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14. ABSTRACT

Traumatic optic neuropathy (TON) is a frequent cause of significant visual loss after combat-related frontal head trauma. There is no clinically proven therapy for improving visual outcomes in TON patients. Identification of new treatment options for TON is of highest priority. Our goal is to conduct studies needed to advance the proposed drug candidate, B-3(+), to clinical development for the TON indication. The following specific aims have been identified in order to attain the study objective: Develop a standard in vitro ADMET package required to support pre-clinical and clinical development of the drug candidate (Specific Aim 1); Conduct systemic and ocular pharmacokinetic studies in beagle dogs; use blood samples collected in PK studies for assessing drug nephrotoxicity (Specific Aim 2); Expand upon existing pre-clinical efficacy data and develop a robust efficacy package that can convincingly justify compound testing in patients with traumatic optic neuropathy (Specific Aim 3).

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION:

Traumatic optic neuropathy (TON) is a frequent cause of significant visual loss after combat-related frontal head trauma. The optic nerve is divided in four anatomical segments: intraocular, intraorbital, intracanalicular and intracranial. In TON, the injury of the optic nerve originates from concussive forces to the head which are transmitted to the optic canal forcing it to move faster than the intracanalicular optic nerve, which thus induce the optic nerve damage. There is no clinically proven therapy for improving visual outcomes in TON patients, and identification of new treatment options for TON is of highest priority. Astrocyte swelling was shown to be the primary trigger inducing tissue damage after closed head injury. Injury-induced astrocyte swelling is assumed to be a significant factor in pathogenesis of TON. Prevention of swelling by inhibiting injury-induced edema in the intracanalicular portion of the optic nerve is a sound strategy for TON treatment. B-3(+) is a fluorenone drug that has demonstrated significant efficacy in preventing mortality in animal models of concussive brain injury and thromboembolic stroke. The overall objective of the proposed project is to conduct the studies needed to support regulatory filings required for our drug candidate to enter clinical development for TON indication.

2. KEYWORDS:

Traumatic optic neuropathy; Astrocyte swelling; Fluorenone drug

3. ACCOMPLISHMENTS:

What were the major goals of the project?

There is no clinically proven therapy for improving visual outcomes in TON patients. Identification of new treatment options for TON is of highest priority. Our goal is to conduct studies needed to advance the proposed drug candidate, B-3(+), to clinical development for the TON indication. The following specific aims have been identified in order to attain the study objective: Develop a standard in vitro ADMET package required to support pre-clinical and clinical development of the drug candidate (Specific Aim 1); Conduct systemic and ocular pharmacokinetic studies in beagle dogs; use blood samples collected in PK studies for assessing drug nephrotoxicity (Specific Aim 2); Expand upon existing pre-clinical efficacy data and develop a robust efficacy package that can convincingly justify compound testing in patients with traumatic optic neuropathy (Specific Aim 3).

What was accomplished under these goals?

As results of studies conducted in Year 2, we completed all studies related to Major Task 1 (Synthesize the drug candidate and implement in vitro ADMET assays). All objectives of Subtasks 1-5 of Major Task 1 are successfully accomplished. Significant progress was made in Year 2 on objectives related to Major Task 2 (Ensure animal protocol approvals and perform three pharmacokinetic studies in beagle dogs). Objectives of Subtasks 2, 5 and 6 of Major task 2 are fully accomplished while objectives of Subtasks 1 and 7 of Major Tasks 7 are accomplished partially. Progress was made in the work on Major Task 3 (Define the range of efficacious drug concentrations in the animal model of optic nerve damage) by successfully completing parts of Subtask 1 related to the guinea pig protocol approval.

A. Completion of characterization of in vitro ADME characteristics of the drug candidate: Assessment of propensity for drug-drug interaction

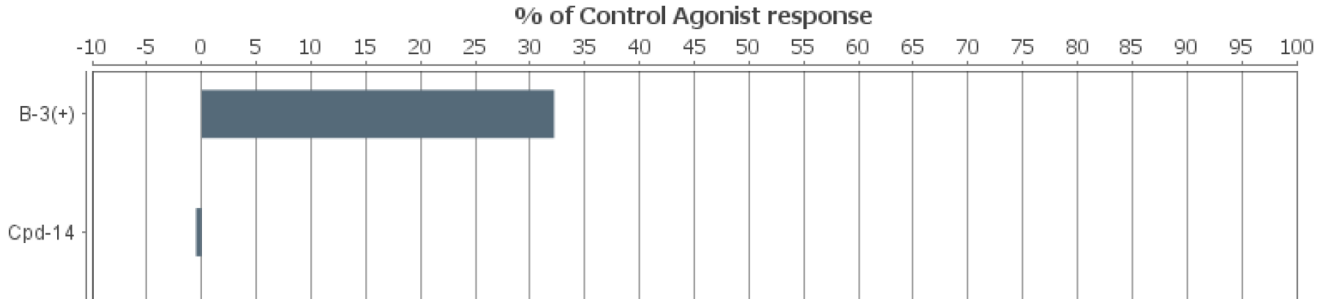
1. Selection of the format for assessing the activity of B-3(+) as a Pregnane X receptor (Major Task1, Subtask 3):

The Pregnane X receptor (PXR) belongs to the nuclear receptor superfamily of transcriptional factors. Its primary role is to act as a sensor for the presence of foreign toxic substances and regulate the expression of proteins involved in the detoxification and clearance of these substances from the body. PXR, upon activation, forms a heterodimer with the retinoid X receptor (RXR). As a complex will bind nuclear response elements that will upregulate specific gene products involved in drug metabolism, the most notable being CYP3A4. Other proteins regulated by PXR include glutathione S-transferase, MDR1 and QATP2. CYP3A4 is a drug metabolizing enzyme, and its significant systemic activation by B-3(+) would be an undesirable attribute as CYP3A4 activation may induce a decrease in the effect of other medicines that are administered to an individual concomitantly with B-3(+). We performed the analysis of several available formats available for the analysis of B-3(+) as an agonist of PXR. Available formats include the assessment of the agonist-induced recruitment of transcriptional activators (functional agonist coactivator assay), characterization of the effect of test compound on radioligand binding, induction of CYP3A4 activity in cultured human hepatocytes and evaluation of the agonistic activity in a cell-based translational activation assay. Following the assessment of all available options, the decision was made to use the cell-based translational transactivation assay for measuring the activity of B-3(+) as a PXR agonist. In this analysis, we will use DPX2™ cells that harbor the human PXR gene (NR1I2) and a luciferase reporter gene linked to two promoters identified in the human CYP3A4 gene. DPX2TM cells (2 x 10^5/ml) are dispensed into the wells of the assay plate to equilibrate overnight at 37°C at 5% CO2 in the incubator. The following day, test compound and a positive control (30 µM rifampicin) and/or vehicle are incubated with the cells for 24 hours at 37°C at 5% CO2 in the incubator. Test compound-induced increase of luminescence (normalized by cell viability, CellTiter-Fluo™) by 50 percent or more (≥50%) relative to the 30 µM rifampicin response will indicate a possible PXR receptor agonist activity. Implementation of the study is planned for the next quarter.

2. Experimental implementation of B-3(+) testing as an activator of Pregnane X receptor (Major Task1, Subtask 3):

Assessment of the activity of the test compound as an agonist of the nuclear receptor PXR is important for characterization of the drug-drug interaction potential of the drug candidate. As described above, we used the cell-based translational transactivation assay for measuring the activity of B-3(+) as a PXR agonist. In this assay, compound-induced increase in luciferase activity is interpreted as an agonistic activity. Test compound-induced increase of luminescence by 50 % relative to the 30 µM rifampicin response indicates a possible PXR receptor agonist activity. B-3(+) was tested at the 10 µM concentration. The outcomes of compound testing are shown in Figure. 1. As shown in Figure 1, B-3(+) activated the luciferase reported gene transcription by 32.2%.

A



B

Compound I.D.	Client Compound I.D.	Test Concentration	% of Control Agonist response 1st	2nd	3rd	Mean
Nuclear Receptor Activation - PXR Human (human, agonist, 1 conc.)						
100057078-1	B-3(+)	1.0E-05 M	22.8	24.5	49.3	32.2
100057078-2	Cpd-14	1.0E-05 M	0.4	-1.9	0.0	-0.5

Figure 1. A, Histogram for human PXR activation by B-3(+) and the unrelated Cpd-14. B, Numeric data for human PXR activation by B-3(+) and the unrelated control Cpd-14

3. Interpretation of results:

Given that the test compound-induced increase of luminescence by 50 % relative to the 30 μM rifampicin response indicates a possible PXR receptor agonist activity, B-3(+) cannot be considered as a PXR agonist. This represents a good drug-like characteristic for B-3(+).

B. Completion of characterization of in vitro ADME characteristics of the drug candidate: Assessment of the activity of B-3(+) as a substrate for efflux pumps.

1. Selection of the format for assessing the activity of B-3(+) as a substrate for the P-glycoprotein transporter (Major Task1, Subtask 4):

P-glycoprotein (Pgp; ABCB1), a member of the ATP-binding cassette (ABC) superfamily, exports structurally diverse hydrophobic compounds from the cell driven by ATP hydrolysis. Activity of the test compound as a substrate for the Pgp transporter may be associated with poor CNS penetration. Even though it is anticipated that B-3(+) will be administered intravitreally, it is important to characterize our drug candidate in the Pgp assay given that very high Pgp substrate activity may need to be mitigated by the increase in the dose of a drug that is administered into the eye. Additionally, FDA guidelines require knowledge of whether a drug candidate is a substrate Pgp. We evaluated the potential study protocols for the analysis of B-3(+) as a Pgp substrate and decided on a reasonable path to conducting such experiments. This assay is performed in MDR1-MDCKII cells. MDR1-MDCKII cells are Madin-Darby canine kidney cells which express the high levels of human MDR1 gene that encodes the Pgp transporter. The cells are cultured to confluency, trypsinized and seeded onto a filter transwell insert at a density of 18,000 cells/well in DMEM cell culture medium. Cells are grown in a humidified atmosphere of 5% CO2 at 37°C. Following an overnight attachment period (24 h after seeding), the cell medium will be replaced with fresh medium in both the apical and basolateral compartments every other day. The cell monolayers are used for transport studies 3 days post seeding after measuring the TEER values (>600 Ohms/cm2). The apical sides and basolateral sides are washed consecutively with HBSS 2.5% (v/v), HEPES (pH 7.4) or HBSS 2.5% (v/v), HEPES 10% (v/v), and Fetal Bovine Serum (pH 7.4) at 37 °C in an incubator under an atmosphere of 5% CO2. Donor working solution is prepared by dilution of DMSO stock of B-3(+) or positive control with transport media to 10 μM. For A → B directional transport, the donor working solution (with B-3(+) or positive control, with or without Pgp inhibitor) is added to the apical (A) compartment and the transport media as receiver working solution is added to the basolateral (B) compartment. For B → A directional transport, the donor working solution (with positive control or B-3(+), with or without Pgp inhibitor) is added to the basolateral (B) compartment and transport media as receiver working solution is added to the apical (A) compartment. The cells are incubated in a humidified atmosphere of 5% CO2 at 37 °C. At the end of the incubation, samples are taken from both donor and receiver compartments and transferred into 96-well assay plates containing internal standard solution (IS) in each well. After centrifugation, the supernatant solutions are transferred to clean 96 well plates and analyzed by LC-MS/MS.

2. Experimental implementation of B-3(+) testing as a substrate for the P-glycoprotein transporter (Major Task1, Subtask 4):

As discussed above, P-glycoprotein (Pgp; ABCB1), a member of the ATP-binding cassette (ABC) superfamily, exports structurally diverse hydrophobic compounds from the cell driven by ATP hydrolysis. Activity of the test compound as a substrate for the Pgp transporter may be associated with poor CNS penetration. Even though it is anticipated that B-3(+) will be administered intravitreally, it is important to characterize this important drug like characteristic of the test compound. The assay was performed in two types of cells: MDCKII and in MDR1-MDCKII. MDR1-MDCKII cells are Madin-Darby canine kidney cells transfected with the MDR1 gene;

as a result, these cells express the high levels of human MDR1 gene that encodes the Pgp transporter. MDCKII represent untransfected Madin-Darby canine kidney strain II cells. Experiments in this cell line allow assessing permeability across epithelial barriers and estimating drug efflux. Cell were cultured on transwells. For MDR1-MDCKII experiments, compound working solution was be prepared by dilution of DMSO stock of B-3(+) or positive control with transport media to 10 µM. For A → B directional transport, the donor working solution (with B-3(+) or positive control, with or without Pgp inhibitor, verapamil) was added to the apical (A) compartment and the transport media as receiver working solution is added to the basolateral (B) compartment. For B → A directional transport, the donor working solution (with positive control or B-3(+), with or without Pgp inhibitor) is added to the basolateral (B) compartment and transport media as receiver working solution is added to the apical (A) compartment. A similar experimental setup, but without the use of the Pgp inhibitor, was used for conducting the study in MDCKII cells. At the end of the incubation, samples are taken from both donor and acceptor compartments, and compound levels were assessed by LC-MS/MS. The outcomes of the B-3(+) permeability study in MDCKII and in MDR1-MDCKII cells are shown in Figure 2.

Compound I.D.	Client Compound I.D.	Test Concentration	Special Condition	Permeability (10^{-6} cm/s)			Percent Recovery(%)		
				1st	2nd	Mean	1st	2nd	Mean
A-B permeability (MDCKII, pH 7.4/7.4)									
100057079-1	B-3(+)	1.0E-05 M		1.22	1.07	1.1	78	89	84
A-B permeability (MDR1-MDCKII, pH 7.4/7.4)									
100057079-1	B-3(+)	1.0E-05 M		0.77	0.92	0.8	89	82	85
A-B permeability (MDR1-MDCKII, pH 7.4/7.4 + verapamil)									
100057079-1	B-3(+)	1.0E-05 M		1.53	1.65	1.6	76	77	76
B-A permeability (MDCKII, pH 7.4/7.4)									
100057079-1	B-3(+)	1.0E-05 M		1.99	2.06	2.0	84	85	84
B-A permeability (MDR1-MDCKII, pH 7.4/7.4)									
100057079-1	B-3(+)	1.0E-05 M		2.01	2.04	2.0	85	84	85
B-A permeability (MDR1-MDCKII, pH 7.4/7.4 + verapamil)									
100057079-1	B-3(+)	1.0E-05 M		1.25	1.37	1.3	81	89	85

Figure 2. The outcomes of permeability testing for B-3(+) in MDCKII and MDR1-MDCKII cells

The outcomes of the control compound testing are summarized in Figure 3:

Compound I.D.	Test Concentration	Special Condition	Permeability (10^{-6} cm/s)			Percent Recovery(%)		
			1st	2nd	Mean	1st	2nd	Mean
A-B permeability (MDCKII, pH 7.4/7.4)								
Colchicine	1.0E-05 M		0.27	0.66	0.5	73	84	78
Labetalol	1.0E-05 M		13.08	14.09	13.6	86	92	89
Propranolol	1.0E-05 M		58.96	44.80	51.9	87	82	84
Ranitidine	1.0E-05 M		1.61	2.13	1.9	89	93	91
A-B permeability (MDR1-MDCKII, pH 7.4/7.4)								
Colchicine	1.0E-05 M		0.53	0.55	0.5	77	75	76
Labetalol	1.0E-05 M		11.15	10.74	10.9	91	91	91
Propranolol	1.0E-05 M		72.61	74.88	73.7	104	105	104
Ranitidine	1.0E-05 M		1.35	1.43	1.4	86	77	81
A-B permeability (MDR1-MDCKII, pH 7.4/7.4 + verapamil)								
Colchicine	1.0E-05 M		0.60	0.54	0.6	65	64	65
B-A permeability (MDCKII, pH 7.4/7.4)								
Colchicine	1.0E-05 M		1.77	2.23	2.0	87	85	86
Propranolol	1.0E-05 M		21.96	20.82	21.4	89	89	89
Ranitidine	1.0E-05 M		4.45	5.61	5.0	91	91	91
B-A permeability (MDR1-MDCKII, pH 7.4/7.4)								
Colchicine	1.0E-05 M		3.71	3.74	3.7	82	89	86
Labetalol	1.0E-05 M		26.49	29.30	27.9	96	96	96
Propranolol	1.0E-05 M		26.70	26.57	26.6	94	95	95
Ranitidine	1.0E-05 M		6.14	5.38	5.8	108	92	100
B-A permeability (MDR1-MDCKII, pH 7.4/7.4 + verapamil)								
Colchicine	1.0E-05 M		0.11	0.13	0.1	49	49	49

Figure 3. The outcomes of permeability testing for control compounds in MDCKII and MDR1-MDCKII cells

3. Interpretation of results:

According to the US FDA Draft Guidance for Industry on Drug–Drug Interaction Studies, for a test compound to be classified as a Pgp substrate, its net flux ratio (NFR), efflux ratio (ER) in MDR1–MDCK cells divided by the ER in parental (nontransfected) MDCK cells (ER′), must be at least 2.0.
The efflux ratio (ER) is calculated from the mean apical to basolateral (A-B) Papp data and basolateral to apical (B-A) Papp data as follows:

$$\text{Efflux Ratio} = \frac{P_{app(B-A)}}{P_{app(A-B)}}$$

In case of B-3(+), the efflux ratios are:

ER in nontrasfected MDCKII=1.76
ER in Pgp-transfected MDR1-MDCKII=0.82

As the ratio of ERs is 0.46 (below 2.0). it must be concluded that B-3(+) does not represent a Pgp substrate

C. Evaluation of systemic PK characteristics for B-3(+) in dogs (Major task 2).

1. Defining the study design (Major Task 2, Subtasks 1 and 2):

Evaluation of ocular pharmacokinetics in large animals, such as dogs, is required for a drug that is intravitreally (IVT) injected. The two objectives of dog PK studies are (1) to conduct evaluation of systemic pharmacokinetics (2) to perform the assessment of ocular pharmacokinetics in beagle dogs, a standard PK and safety species. The purpose of the systemic PK study is to evaluate pharmacokinetics parameters of the investigational test article B-3(+) in male Beagle dogs after a single standard oral (PO) (2 mg/kg) and a single intravenous (IV) (0.5 mg/kg) dose. The dog PK study was decided to be a crossover study to be performed in a total of 3 dogs. The systemic PK study consists of the oral gavage and intravenous administration legs. The drug is highly soluble in buffered saline, and dogs receive a single IV dose of test compound at 0.5 mg/kg or a single PO dose of a test compound of at 2 mg/kg. The dosing volume will be 4.0 ml/kg for the oral administration and 1.25 ml/kg for IV administration. Similar to a typical PK experiment, 3 animals per administration route will be used. The overview of the experimental plan for systemic PK study is shown below:

Group	Test Article	Dose Level (mg/kg)	Dose Conc. (mg/ml)	Dosing Volume (ml/kg)	Dose Route	No. of Animals	Blood Collection Time Point (hr)
1	B-3(+)	0.5	0.4	1.25	iv	3	Pre-dose, 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48
Washout (minimum 7 days							
2	B-3(+)	2	0.5	4.0	po	3	Pre-dose, 0.25, 0.5, 1, 2, 4, 8, 10, 12, 24, 36, 48

Each animal may be bled by any available vein (jugular, cephalic, or saphenous). Blood will not be collected from the vein in which the drug was administered. For the IV dose blood will be collected at pre- dose, 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36 and 48 hr. For the oral
dose blood will be collected at pre-dose, 0.25, 0.5, 1, 2, 4, 8, 10, 12, 24, 36 and 48 hr. A maximum of 2 ml of whole blood is collected at each timepoint. Animal protocols covering the dog PK studies were submitted to ACURO, and, following the revision, were approved allowing the study to begin.

2. Experimental implementation of B-3(+) testing in dog PK studies (Major Task 2, Subtasks 4, 6):

B-3(+), 0.5 mg/kg (concentration 0.4 mg/mL at a dose volume of 1.25 mL/kg) was administered to Leg 1 with a slow bolus for the intravenous administration. The intravenous dose was administered via the cephalic vein. The oral dose of 5.0 mg/kg (concentration 0.5 mg/mL at a dose volume of 10 mL/kg) was administered using an oral gavage tube followed by 10 mL flush with water. The animals were restrained per ASC SOPs.

Table 1. Study Design (Crossover)

Leg #	Test Article	Dose Route	N= Male	Dose (mg/kg)	Dose Conc. (mg/mL)	Dose Volume (mL/kg)	Vehicle	Blood Sampling Time Points
1	B-3(+)	IV (slow bolus)	3	0.5	0.4	1.25	5%Dextrose/ 0.45% Normal saline	Pre-dose, 5, 15, 30 min, 1, 2, 4, 8, 24 and 48 hours post dose
Minimum 7 Days Washout								
2	B-3(+)	PO	3	5	0.5	10	Water	Pre-dose, 15, 30 min, 1, 2, 4, 8, 24 and 48 hours post dose

Each blood sample (approx. 2 mL) was collected from the dog's cephalic vein in a K_2ETDA collection tube and gently inverted several time to mix. The samples were kept on ice until centrifugation at 4°C for 5 minutes at 3,000 x g. Approximately 1 mL of plasma was separated by centrifugation. The resulting plasma samples were stored at -80 C until bioanalysis was conducted.

All collected plasma samples were noted as normal and free of hemolysis.

Plasma samples were extracted and analyzed using the methods described in Appendix C. Individual and mean concentrations and pharmacokinetic parameters for plasma concentrations of B-3(+) are shown in Tables 5 - 6. All data are expressed as ng/mL of B-3(+). Samples that were below the limit of quantification (1.0 ng/mL in plasma) were excluded from the calculation of mean values.

3. Data analysis and interpretation of results (Major Task 2, Subtask 7) :

Body Weights and General Observations:

Body weights during the study are presented in Table 2:

Table 2. Body Weight (kg)

Leg	Animal ID	Weight
1	3909833	7.5
	3914268	11.5
	3902804	11.8
2	3909833	7.2
	3914268	11.9
	3902804	11.6

Most animals in Legs 1 and 2 were observed as normal for the duration. In Leg 1, mild salivation was noted in animal #3909833 shortly following dosing. Two of the Leg 2 animals (3909833 and 3902804) experienced emesis up to the 30 minute time point which resolved after this point.

Table 3 summarizes General Health Observations for tested animals (salivation and emesis).

Table 3. General Health Observations

Animal ID	Leg	Time Point	Observations
3909833	1	During dosing	Mild salivation
3909833	2	Immediately post dose	~15 mL emesis
3902804	2	One minute post dose	~10 mL clear emesis
3909833	2	15	~30 mL clear emesis and Retching
3902804	2	15	Retching
3902804	2	30	Retching

Dosing Solution Analysis:

The concentrations of the IV and PO dosing solutions for Legs 1 and 2 were analyzed by LC-MS/MS. The measured dosing solution concentration is shown in Table 4. The dosing solutions were diluted into blank beagle plasma and analyzed in triplicate. All concentrations are expressed as mg/mL of B-3(+). The nominal dose concentration was used in all calculations.

Table 4. Measured Dosing Solution Concentrations (mg/mL)

Test Article	Leg	Route of Administration	Nominal Dosing Conc. (mg/mL)	Measured Dosing Solution Conc. (mg/mL)	% of Nominal
B-3(+)	1	IV	0.4	0.360	90.0%
B-3(+)	2	PO	0.5	0.488	97.6%

Quantitative Plasma Sample Analysis:

Plasma samples were extracted and successfully analyzed. Individual and mean concentrations are shown in Tables 5 and 6. The LLOQ was established as 1 ng/mL for B-3(+) in plasma. Concentrations versus time data are plotted in Figures 4 - 7.

Pharmacokinetic Analysis:

Pharmacokinetic parameters were determined with Phoenix WinNonlin (v8.0) software using a non-compartmental model. Pharmacokinetic parameters calculated from the time course of the plasma concentration are presented in Tables 5 - 6.

Table 5. Pharmacokinetic Parameters and Plasma Concentrations (ng/mL) for B-3(+) after IV Administration (0.5 mg/kg, slow bolus) in Male Beagle dogs (Leg 1)

Leg 1, IV (0.5 mg/kg)					
Time Point (hr)	**Animal #**			**Mean**	**SD**
	3909833	3914268	3902804		
0 (pre-dose)	BLOQ	BLOQ	BLOQ	BLOQ	NA
0.0833	1582	1236	1294	1370	185
0.25	614	339	463	472	138
0.5	238	165	231	211	40.3
1	**131**	**90.6**	**118**	113	20.5
2	**56.0**	**52.1**	**40.9**	49.7	7.83
4	**33.3**	**36.6**	**27.3**	32.4	4.71
8	**1.95**	**9.26**	**1.08**	4.10	4.49
24	BLOQ	BLOQ	BLOQ	BLOQ	NA
48	BLOQ	BLOQ	BLOQ	BLOQ	NA
Dose (mg/kg)	0.500	0.500	0.500	0.500	NA
C_0 (ng/mL)	2538	2358	2163	2353	187
C_{max} (ng/mL)	3163	2471	2588	2741	370
t_{max} (hr)	0.0833	0.083	0.0833	0.0833	NA
$t_{1/2}$ (hr)	1.19	2.23	1.08	1.50	0.632
MRT_{last} (hr)	0.98	1.32	0.95	1.09	0.207
Cl_obs (L/kg/hr)	0.617	0.725	0.746	0.696	0.069
Vss_obs (L/kg)	0.627	1.27	0.727	0.87	0.346
AUC_{last} (hr·ng/mL)	807	660	669	712	82.3
AUC_{∞} (hr·ng/mL)	810	689	671	723	75.6
Dose-normalized Values[1]					
AUC_{last}/D (hr·kg·ng /mL/mg)	1613	1319	1338	1423	165
AUC_{∞}/D (hr·kg·ng /mL/mg)	1620	1379	1341	1447	151

C_{max}: maximum plasma concentration; t_{max}: time of maximum plasma concentration; $t_{1/2}$: half-life, data points used for half-life determination are in bold; MRT_{last}: mean residence time, calculated to the last observable time point; AUC_{last}: area under the curve, calculated to the last observable time point; AUC_{∞}: area under the curve, extrapolated to infinity. NA: not applicable; BLOQ: below the limit of quantitation (1 ng/mL). Data points in bold were used for half-life determination. Cl_obs: Clearance was calculated as Dose/AUC_{∞}. Vss_obs: Volume of distribution was calculated as MRT_{∞}*Cl. [1]Dose-normalized by dividing the parameter by the nominal dose in mg/kg.

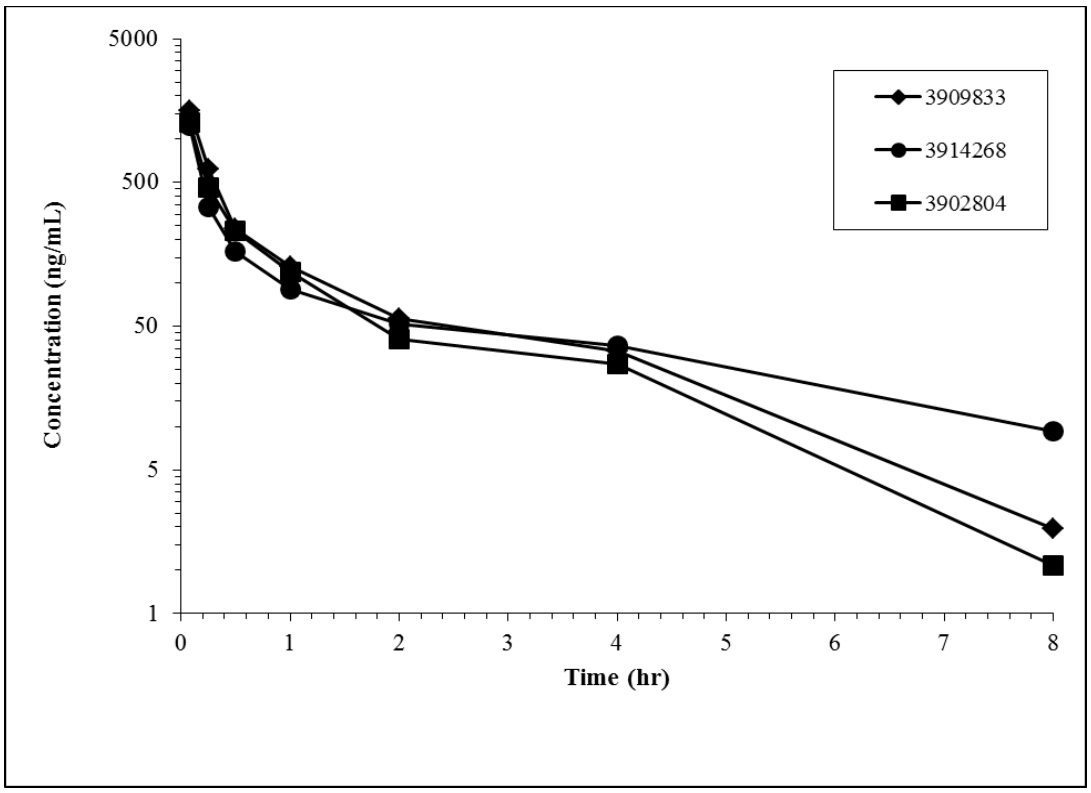


Figure 4. Individual Plasma Concentrations (ng/mL) of B-3(+) versus Time after IV Administration (0.5 mg/kg, slow bolus) in Male Beagle dogs (Leg 1)

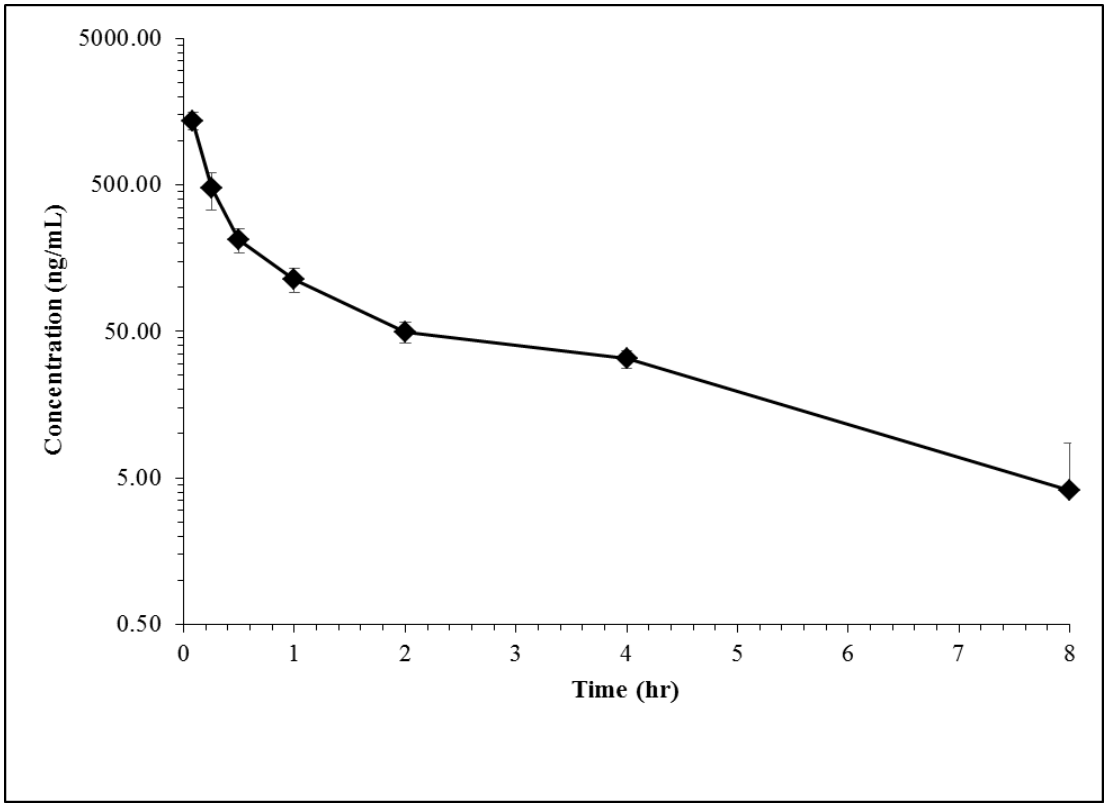


Figure 5. Mean Plasma Concentration (ng/mL) of B-3(+) versus Time after IV Administration (0.5 mg/kg, slow bolus) in Male Beagle dogs (Leg 1)

Table 6. Pharmacokinetic Parameters and Plasma Concentrations (ng/mL) for B-3(+) after PO Administration (5.0 mg/kg) in Male Beagle dogs (Leg 2)

Leg 2, PO (5 mg/kg)					
Time Point (hr)	**Animal #**			**Mean**	**SD**
	3909833	3914268	3902804		
0 (pre-dose)	BLOQ	BLOQ	BLOQ	BLOQ	NA
0.25	1069	1981	1279	1443	478
0.5	1276	2064	2251	1864	518
1	1193	2271	2381	1948	656
2	**1034**	1138	2417	1529	770
4	**627**	**309**	**547**	494	166
8	**37.2**	**142**	**25.0**	68.1	64.4
24	**2.84**	**5.62**	**8.35**	5.60	2.75
48	BLOQ	BLOQ	**1.30**	BLOQ	NA
Dose (mg/kg)	5.00	5.00	5.00	5.00	NA
C_{max} (ng/mL)	1276	2271	2417	1988	621
t_{max} (hr)	0.500	1.00	2.00	1.17	0.764
$t_{1/2}$ (hr)	2.67	3.45	ND[2]	3.06	0.556
MRT_{last} (hr)	2.81	3.08	2.64	2.84	0.221
AUC_{last} (hr·ng/mL)	5467	7070	8647	7061	1590
AUC_{∞} (hr·ng/mL)	5478	7098	ND[2]	6288	NA
Dose-normalized Values[1]					
AUC_{last}/D (hr·kg·ng /mL/mg)	1093	1414	1729	1412	318
AUC_{∞}/D (hr·kg·ng /mL/mg)	1096	1420	ND[2]	1258	NA
Bioavailability	75.7%	98.1%	NA	86.9%	NA

C_{max}: maximum plasma concentration; t_{max}: time of maximum plasma concentration; $t_{1/2}$: half-life, data points used for half-life determination are in bold; MRT_{last}: mean residence time, calculated to the last observable time point; AUC_{last}: area under the curve, calculated to the last observable time point; AUC_{∞}: area under the curve, extrapolated to infinity. NA: not applicable; BLOQ: below the limit of quantitation (1 ng/mL). Data points in bold were used for half-life determination. [1]Dose-normalized by dividing the parameter by the nominal dose in mg/kg. [2]Not determined because the line defining the terminal elimination phase had an r^2 of <0.85.

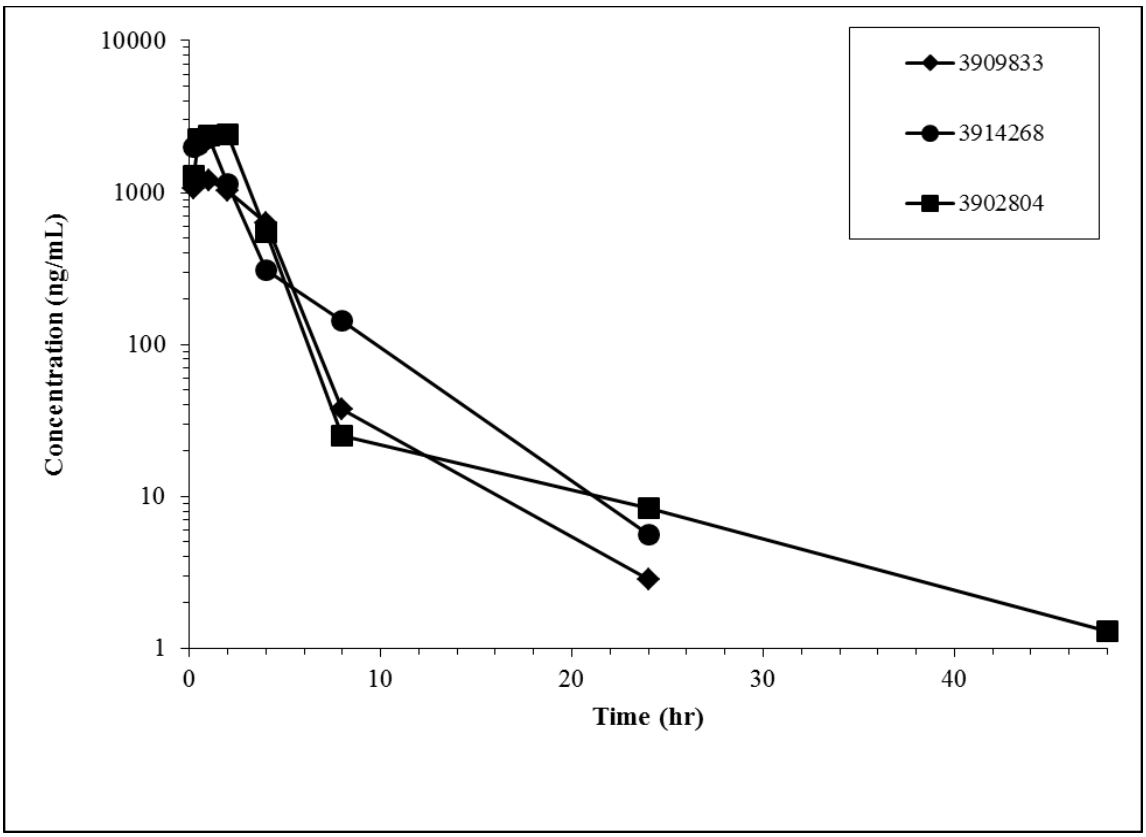


Figure 6. Individual Plasma Concentrations (ng/mL) of B-3(+) versus Time after PO Administration (5.0 mg/kg) in Male Beagle dogs (Leg 2)

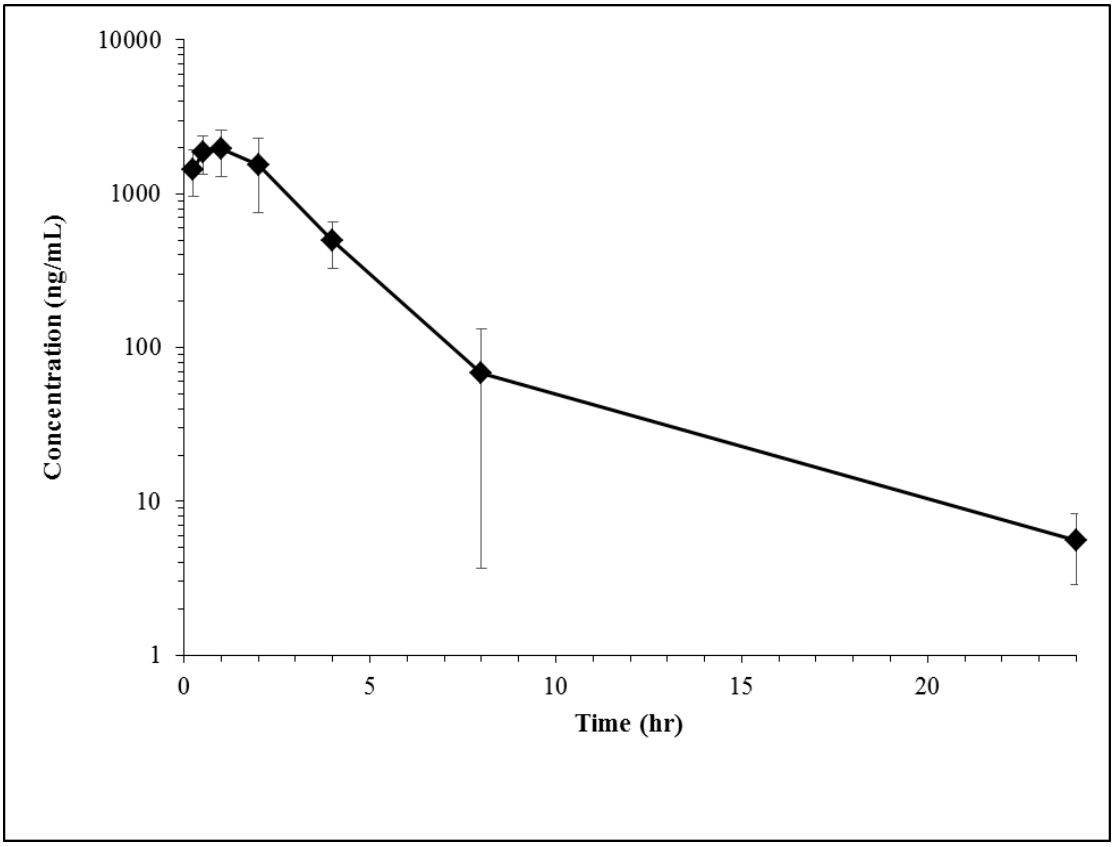


Figure 7. Mean Plasma Concentration (ng/mL) of B-3(+) versus Time after PO Administration (5.0 mg/kg) in Male Beagle dogs (Leg 2)

4. Summary on dog systemic PK data (Major Task 2, Subtask 7) :

In this study, the exposure of test article B-3(+) was evaluated in Male Beagle dogs following intravenous (IV) (slow bolus) dosing at 0.5 mg/kg in Leg 1 and Oral (PO) administration at 5 mg/kg in Leg 2 with a 7 days washout period in between each leg. A large majority of time points were observed as normal with no adverse effects from administration of the test article in both legs. In Leg 1, mild salivation was noted in one animal at the time of dosing. Two of the Leg 2 animals experienced emesis and retching up to the 30 minute time point which resolved after this time point.

Blood samples were collected up to 48 hours post dose. Plasma concentrations of B-3(+) were determined by LC-MS/MS. Pharmacokinetic parameters were determined using Phoenix WinNonlin (v8.0).

Table 7. Summary of Mean Pharmacokinetic Parameters for B-3(+) after 0.5 mg/kg IV and 5 mg/kg PO Administration in Male Beagle dogs

Summary	Leg 1 IV	Leg 2 PO
Dose (mg/kg)	0.500	5.00
C_{max} (ng/mL)	2741	1988
t_{max} (hr)	0.0833	1.17
$t_{1/2}$ (hr)	1.50	3.06
MRT_{last} (hr)	1.09	2.84
AUC_{last} (hr·ng/mL)	712	7061
AUC_{∞} (hr·ng/mL)	723	6288
Bioavailability	NA	86.90%

C_{max}: maximum plasma concentration; t_{max}: time of maximum plasma concentration; $t_{1/2}$: half-life; MRT_{last}: mean residence time, calculated to the last observable time point; AUC_{last}: area under the curve, calculated to the last observable time point; AUC_{∞}: area under the curve extrapolated to infinity. Bioavailability: calculated as the ratio of dose-normalized AUC_{∞} for PO/IV

Both legs gave well defined plasma versus time curves enabling the calculation of terminal elimination parameters of $t_{1/2}$ and AUC_{∞}, with the exception of Leg 2 animal # 3902804. In this instance the AUC_{last} was possible to calculate but the R^2 of the regression line defining the terminal elimination phase was less than 0.85. Per ASC SOP the resulting parameters of $t_{1/2}$ and AUC_{∞} for this animal were not calculated.

Leg 1 intravenous B-3(+) dosing of 0.50 mg/kg (0.4 mg/mL in 5% Dextrose/ 0.45% Normal saline), showed quantifiable plasma concentrations of B-3(+) to 8 hours. Mean T_{max} for this leg was 0.0833 hr corresponding to a mean C_{max} of 2741 ng/mL (370).

Leg 2 oral B-3(+) dosing of 5.00 mg/kg (0.5 mg/mL in water) showed quantifiable plasma concentrations of B-3(+) to 24 hours. Mean T_{max} for this leg was 1.17 hr (0.764) corresponding to a mean C_{max} of 1988 ng/mL (621). Overall bioavailability for B-3(+) calculated from the study was 86.9 % as calculated from the PO dose normalized AUC_{∞} of 1258 hr·kg·ng /mL/mg by the IV dose normalized AUC_{∞} of 1447 hr·kg·ng /mL/mg (151).

D. Define the range of efficacious drug concentrations in the animal model of optic nerve damage (Major task 3).

Drafting the animal protocols for conducting the required studies in guinea pigs (Major Task 3, Subtasks 1):

After several rounds of reviews, the animal protocol related to testing the drug candidate in the guinea pig model of optic nerve damage was successfully approved by the Columbia University IACUC committee. The protocol is being transferred to ACURO for their review and approval

What opportunities for training and professional development has the project provided?
Nothing to Report

How were the results disseminated to communities of interest?
Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
We will continue to implement our research plan as outlined in the Statement of Work in order to accomplish the study objectives

4. **IMPACT:**
We successfully completed all objectives of Major Task 1 over the period of Grant Year 2. We built a strong foundation for successful completion of Major Tasks 2 and 3 during the remaining grant period.

What was the impact on the development of the principal discipline(s) of the project?

We defined critical pharmacokinetic characteristics of the drug candidate in dogs. We completed the analysis of the drug candidate in all relevant in vitro ADME assays. Drug characteristics that we elucidated are fully compatible with the future use of B-3(+) as a therapy for TON.

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

A delay in drafting and reviewing the animal protocol was encountered due to the general slowing of business operations in the first half of 2021. The work on the protocols has resumed and we will attempt to complete all remaining animal studies outlined in the proposal in a timely fashion.

Changes that had a significant impact on expenditures
Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report

Significant changes in use or care of human subjects
Nothing to Report

Significant changes in use or care of vertebrate animals
Nothing to Report

Significant changes in use of biohazards and/or select agents
Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  - Nothing to Report

  Journal publications.
  Nothing to Report

  Books or other non-periodical, one-time publications.
  Nothing to Report

  Other publications, conference papers and presentations.
  Nothing to Report

- **Website(s) or other Internet site(s)**
  Nothing to Report

- **Technologies or techniques**
  Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Nothing to Report

- **Other Products**
  Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Konstantin Petrukhin, Ph.D.
Project Role:	PI/PD
Researcher Identifier (eRA Commons):	KEPET4
Nearest person month worked:	1.9 (16% effort)
Contribution to Project:	Dr. Petrukhin has designed experiments and analyzed the data
Name:	Boglarka Racz, Ph.D.
Project Role:	Associate Research Scientist
Researcher Identifier (eRA Commons):	BORACZ
Nearest person month worked:	1.9 (16% effort)
Contribution to Project:	Dr. Racz was responsible for implementation of studies and participated in data analysis
Name:	Jeffrey Liebmann, M.D.
Project Role:	Co-Investigator
Researcher Identifier (eRA Commons):	LIEBMANNCU
Nearest person month worked:	0.6 (5% effort)
Contribution to Project:	Dr. Liebmann contributed to designing experiments and analyzing the data

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

9. APPENDICES:

Nothing to Report