compared with the monkey. After 1 h in rats and approximately 4 h in monkey, little radioactivity was observed in bladder or renal pelvis, whereas significant amounts of radioactivity could still be observed in the kidney cortex. Cortical concentration has been reported for numerous radiolabeled versions of peptide drugs including octreotide, bombesin, exendin, and gastrin (Gotthardt et al., 2007). The mechanism of cortical retention has been most thoroughly described for octreotide. Tubular reabsorption of the cationic octapeptide is mediated by megalin, a scavenger receptor expressed in the proximal kidney tubule (de Jong et al., 2005). Mice with a kidney-specific disruption of the receptor lack the cortical retention of radiolabeled octreotide seen in wild-type mice. Renal uptake of octreotide is partially mediated by charge and can be disrupted by coinjection of the positively charged amino acids L-lysine and L-arginine (Bodei et al., 2003). ShK-186 carries a net +6 charge at physiological pH, so its cortical retention may be mediated by a similar mechanism.

Another contributing factor to the long PD effect of ShK-186 along with an extended terminal half-life could be its slow dissociation from the Kv1.3 channel. Studies with PBMCs show that there is essentially no difference between continuous ShK-186 treatment during thapsigargin stimulation and treatment up to 16 h before stimulation. Although we did not formally measure an off-rate of the drug on the Kv1.3 channel by using traditional methods, we did show that ShK-186's PD effect can persist for at least 72 h after its brief exposure to PBMCs. The receptor dissociation half-life of some animal toxins has been reported to be as long as 1 week (Berg and Hall, 1975, Chang and Huang, 1975). Future receptor binding studies using the radiolabeled analog will allow determination of the off-rate of ShK-186 from Kv1.3.

Absorption, distribution, metabolism, and excretion studies with ^{111}In -labeled ShK-221 suggest that a single dose of peptide can provide therapeutically meaningful blood concentrations for up to 5 days in rats and 7 days in monkeys. These data are consistent with data from the rat DTH model where a single administered dose of ShK-186 provided a statistically significant reduction in ear swelling for up to 5 days. Likewise, dose administration every 2 to 3 days in the CR-EAE and PIA models were as effective in ameliorating disease as daily ShK-186 administration. Because ShK-186's therapeutic effects are long-lasting, we anticipate administering the peptide to humans once weekly or less frequently. From a therapeutic and commercial perspective, ShK-186 will compete favorably with pills taken daily or multiple times a day or injectables.

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Authorship Contributions

Participated in research design: Tarcha, Chi, Muñoz-Elías, Bailey, Ruppert, Boley, Beeton, Slauter, Knapp, Pennington, Chandy, and Iadonato.

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Contributed new reagents or analytic tools: Tarcha, Bailey, and Pennington.

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Wrote or contributed to the writing of the manuscript: Tarcha, Chi, Muñoz-Elías, Chandy, and Iadonato.

References

- Audebert F, Urtizberea M, Sabouraud A, Scherrmann JM, and Bon C (1994) Pharmacokinetics of *Vipera aspis* venom after experimental envenomation in rabbits. *J Pharmacol Exp Ther* **268**:1512–1517.
- Azam P, Sankaranarayanan A, Homerick D, Griffey S, and Wulff H (2007) Targeting effector memory T cells with the small molecule Kv1.3 blocker PAP-1 suppresses allergic contact dermatitis. *J Invest Dermatol* **127**:1419–1429.
- Barral-Netto M, Schrieffer A, Vinhas V, and Almeida AR (1990) Enzyme-linked immunosorbent assay for the detection of *Bothrops jararaca* venom. *Toxicon* **28**:1053–1061.
- Beeton C and Chandy KG (2007) Induction and monitoring of active delayed type hypersensitivity (DTH) in rats. *J Vis Exp* **6**:237.
- Beeton C, Pennington MW, Wulff H, Singh S, Nugent D, Crossley G, Khaytin I, Calabresi PA, Chen CY, Gutman GA, et al. (2005) Targeting effector memory T cells with a selective peptide inhibitor of Kv1.3 channels for therapy of autoimmune diseases. *Mol Pharmacol* **67**:1369–1381.
- Beeton C, Wulff H, Barbaria J, Clot-Faybesse O, Pennington M, Bernard D, Cahalan MD, Chandy KG, and Béraud E (2001) Selective blockade of T lymphocyte K^+ channels ameliorates experimental autoimmune encephalomyelitis, a model for multiple sclerosis. *Proc Natl Acad Sci U S A* **98**:13942–13947.
- Beeton C, Wulff H, Standifer NE, Azam P, Mullen KM, Pennington MW, Kolski-Andreaco A, Wei E, Grino A, Counts DR, et al. (2006) Kv1.3 channels are a therapeutic target for T cell-mediated autoimmune diseases. *Proc Natl Acad Sci U S A* **103**:17414–17419.
- Berg DK and Hall ZW (1975) Loss of α -bungarotoxin from junctional and extrajunctional acetylcholine receptors in rat diaphragm muscle in vivo and in organ culture. *J Physiol* **252**:771–789.
- Bodei L, Cremonesi M, Zoboli S, Grana C, Bartolomei M, Rocca P, Caracciolo M, Mäcke HR, Chinol M, and Paganelli G (2003) Receptor-mediated radionuclide therapy with $^{90\text{Y}}$ -DOTATOC in association with amino acid infusion: a phase 1 study. *Eur J Nucl Med Mol Imaging* **30**:207–216.
- Cahalan MD and Chandy KG (2009) The functional network of ion channels in T lymphocytes. *Immunol Rev* **231**:59–87.
- Castañeda O, Sotolongo V, Amor AM, Stöcklin R, Anderson AJ, Harvey AL, Engström A, Wernstedt C, and Karlsson E (1995) Characterization of a potassium channel toxin from the Caribbean sea anemone *Stichodactyla helianthus*. *Toxicon* **33**:603–613.
- Chang CC and Huang MC (1975) Turnover of junctional and extrajunctional acetylcholine receptors of the rat diaphragm. *Nature* **253**:643–644.
- Cheong A, Li J, Sukumar P, Kumar B, Zeng F, Riches K, Munsch C, Wood IC, Porter KE, and Beech DJ (2011) Potent suppression of vascular smooth muscle cell migration and human neointimal hyperplasia by Kv1.3 blockers. *Cardiovasc Res* **89**:282–289.
- Chi V, Pennington MW, Norton RS, Tarcha EJ, Londono LM, Sims-Fahey B, Upadhyay SK, Lakey JT, Iadonato S, Wulff H, et al. (2012) Development of a sea anemone toxin as an immunomodulator for therapy of autoimmune diseases. *Toxicon* **59**:529–546.
- Dart RC, Seifert SA, Boyer LV, Clark RF, Hall E, McKinney P, McNally J, Kitchens CS, Curry SC, Bogdan GM, et al. (2001) A randomized multicenter trial of crotaline polyvalent immune Fab (ovine) antivenom for the treatment for crotaline snakebite in the United States. *Arch Intern Med* **161**:2030–2036.
- de Jong M, Barone R, Krenning E, Bernard B, Melis M, Visser T, Gekle M, Willnow TE, Walrand S, Jamar F, et al. (2005) Megalin is essential for renal proximal tubule reabsorption of ^{111}In -DTPA-octreotide. *J Nucl Med* **46**:1696–1700.
- Feurer C, Prentice DE, and Cammisuli S (1985) Chronic relapsing experimental allergic encephalomyelitis in the Lewis rat. *J Neuroimmunol* **10**:159–166.
- Gilhar A, Bergman R, Assay B, Ullmann Y, and Etzioni A (2011) The beneficial effect of blocking Kv1.3 in the psoriasiform SCID mouse model. *J Invest Dermatol* **131**:118–124.
- Gillett A, Marta M, Jin T, Tuncel J, Leclerc P, Nohra R, Lange S, Holmdahl R, Olsson T, Harris RA, et al. (2010) TNF production in macrophages is genetically determined and regulates inflammatory disease in rats. *J Immunol* **185**:442–450.
- Gotthardt M, van Eerd-Vismale J, Oyen WJ, de Jong M, Zhang H, Rolleman E, Maecke HR, Béhé M, and Boerman O (2007) Indication for different mechanisms of kidney uptake of radiolabeled peptides. *J Nucl Med* **48**:596–601.
- Grgic I, Wulff H, Eichler I, Flothmann C, Köhler R, and Hoyer J (2009) Blockade of T-lymphocyte $\text{KCa}3.1$ and Kv1.3 channels as novel immunosuppression strategy to prevent kidney allograft rejection. *Transplant Proc* **41**:2601–2606.
- Gutiérrez JM, León G, and Lomonte B (2003) Pharmacokinetic-pharmacodynamic relationships of immunoglobulin therapy for envenoming. *Clin Pharmacokinet* **42**:721–741.

- Hu L, Gocke AR, Knapp E, Rosenzweig JM, Grishkan IV, Baxi EG, Zhang H, Margolick JB, Whartenby KA, and Calabresi PA (2012) Functional blockade of the voltage-gated potassium channel Kv1.3 mediates reversion of T effector to central memory lymphocytes through SMAD3/p21cip1 signaling. *J Biol Chem* **287**:1261–1268.
- Hu L, Pennington M, Jiang Q, Whartenby KA, and Calabresi PA (2007) Characterization of the functional properties of the voltage-gated potassium channel Kv1.3 in human CD4⁺ T lymphocytes. *J Immunol* **179**:4563–4570.
- Hyodo T, Oda T, Kikuchi Y, Higashi K, Kushiya T, Yamamoto K, Yamada M, Suzuki S, Hokari R, Kinoshita M, et al. (2010) Voltage-gated potassium channel Kv1.3 blocker as a potential treatment for rat anti-glomerular basement membrane glomerulonephritis. *Am J Physiol Renal Physiol* **299**:F1258–F1269.
- Ismail M, Aly MH, Abd-Elsalam MA, and Morad AM (1996) A three-compartment open pharmacokinetic model can explain variable toxicities of cobra venom and their alpha toxins. *Toxicon* **34**:1011–1026.
- Keith AB, Arnon R, Teitelbaum D, Caspary EA, and Wisniewski HM (1979) The effect of Cop 1, a synthetic polypeptide, on chronic relapsing experimental allergic encephalomyelitis in guinea pigs. *J Neurol Sci* **42**:267–274.
- Koo GC, Blake JT, Talento A, Nguyen M, Lin S, Sirotina A, Shah K, Mulvany K, Hora D Jr, Cunningham P, et al. (1997) Blockade of the voltage-gated potassium channel Kv1.3 inhibits immune responses in vivo. *J Immunol* **158**:5120–5128.
- Lassmann H and Wisniewski HM (1979) Chronic relapsing experimental allergic encephalomyelitis: effect of age at the time of sensitization on clinical course and pathology. *Acta Neuropathol* **47**:111–116.
- Lorentzen JC, Issazadeh S, Storch M, Mustafa MI, Lassman H, Linington C, Klareskog L, and Olsson T (1995) Protracted, relapsing and demyelinating experimental autoimmune encephalomyelitis in DA rats immunized with syngeneic spinal cord and incomplete Freund's adjuvant. *J Neuroimmunol* **63**:193–205.
- Matheu MP, Beeton C, Garcia A, Chi V, Rangaraju S, Safrina O, Monaghan K, Uemura MI, Li D, Pal S, et al. (2008) Imaging of effector memory T cells during a delayed-type hypersensitivity reaction and suppression by Kv1.3 channel block. *Immunity* **29**:602–614.
- Pennington MW, Beeton C, Galea CA, Smith BJ, Chi V, Monaghan KP, Garcia A, Rangaraju S, Giuffrida A, Plank D, et al. (2009) Engineering a stable and selective peptide blocker of the Kv1.3 channel in T lymphocytes. *Mol Pharmacol* **75**:762–773.
- Pennington MW, Mahnir VM, Khaytin I, Zaydenberg I, Byrnes ME, and Kem WR (1996) An essential binding surface for ShK toxin interaction with rat brain potassium channels. *Biochemistry* **35**:16407–16411.
- Raine CS, Snyder DH, Stone SH, and Bornstein MB (1977) Suppression of acute and chronic experimental allergic encephalomyelitis in Strain 13 guinea pigs. A clinical and pathological study. *J Neurol Sci* **31**:355–367.
- Rangaraju S, Chi V, Pennington MW, and Chandy KG (2009) Kv1.3 potassium channels as a therapeutic target in multiple sclerosis. *Expert Opin Ther Targets* **13**:909–924.
- Rintisch C, Förster M, and Holmdahl R (2009) Detection of arthritis-susceptibility loci, including Ncf1, and variable effects of the major histocompatibility complex region depending on genetic background in rats. *Arthritis Rheum* **60**:419–427.
- Rus H, Pardo CA, Hu L, Darrah E, Cudrici C, Niculescu T, Niculescu F, Mullen KM, Allie R, Guo L, et al. (2005) The voltage-gated potassium channel Kv1.3 is highly expressed on inflammatory brain infiltrates in multiple sclerosis brain. *Proc Natl Acad Sci U S A* **102**:11094–11099.
- Seifert SA and Boyer LV (2001) Recurrence phenomena after immunoglobulin therapy for snake envenomations: Part 1. Pharmacokinetics and pharmacodynamics of immunoglobulin antivenoms and related antibodies. *Ann Emerg Med* **37**:189–195.
- Soler D, Humphreys TL, Spinola SM, and Campbell JJ (2003) CCR4 versus CCR10 in human cutaneous TH lymphocyte trafficking. *Blood* **101**:1677–1682.
- Wulff H, Calabresi PA, Allie R, Yun S, Pennington M, Beeton C, and Chandy KG (2003) The voltage-gated Kv1.3 K⁺ channel in effector memory T cells as new target for MS. *J Clin Invest* **111**:1703–1713.
- Wulff H, Miller MJ, Hansel W, Grissmer S, Cahalan MD, and Chandy KG (2000) Design of a potent and selective inhibitor of the intermediate-conductance Ca²⁺-activated K⁺ channel, IKCa1: a potential immunosuppressant. *Proc Natl Acad Sci U S A* **97**:8151–8156.

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