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SINGLE INJECTION DOSE-RANGE-FINDING STUDY OF A CANINE PD-1 SINGLE DOMAIN ANTIBODY (SDAB) IN DOGS

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ABSTRACT

Single domain antibodies (SDAb) represent an alternative immunotherapeutic platform for veterinary use. Several SDAb targeting canine PD-1 were previously identified with potential for canine cancer immunotherapy. A lead SDAb, clone STX-1B5, with nanomolar binding to canine PD-1 was selected for evaluation of tolerability in dogs. The anti-PD-1 SDAb was expressed in wild-type *Komagataella phaffii* (*Pichia pastoris*) at a 100 mg scale, purified by tangential flow filtration, and quality confirmed controlled for host-cell protein, host-cell DNA, and endotoxin. Single 1.0 mL subcutaneous (SQ) injections of 0.15 to 1.12 mg/kg were administered to three adult Beagle dogs (1 male and 2 female) one per week to allow observations from each dose level prior to dose escalation. The dogs were monitored by a veterinarian on the day of dosing and daily for three days. Administering 1.0 ml of STX-1B5 subcutaneously at 0.146, 0.383 or 1.154 mg/kg in young adult male or female Beagle dogs was well tolerated and did not result in adverse test article-related clinical observations.

KEYWORDS: immunotherapeutic; PD-1; single domain antibody; yeast; canine; cancer.

INTRODUCTION

In Veterinary Medicine cancer is the leading cause of death for over 80% of dog breeds and all mix-breed dogs[1], but treatment choices are limited. We have developed a single-domain antibody (SDAb) agent, specific for the canine checkpoint protein Programmed Cell Death Protein 1 (PD-1) that functions similarly to existing human monoclonal Ab therapies.[2] It interacts with the canine PD-1 molecule at overlapping but distinct points compared to the human medications, and effectively blocks PD-1/PDL-1 interaction. As such, it shows promise either as monotherapy for checkpoint inhibition or potentially in combination with other checkpoint inhibitors or treatments.

Single-domain antibodies are small molecules (12-15 kD) that are derived from a unique heavy-chain only antibody produced by camelids. Single-domain

antibodies exhibit similar target affinity and drug-like targeting properties as monoclonal antibodies.[3,4] SDAbs can also exhibit improved safety, being less immunogenic and not trigger complement mediated cytotoxicity compared to monoclonal antibodies.[5,6] We sought to initiate studies to assess the safety of our candidate canine cancer immunotherapeutic SDAb agent in animals.

MATERIALS AND METHODS

Yeast Production and Analysis of SDAb. Anti-PD-1 SDAb sequences were expressed in wild-type *Komagataella phaffii* (*Pichia pastoris*) (NRRL Y-11430) as described (1) at Sunflower Therapeutics, Medford, MA, USA. Samples of cultivation supernatant were analyzed for protein expression using SDS-PAGE. SDS-PAGE was carried out under reducing conditions using Novex 12% Tris-Glycine Gels (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommended protocol and stained using Coomassie Instant Blue Protein Stain (Abcam). SDAb purification was carried out on an AKTA pure purification system (Cytiva, Marlborough, MA,USA) according to the process described previously. Impurities were all below limits (host-cell protein, DNA, endotoxin) indicated in **Table 1**.

Table 1: Quality Control Indices.

Target Concentration	A280 (mg/mL)	Host Cell Protein (PPM)	HC DNA (PPM)*	Endotoxin (EU/mL)
Recommended Limit		<1000 PPM	< 100 PPM	< 20 EU/mL
1.2 mg/mL	1.2	312	<LoD	< 1
3.6 mg/mL	3.8	355	8	< 1
12 mg/mL	11	100	8.5	1.8

Mouse Injection and Monitoring for Acute Toxicity. Prior to the canine study, 3 healthy male mice were injected subcutaneously in the neck with 72 mcg of STX-1B5 in 300 mcl buffer. Test animals weighed an average of 38 g, resulting in an average dose of 1.89 mg/kg. This dose was approximately triple the anticipated clinical high dose (0.6 mg/kg) to be used in the dogs. Mice were monitored at given time points (0, 10 min, 20 min, 30 min, 1 hr, 6 hr and 24 hr) after injection. Mice were assessed for changes in weight, temperature, outward physiological readouts (tremors, convulsions, salivation, diarrhea, lethargy, sleepiness, dyspnea, ruffled fur and morbidity) and behavior (reduced activity, excessive licking, ungrooming, abnormal stance, grunting/vocalization and loss of appetite and drinking). Temperature was determined using an infrared thermometer. The mice were sacrificed 24 hours after injection and blood serum collected for biochemical analysis.

Beagle Dogs. The study was conducted at the Mountain West Research (dba CARE Research) facility (Fort Collins, CO). The procedures used were conducted in accordance with all state, local, and federal laws and regulations, and abided by USDA guidelines for animal care and handling. Mountain West Research (MWR) is an USDA certified, and OLAW accredited facility (USDA Certificate Number 84-R-0093, OLAW Assurance Number A4678-01). All relevant MWR SOPs and IACUC requirements were followed in the execution of this study. Dose volumes were 1.0 ml. The first dog in the study was dosed at 1.2 mg. Dog number two was dosed at 3.77 mg. The third dog was dosed at 11.0 mg, resulting in doses of 0.146 to 1.154 mg/kg. The optimal target dose is estimated to be 0.3 to 0.6 mg/kg. Detailed clinical observations were conducted twice daily with additional pen-side general health/mortality/morbidity observations conducted by Animal Care staff at least once daily. Body weights, food consumption, and body temperatures were recorded once daily. ECGs, respiration rates and blood pressure, were recorded and then evaluated by a veterinarian the morning following dose and approximately 72 hours later. Blood was collected prior to dosing and approximately 72 hours post-dose and divided into K_2EDTA tubes (~1-2 ml) for CBC and Red Top tubes (~3-4 ml). Red-Top tubes for serum chemistry analysis. All animal housing and research procedures involving live animals were conducted at MWR. The standards for animal husbandry and care followed are those found in the Guide for the Care and Use of Laboratory Animals, 8th Edition, Revised 2011.[7]

Animal Welfare. The study design and animal usage were reviewed and approved by the MWR Research Institutional Animal Care and Use Committee (IACUC #2531) for compliance with regulations prior to study initiation. Animal welfare for this study was in compliance with the U.S. Department of Agriculture's (USDA) Animal Welfare Act (9 CFR Parts 1, 2, and 3), the Guide for the Care and Use of Laboratory Animals[8], and MWR SOPs.

RESULTS AND DISCUSSION

Mouse Safety Assessment. Following injection, no changes in physical examination parameters, outward physiological readouts, behavior or serum analytes were observed over 24 hours (data not shown and Table 2). These results suggested that STX-1B5 was well tolerated by the mice and enabled the dog study.

Table 2: Mouse Blood Serum (24 hours)

	ALT (U/L)	AST (U/L)	BUN (mg/dL)	Creatinine(mg/dL)
Normal[9]	22-133	46-221	2-71	0.1-1.8
Mouse A	36	92	24	<0.2
Mouse B	32	53	21	0.2
Mouse C	25	48	21	0.2

Dog Safety Assessment. All animals appeared normal on all clinical observations with one exception. Blood was observed in the pen of the dog receiving the lowest dose the morning after injection. The animal did not have any obvious wounds and did not appear to be in heat. No further blood was noted on subsequent observations. This observation was transient and is expected not to be related to the test article.

Electrocardiograms. All animals had normal sinus rhythm at all time points.

Blood Pressure. Blood pressure data did not demonstrate biologically relevant changes (Table 3). On Day 2, STX-1B5 0.146 mg/kg had elevated blood pressure. Blood pressure was evaluated 3 times with similar values.

Table 3: Blood Pressure (mmHg)

Animal Number	Day 0			Day 2			Day 4		
	Systolic	Diastolic	Mean	Systolic	Diastolic	Mean	Systolic	Diastolic	Mean
STX-1B5 (0.146 mg/kg)	141	51	84	261	230	245	151	73	99
STX-1B5 (0.383 mg/kg)	154	105	121	117	82	96	155	111	125
STX-1B5 (1.154 mg/kg)	192	91	124	170	125	140	131	70	93

Respiration Rates. Respiration data did not demonstrate biologically relevant changes (Table 4). Minor fluctuations were observed in all animals. Elevated respiration rates are due to animals panting.

Table 4: Respiration Rate (breaths/minute)

Animal Number	Day 0	Day 2	Day 4
STX-1B5 (0.146 mg/kg)	28	32	116
STX-1B5 (0.383 mg/kg)	24	16	20
STX-1B5 (1.154 mg/kg)	76	116	104

Body Weights. Body weight data did not demonstrate biologically relevant changes (Table 5). Minor fluctuations were observed in all animals.

Table 5: Body Weight (kg)

Animal Number	Day 0	Day 1	Day 2	Day 3	Day 4
STX-1B5 (0.146 mg/kg)	8.2	8.2	8.2	8.0	8.2
STX-1B5 (0.383 mg/kg)	8.8	9.4	9.4	9.4	9.4
STX-1B5 (1.154 mg/kg)	10.2	10.4	10.8	10.2	10.0

Body Temperature. Body temperature data did not demonstrate biologically relevant changes (Table 6). Minor fluctuations were observed in all animals. Normal body temperature in dogs ranges form 100.5°F to 102.5°F. STX-1B5 1.154 mg/kg had slightly low temperature (99.9°F) on Day 0 and STX-1B5 0.146 mg/kg had slightly elevated temperature (102.9°F) on Day 4.

Table 6: Temperature (°F)

Animal Number	Day 0	Day 1	Day 2	Day 3	Day 4
STX-1B5 (0.146 mg/kg)	102.2	101.9	101.9	101.6	102.9
STX-1B5 (0.383 mg/kg)	102.0	101.6	101.6	101.4	100.9
STX-1B5 (1.154 mg/kg)	99.9	100.6	101.2	101.1	101.3

Hematology. There were no notable changes in hematology values. All hematology values were within normal limits for all animals at all timepoints with a few exceptions. Slightly low CHCM 32 g/dL(normal 33-36) was recorded for all animals at both baseline and ~72-hours post-dose. Slightly low MCHC, 32 g/dL(normal 33 to 36), was recorded for STX-1B5 0.383 mg/kg at ~72-hours post-dose and for STX-1B5 1.154 mg/kg at baseline.

Clinical Chemistry. There were no notable changes in clinical chemistry values. All clinical chemistry values were within normal limits for all animals at all timepoints with a few exceptions. STX-1B5 0.146 mg/kg

had slightly low creatine 0.54 mg/dL (normal 0.6-1.6) at ~72 hours post-dose. STX-1B5 0.383 mg/kg had slightly low cholesterol (normal 130-300) of 121 mg/dL at baseline and 127 mg/dL at ~72 hours post-dose. STX-1B5 1.154 mg/kg had slightly low cholesterol (normal 130-300) of 122 mg/dL at baseline and 121 mg/dL at ~72 hours post-dose.

The purpose of this Non-GLP Pilot Discovery study was to investigate the potential toxicity of a single subcutaneous (SQ) injection of a proprietary SDAb (STX-1B5) being developed for the treatment of cancers in dogs. The agent was well-tolerated and no evidence of toxicity was found. To the best of our knowledge, this is also the first report of administration of a yeast-produced, camelid-derived single-domain antibody (SDAb) to dogs. Thus, in addition to providing an important milestone in the path to develop an affordable, effective immunotherapeutic for cancer in dogs, this provides some evidence for the overall safety of yeast-produced SDAb in that species. This should facilitate scientific development in both those directions.

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Conflicts of Interest. The authors D.V.M., C.E.R., S.B. and C.K.C. are co-founders of BioTesserae, Inc., which holds a patent license for STX-1B5 from Oregon State University. Author P.L.I is also affiliated with BioTesserae, Inc. Author L.C. were employed by the company Sunflower Therapeutics. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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