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EXPERIMENTAL THERAPEUTICS

Assessing the Safety, Tolerability, Pharmacokinetics, and Biodistribution of Novel Oral Formulations of Amphotericin B following Single- and Multiple-Dose Administration to Beagle Dogs

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ABSTRACT The purpose of this study was to assess the safety, tolerability, pharmacokinetics (PK), and biodistribution of novel oral amphotericin B (AmpB) formulations following single- and multiple-oral-dose administration to healthy beagle dogs. The liquid formulation of AmpB was administered to three male dogs, and the capsule formulations of AmpB were administered to each of two groups of six male dogs. Blood was collected for pharmacokinetic evaluation on days 1, 2, and 3 (up to 72 h postdosing). Dogs receiving the capsule formulations further received a single oral dose of 100 mg once daily for three more days, and on the 4th day, blood samples were taken at 24 h postdosing and the dogs were humanely sacrificed with the removal of organs, from which tissue samples were taken for analysis of the AmpB content. Multiple-dose studies were completed for 7 or 14 days with daily doses of up to 1,000 mg/day with the capsule formulations. All oral formulations of AmpB following both single- and multiple-dose administration were well tolerated in the dogs, and there were no relevant adverse signs observed, such as changes in hematologic, coagulation, or biochemistry parameters; loss of weight; changes in food or water intake; or signs of gastrointestinal distress. The oral absorption of AmpB from the liquid formulation and the capsule formulations were similar, with no significant differences. The tissue distributions of AmpB were similar following repeated doses of the two capsule formulations to dogs. Following 14 days of treatment with the iCo-010 liquid formulation and the iCo-019 and iCo-022 capsule formulations, the range of values of the maximum observed plasma concentration (C_{max}) was 53.2 to 62.3, 24.9 to 66.4, and 36.7 to 85.2 ng/ml, respectively; the range of values of the time to C_{max} was 4 to 12, 4 to 24, and 2 to 24 h, respectively; and the range of values of the area under the plasma concentration-time curve from time zero to the time of the last quantifiable concentration was 2,635 to 3,071, 1,053 to 2,517, and 1,443 to 3,713 ng · h/ml, respectively. We have developed a safe novel oral AmpB formulation suitable for future efficacy studies.

KEYWORDS oral amphotericin B, safety, tolerability, pharmacokinetics, systemic fungal infections, beagle dogs, GLP toxicity studies, amphotericin B, biodistribution, oral drug delivery

Amphotericin B (AmpB) is a polyene macrolide antibiotic that acts by binding to sterols in the plasma membranes of fungi, causing the cells to leak, eventually leading to fungal cell death (1, 2). Amphotericin B is indicated for the treatment of several fungal and parasitic infections, including leishmaniasis, invasive aspergillosis, blastomycosis, candidiasis, coccidiomycosis, cryptococcal meningitis, cryptococcosis,

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histoplasmosis, mucormycosis, sporotrichosis, and others (1, 2). Amphotericin B was approved by the FDA in 1971 and is currently widely used as the treatment of choice for serious fungal infections. It was first isolated from *Streptomyces nodosus* in 1955 and acts through binding to the sterol component of fungal cell membranes, leading to alterations in cell permeability and, ultimately, cell death (1, 2).

Amphotericin B has a higher affinity for ergosterol, which is found in the membranes of fungi and some protozoan parasites, and a lower affinity for cholesterol, which is found in mammalian cell membranes. However, this selectivity is not absolute, and some cytotoxicity toward mammalian cells is observed (3–7). Initial data from both cell lines and *in vivo* research indicate that it is highly efficacious within the dosage range required for the treatment of disseminated fungal infections and diseases, such as leishmaniasis (1, 2, 8–13).

Amphotericin B is insoluble in water and is formulated for intravenous (i.v.) use by complexing it with lipotrophic molecules, such as deoxycholate, liposomes, or lipid complexes (1, 2). Amphotericin B is available in multiple forms and concentrations generically and under the brand names Amphocin and Fungizone (deoxycholate), AmBisome (liposome), Abelcet (lipid complex), and Amphotec (cholesteryl sulfate complex) (2). The recommended dose varies by product and by disease entity; AmpB is given intravenously, and the usual dosage is up to 0.5 to 1.0 mg/kg of body weight daily. Many fungal infections require prolonged therapy, for 1 month or as many as 9 months (1, 2, 8–13).

Since all marketed forms of AmpB are administered i.v. (14–20), most of the toxicological evaluation to date has been completed with the i.v. route of administration. Lipid-based formulations have reduced toxicity, illustrated by acute toxicity studies in rodents which showed that liposomal complex AmpB (AmBisome) is dramatically less toxic than the conventional colloidal dispersion AmpB (Fungizone) or the other colloidal suspension AmpB (ABCD) and lipid complex AmpB (ABLC; AmBisome; Gilead). Toxicological studies in mice, rats, and dogs have shown prolonged exposure to i.v. AmpB to be nephrotoxic and locally irritating. Histopathology revealed that the kidney pelvis and the urinary bladder were target organs of toxicity. The lesions consisted of hyperplasia of the epithelium lining the renal pelvis and the bladder.

Amphotericin B is poorly tolerated in many patients and produces a range of toxic effects, some of which are immediate and some of which are of slower onset. The compound is currently administered as a slow infusion, and this can produce immediate side effects, including fever, chills, rigors, nausea, vomiting, hyperpyrexia, severe malaise, hypotension, thrombophlebitis, cardiac enlargement, anemia, and hepatitis. The reactions are most common in the first week of administration and vary considerably between patients, sometimes leading to the drug being discontinued. Adverse effects may diminish as therapy progresses, but in some patients, the dose has to be reduced or the therapy has to be discontinued. Nephrotoxicity is the commonest subacute adverse reaction seen with AmpB, with most patients suffering some impairment of renal function. This is common in those receiving more than 0.5 mg/kg/day AmpB. Kidney damage is indicated by rising serum creatinine and urea levels and is often accompanied by hypokalemia. Therapy often has to be interrupted or discontinued, and renal function may return to normal levels, but in some patients, even when therapy is discontinued, irreversible damage may occur.

AmpB administered intravenously at therapeutic doses has also been associated with multiple-organ damage. Kidney damage is a frequently reported side effect and can be severe and/or irreversible. Less kidney toxicity has been reported with liposomal formulations (such as AmBisome), and it has become preferred for patients with preexisting renal injury. In the liver, increased liver enzyme levels and hepatotoxicity (up to and including liver failure) are common. In the circulatory system, several forms of anemia and other blood dyscrasias (such as leukopenia and thrombocytopenia), serious cardiac arrhythmias (including ventricular fibrillation), and even cardiac failure have been reported. Skin reactions, including serious forms, are also possible.

To overcome these challenges, the development of an oral formulation of AmpB

TABLE 1 Study outline for the formulation comparison studies

Dose formulation	Administration route	AmpB dose	Dosing days	No. of dogs	Comments
iCo-019, 100 mg capsule (lot no. L268-01019)	Oral, single dose per day	One capsule of 100 mg per dog	1 and 4 to 6	6	A total of 4 doses were administered (PK were determined with plasma samples after dosing on days 1 and 24 h after 4th dose); tissue distribution was determined 24 h after 4th dose
iCo-022, 100 mg capsule (lot no. L268-01023)	Oral, single dose per day	One capsule of 100 mg per dog	1 and 4 to 6	6	Same as for the iCo-019 100 mg capsule
iCo-010, liquid formulation (5 mg/ml of AmpB)	Oral, single dose	20 ml by oral gavage (100 mg) per dog	1	3	PK were determined with plasma samples after dosing on day 1; no tissue distribution evaluation

that is cost-effective, easy to administer, and nontoxic yet that retains pharmacological activity and the ability to be stored at room temperature would be ideal (1, 2, 8, 9). To date, several oral formulations of AmpB have been investigated, but very few have advanced to clinical trials (21–38).

Oral AmpB was originally developed to address the challenges associated with the existing i.v. formulations in treating systemic fungal infections (14–20, 39) and individuals with visceral leishmaniasis (VL) (1, 2, 13) in the developing world. The rates of opportunistic fungal infections, such as candidiasis, histoplasmosis, and aspergillosis, are climbing, particularly for patients who have cancer, diabetes, or HIV infection/AIDS or who are organ transplant recipients. The ability to self-administer the drug for either treatment or maintenance of treatment for systemic fungal infections would significantly increase the quality of life of these patients and avoid the toxicity associated with intravenous administration. It would also increase the accessibility of this treatment in many geographical areas. Thus, showing the safety and tolerability of new oral AmpB formulations following single- and multiple-dose therapy in healthy beagle dogs is an important first step for its clinical development. Initial data from both cell line and *in vivo* research indicate that it is highly efficacious and exhibits low toxicity within the dosage range required for the treatment of diseases such as disseminated fungal infections and leishmaniasis (12, 13, 40, 41).

Our laboratories have developed a lipid-based self-emulsifying drug delivery system for AmpB to permit oral administration of this poorly bioavailable drug, with an additional aim of lessening its nephrotoxicity while maintaining optimal antifungal and antileishmanial activity (10–13, 40). An additional goal was to develop a stable formulation of AmpB that could withstand tropical temperatures and humidity (12, 40).

Based on the preliminary studies completed in our laboratories to date, the purpose of this study was to determine the safety, tolerability, pharmacokinetics (PK), and biodistribution of AmpB following a single oral dose and multiple oral doses of our novel oral AmpB formulations to beagle dogs.

RESULTS

Formulation comparison study. Oral administration of AmpB at a dose of 100 mg in all formulations (Table 1) was well tolerated by the dogs, and no relevant adverse clinical signs or changes in body weight were observed (Table 2). Following oral dosing with amphotericin B in three different formulations, the mean plasma levels of amphotericin B initially rose rapidly and in a similar manner (up to 2 h postdosing), and

TABLE 2 Summary of body weight and body weight changes

Dose formulation	No. of dogs	Mean body wt ± SD (kg)			Mean body wt change from days 1 to 7 ± SD (kg)
		Day 1	Day 4	Day 7	
iCo-010	3	10.6 ± 0.9	10.6 ± 0.9	10.7 ± 0.9	0.1 ± 0.1
iCo-019	6	10.7 ± 0.6	10.7 ± 0.7	10.8 ± 0.7	0.1 ± 0.2
iCo-022	6	10.8 ± 1.0	10.8 ± 1.1	10.9 ± 1/1	0.1 ± 0.2

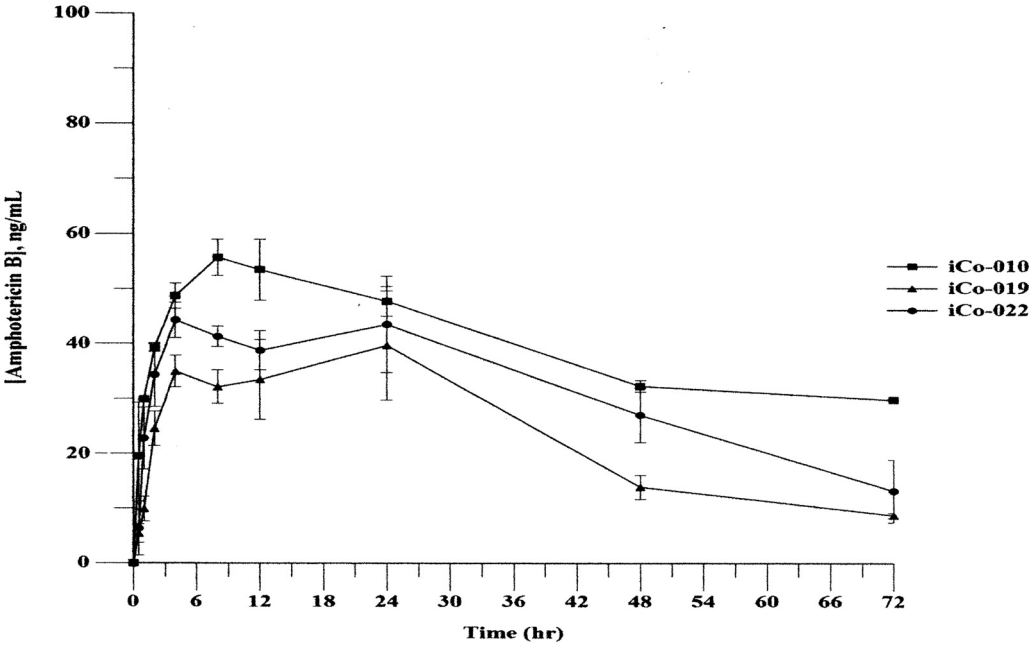

FIG 1 Mean plasma levels of amphotericin B following oral dosing with 100 mg of amphotericin B in three different formulations. The mean plasma levels of amphotericin B in dogs following oral dosing with 100 mg amphotericin B in the iCo-010, iCo-019, and iCo-022 formulations are shown. The data are the mean ± SE from three determinations for the formulation.

then they rose at a lower rate to attain a plateau (6 to 24 h postdosing) and dropped slowly thereafter, resulting in a large extrapolation of the area under the plasma concentration-time curve (AUC) from time zero to the time of the last quantifiable concentration ($AUC_{0-Tlast}$) to infinity (Fig. 1 and Table 3). The mean maximum observed plasma concentration (C_{max}), the time to C_{max} (T_{max}), and $AUC_{0-Tlast}$ values obtained with the three formulations were not significantly different from each other. for the iCo-010 liquid formulation and the iCo-019 and iCo-022 capsule formulations, the range of values of C_{max} was 53.2 to 62.3, 24.9 to 66.4, and 36.7 to 85.2 ng/ml, respectively; the range of values of T_{max} was 4 to 12, 4 to 24, and 2 to 24 h, respectively; and the range of values of $AUC_{0-Tlast}$ was 2,635 to 3,071, 1,053 to 2,517, and 1,443 to 3,713 ng · h/ml, respectively. The mean plasma terminal elimination half-life ($t_{1/2}$) for amphotericin B from the three different formulations ranged from 25.9 to 58.4 h (Table 3).

The distribution of AmpB among tissues and intestinal contents was similar following repeated dosing with amphotericin B in the iCo-019 and iCo-022 formulations. Gastrointestinal tissues and contents contained the highest levels, with intestinal content levels ranging from 1,938.8 to 3,106.6 ng/g (wet weight [w.w.]) of sample and

TABLE 3 Pharmacokinetic parameters of amphotericin B following oral dosing of 100 mg amphotericin B in three different oral formulations (iCo-010, iCo-019, iCo-022) in beagle dogs[a]

Formulation	T_{max} (h)	C_{max} (ng/ml)	$AUC_{0-Tlast}$ (ng · h/ml)	$t_{1/2}$ (h)	MRT-Last (h)
iCo-010 ($n = 3$)[b]	8.0 ± 2.3	57.4 ± 2.6	2,879 ± 128	58.4 ± 1.8	31.7 ± 0.4
iCo-019 ($n = 6$)	14.0 ± 4.5	46.4 ± 7.1	1,700 ± 291	25.9 ± 4.8	26.7 ± 5.0
iCo-022 ($n = 6$)	8.3 ± 3.3	52.5 ± 7.2	2,146 ± 369	55.3 ± 15.2	27.3 ± 1.8

[a]Data are presented as the mean ± standard error of the mean. Abbreviations; T_{max}, time to reach the maximum observed plasma concentration; C_{max}, maximum observed plasma concentration; $AUC_{0-Tlast}$, area under the concentration-time curve from time zero to the time of the last quantifiable concentration; $t_{1/2}$, terminal elimination half-life; MRT-Last, mean residence time to the last time point measured; *n*, number of beagle dogs.

[b]Data for all parameters are for 3 dogs each, except for $t_{1/2}$, for which data are for 2 dogs.

TABLE 4 Tissue levels of amphotericin B 24 h after 4th dose of either iCo-019 or iCo-022[a]

	iCo-019			iCo-022		
	Level (ng/g [w.w.]) in tissue sample			Level (ng/g [w.w.]) in tissue sample		
Tissue sample	Mean	SE	*Kp*1	Mean	SE	*Kp*1
Brain						
Cerebrum	2.9	1.1	0.05	4.0	1.2	0.08
Cerebellum	2.3	0.8	0.04	5.0	1.8	0.10
Medulla	3.6	0.9	0.06	2.8	0.6	0.05
Heart	0.0	0.0	0.00	2.5	0.7	0.05
Kidney						
Cortex	78.3	27.1	1.40	72.9	21.0	1.40
Medulla	93.8	49.5	1.67	157.8	40.0	3.03
Liver	25.4	10.4	0.45	30.9	7.9	0.59
Lung	7.2	2.3	0.13	8.7	1.8	0.17
Spleen	5.3	4.3	0.10	6.0	1.6	0.12
Testes	7.4	2.6	0.13	7.6	1.4	0.15
Mesenteric lymph node	42.9	14.6	0.77	21.7	4.8	0.42
Duodenum	69.3	32.2	1.24	69.0	31.7	1.33
Jejunum	533.7	174.9	9.52	382.1	256.9	7.34
Ileum	422.3	142.4	7.54	346.1	165.6	6.66
Colon	703.3	427.5	12.55	481.3	131.2	9.25
Intestinal contents	1,938.8	785.4	n.r.	3,106.6	1,235.9	n.r.

[a]The plasma concentrations of amphotericin B at 24 h following dosing with formulation prototypes iCo-019 and iCo-022 were 56.0 ± 6.9 ng/ml and 52.3 ± 4.6 ng/ml, respectively. Values are presented as the mean ± standard error of the mean for 6 dogs and included plasma concentrations above the limit of detection but below the limit of quantification and values above the upper limit of quantification; samples with no peak detected were included in the means as values of zero.
Abbreviations: n.r., not reported; Kp_1, tissue partition coefficient, which was calculated by dividing the mean tissue levels by the mean plasma levels of amphotericin B observed following repeated dosing with prototype formulations iCo-019 and iCo-022 and assuming that 1 g of tissue represents 1 ml of tissue volume.

with tissue levels and partition coefficients ranging from 69.0 to 703.3 ng/g (w.w.) of tissue and 1.24 to 12.55, respectively (Table 4).

Among nongastrointestinal tissues, the kidney cortex and medulla followed by the liver and mesenteric lymph node had the highest levels, with tissue levels and partition coefficients ranging from 21.7 to 157.8 ng/g (wt/wt) and 0.42 to 3.03, respectively. The remaining tissues had very low levels of amphotericin B, with tissue levels and partition coefficients ranging from 0.0 to 7.6 ng/g (wt/wt) and 0.00 to 0.17, respectively (Table 4).

In conclusion, oral dosing of the 100 mg AmpB contained in the iCo-010, iCo-019, and iCo-022 formulations was well tolerated by the dogs. The oral absorption of AmpB from iCo-010 (the liquid formulation) and iCo-019 and iCo-022 (the capsule formulations) was similar, with no significant differences between the formulation groups being noted for C_{max}, T_{max}, and $AUC_{0-Tlast}$. The tissue distribution of AmpB following dosing with iCo-019 and iCo-022 was similar, with the highest levels being found in gastrointestinal tissues, followed by the kidney, liver, and mesenteric lymph node; lower levels were observed in the lung, spleen, and testis, and very low levels were observed in regions of the brain and the heart.

Multiple-dose studies. (i) Seven-day treatment studies. The 7-day multiple-dose treatment study evaluated the toxicity and toxicokinetics following 7 days of twice-a-day repeated oral dosing of a novel capsule formulation of AmpB in beagle dogs and to determine the AmpB levels in several tissues of interest following repeated exposure of three dose levels of AmpB in a novel capsule formulation (Table 5). Three groups of beagle dogs were administered the test item at the following dose levels: 200, 600, and 1,000 mg/day.

All the animals gained body weight, and food consumption was normal for all animals over an 8-day observation period (Table 6). Clinical pathology parameters were normal for all dogs (Tables 7 to 9), but some of the clinical chemistry and hematology parameters were out of range for some animals, and this was also observed in the prestudy period; thus, these are incidental findings. Urinalysis results were normal for

TABLE 5 Summary of amphotericin B treatment schedule[a]

Treatment group[b]	Dose level (mg/day)	Dose level (mg/single dose)	No. of capsules for each single dose
Control	0	0	5
Low dose	200	100	1
Middle dose	600	300	3
High dose	1,000	500	5

[a]The frequency of dosing was twice daily with 12 h ± 0.5 h between doses for 7 consecutive days.
[b]Each group contained four dogs (two dogs of each sex). Controls were dosed with five capsules containing formulation ingredients without amphotericin B.

all animals in all groups at the end of the study, except for one female dog in the high-dose group, which had a larger amount of proteinuria, but histologically, no abnormal finding was noted in the kidney for this animal. No abnormal gross findings were observed in any of the dogs at necropsy. Organ weights were within the expected range for all animals. In liver and kidneys, a few background lesions were seen histologically in test and control group animals, but these findings were considered of no toxicological relevance.

As expected, on the first day of dosing with 200, 600, or 1,000 mg/kg/day of AmpB, T_{max} was longer after the first daily dose than after the second dose and C_{max} was higher after the second dose than after the first dose. The AUC after the second daily dose was higher than that after the first dose (Table 10). On the 7th day of dosing, predose levels of amphotericin B were measured in all dogs, and as a consequence, the AUC from time zero to 12 h (AUC_{0-12}) was higher following the 7th day of dosing than following the first day of dosing (Table 11). In general, the plasma levels for males and females of each group were comparable, despite the body weight differences. The highest tissue levels of amphotericin B were found in kidneys, especially in the kidney medulla. The tissue levels of amphotericin B appeared to be dose dependent (Table 12).

In conclusion, analysis of all generated data, including clinical observations, body weights, food consumption, clinical pathology, gross necropsy, organ weights, and histopathology (kidneys and liver), revealed no test item-related toxicity in dogs that were treated orally with amphotericin B at dose levels of up to 1,000 mg/day, equivalent to 120.5 to 161.3 mg/kg/day.

(ii) Fourteen-day treatment studies. The 14-day multiple-dose treatment study assessed the toxicity and toxicokinetics of AmpB when administered orally to dogs daily in a capsule formulation for a 14-day period. Three groups of dogs were dosed with AmpB supplied in gelatin capsules at dose levels of 200, 400, and 600 mg/day for 14 days. This study also assessed the progression or regression of any effects following a 14-day treatment-free recovery period in animals in the vehicle control and high-dose groups.

All dogs completed the 14-day treatment period and survived until the scheduled termination for necropsy. The primary clinical sign noted was soft feces, observed in

TABLE 6 Summary of body weights, body weight changes, and food consumption[a]

Treatment group/sex	Dose level (mg/day)	Mean body wt (kg) ± SD		Body wt change (kg) ± SD from days 1 to 7	Mean food consumption (kg) ± SD from days 1 to 7
		Day 1	Day 7		
Group 1/M	0	8.1 ± 0.4	8.6 ± 0.8	+0.4 ± 0.4	2.1 ± 0.2
Group 2/M	200	8.2 ± 0.1	8.8 ± 0.4	+0.6 ± 0.3	2.1 ± 0.2
Group 3/M	600	7.9 ± 1.0	8.2 ± 0.8	+0.3 ± 0.1	1.8 ± 0.3
Group 4/M	1,000	7.9 ± 0.8	8.3 ± 0.8	+0.4 ± 0.0	2.2 ± 0.6
Group 1/F	0	6.2 ± 0.6	6.4 ± 0.6	+0.3 ± 0.0	1.6 ± 0.0
Group 2/F	200	5.6 ± 0.8	5.8 ± 0.9	+0.2 ± 0.1	1.3 ± 0.2
Group 3/F	600	5.8 ± 0.1	6.2 ± 0.1	+0.4 ± 0.3	1.6 ± 0.4
Group 4/F	1,000	6.2 ± 0.2	6.3 ± 0.3	+0.2 ± 0.1	1.4 ± 0.0

[a]The frequency of dosing was twice daily with 12 h ± 0.5 h between doses for 7 consecutive days. Each group contained two dogs. Controls were dosed with five capsules containing formulation ingredients without amphotericin B. Abbreviations: M, male; F, female.

TABLE 7 Hematology data following iCo-019 dosing at 0, 200, 600, and 1,000 mg/day for 7 consecutive days[a]

	Value for the following dose and day:							
	Control		200 mg/day		600 mg/day		1,000 mg/day	
Parameter	Day −3	Day 8	Day −3	Day 8	Day −3	Day 8	Day −3	Day 8
RBC (10e12/liter)	6.83 ± 0.52	6.76 ± 0.64	7.07 ± 0.7	6.5 ± 0.7	6.5 ± 0.7	5.9 ± 0.6	6.9 ± 0.5	6.2 ± 0.4
HEMO (g/liter)	154 ± 9.4	151.8 ± 10.7	158 ± 16	147 ± 17	145 ± 13	131 ± 10	153.5 ± 12.9	139 ± 4.5
HEMA (%)	47.2 ± 2.6	46.8 ± 3.3	49.3 ± 5.3	45.8 ± 5.3	44.9 ± 3.7	40.9 ± 3.6	47.6 ± 3.8	42.5 ± 1.5

[a]Data are presented as the mean ± SD ($n = 4$ in each dosing group; 2 males and 2 females combined). Abbreviations: RBC, red blood cells; HEMO, hemoglobin concentration; HEMA, hematocrit.

one male administered the empty capsule, two females in the low-dose group, one female in the middose group, and three males in the high-dose group. Episodes of soft feces occurred on 1 to 2 occasions for the control, low-dose, and mid-dose groups and 3 to 4 occasions for the high-dose group. Other clinical signs of note included vomiting (observed in one male administered the excipient in a capsule), diarrhea (observed in two males in the high-dose group), and mucoid feces (observed in two males in the high-dose group). These sporadic findings are commonly observed in colony dogs, are considered incidental, and were unlikely to have been related to the test item treatment.

Animals in all dose groups showed minor body weight losses during the second week of treatment, and only the male high-dose group mean weight change (−0.1 kg) was statistically significant compared to that for the empty-capsule treatment group. This observation was not associated with any clinical-pathological findings, and it was considered to be related to the stress associated with multiple-capsule dosing and intensive handling.

Ophthalmological findings prestudy and at the end of treatment did not reveal any ophthalmological findings attributed to the treatment with the test item.

Evaluation of electrocardiograms (ECGs) and clinical pathology data (hematology, coagulation, serum chemistry, and urinalysis) did not reveal any findings clearly attributable to the test item.

Following dosing with iCo-019 on day 1, there was delayed absorption of amphotericin B, as amphotericin B was not found in plasma until 2 h postdosing in all but one dog receiving the low dose. Maximum plasma levels for individual dogs peaked at between 2 and 24 h on days 1 and 14 of dosing. iCo-019 administered to male and female dogs over the dose range of 200 to 600 mg/day resulted in the development of plasma concentrations of AmpB on day 1 and day 14 of dosing which did not display dose dependence (Fig. 2 and 3).

The mean $AUC_{0-Tlast}$ and C_{max} values were similar between male and female dogs, indicating a lack of sex-related differences in systemic exposure. In male dogs, the mean $AUC_{0-Tlast}$ ratio values for day 14 to day 1 ranged from 1.54 to 3.60 and the C_{max} ratio values ranged from 1.45 to 2.04, while in female dogs, the ranges were 1.29 to 1.78 and 1.27 to 1.75, respectively (Table 13).

There were no findings observed upon gross necropsy of the animals at the end of the treatment period or in the recovery period.

TABLE 8 Coagulation data following iCo-019 dosing at 0, 200, 600, and 1,000 mg/day for 7 consecutive days[a]

	Value for the following dose and day:							
	Control		200 mg/day		600 mg/day		1,000 mg/day	
Parameter	Day −3	Day 8	Day −3	Day 8	Day −3	Day 8	Day −3	Day 8
PT (s)	7.7 ± 0.3	7.9 ± 0.3	7.5 ± 0.4	8.1 ± 0.6	7.7 ± 0.6	8.1 ± 0.5	7.8 ± 0.3	8.0 ± 0.4
APTT (s)	21.4 ± 0.7	21.6 ± 1.1	19.9 ± 0.4	20.7 ± 1.0	22.2 ± 1.7	23.6 ± 2.9	20.9 ± 1.0	20.7 ± 2.2
Fibrinogen concn (mg/dl)	174 ± 37	169 ± 47	188 ± 38	161 ± 15	192 ± 38	187 ± 32	173 ± 34	178 ± 41

[a]Data are presented as the mean ± SD ($n = 4$ in each dosing group; 2 males and 2 females combined). Abbreviations: PT, prothrombin time; APTT, activated partial thromboplastin time.

TABLE 9 Biochemistry data (kidney and liver function) following iCo-019 dosing at 0, 200, 600, and 1,000 mg/day for 7 consecutive days[a]

	Value for the following dose and day:							
	Control		200 mg/day		600 mg/day		1,000 mg/day	
Parameter	Day −3	Day 8	Day −3	Day 8	Day −3	Day 8	Day −3	Day 8
BUN (mmol/liter)	4.0 ± 0.7	5.6 ± 0.3[b]	4.6 ± 1.1	6.3 ± 1.4	4.9 ± 1.5	6.8 ± 1.7	3.9 ± 1.2	6.3 ± 1.1[b]
CREA (μmol/liter)	46 ± 5	50 ± 3	53 ± 7	54 ± 6	47 ± 5	52 ± 6	48 ± 9	51 ± 6
AST (U/liter)	37 ± 6	39 ± 4	32 ± 6	40 ± 4	33 ± 7	37 ± 3	41 ± 11	47 ± 5
ALT (U/liter)	51 ± 9	48 ± 7	42 ± 3	42 ± 4	42 ± 3	43 ± 6	45 ± 2	44 ± 7

[a]Data are presented as the mean ± SD ($n = 4$ in each dosing group; 2 males and 2 females combined). Abbreviations: BUN, blood urea nitrogen; CREA; serum creatinine; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
[b]$P < 0.05$ versus day −3.

At the end of the treatment period, the mean thymus weights of males receiving the middose and the high dose were significantly lower ($P < 0.05$) than those of the control animals administered the empty capsules. The mean male thymus/body weight ratios were also significantly lower in the middose group ($P < 0.05$) and the high-dose group ($P < 0.01$) than in the control group.

At the end of the treatment period, the mean pituitary gland weight of females receiving the high dose was significantly heavier ($P < 0.05$) than that of the control animals administered the empty capsules.

There were no histological findings that were clearly toxicologically significant in any of the animal tissues scheduled to be examined at day 15 in the main study or at day 29 in the recovery study. Notably, target organ adverse responses to amphotericin B were not observed in the kidneys or liver. Minor conditions observed in the kidneys, namely, focal mineralization in medullary tubules, focal basophilia in cortical tubules, or focal lymphocytic infiltrates in the cortex, were observed in some animals in the control and test groups. Thus, only background changes were observed in kidneys in this study, and their severity and frequency were unrelated to amphotericin B for the animals in the study.

Administration of amphotericin B at dose levels of up to 600 mg/day for 14 days was well tolerated by the young male and female beagle dogs used in this study. The no-observed-adverse-effect level (NOAEL) was considered to be 600 mg per day (equivalent to 60.0 to 93.8 mg per kg per day).

DISCUSSION

As we discussed in our recent reviews (1, 2), AmpB is a polyene macrolide antibiotic administered intravenously in the treatment of a variety of systemic fungal infections, including candidiasis, aspergillosis, fusariosis, and zygomycosis (14). In addition, AmpB has exhibited antiparasitic activity for certain protozoan infections, including leishmaniasis as well as primary amoebic meningoencephalitis (15). Prior to the development of lipid-based formulations, the commercially available formulation used in the clinic was Fungizone, a conventional micellar form of AmpB in a complex with deoxycholate (16). Unfortunately, the conventional form is associated with renal toxicity, which led to the development of other nonconventional formulations (17). Nonconventional or lipid-

TABLE 10 Day 1 toxicokinetic parameters[a]

	Value after:					
	First dose			Second dose		
Dose (mg/day)	C_{max} (ng/ml)	T_{max} (h)	AUC_{0-12} (ng · h /ml)	C_{max} (ng/ml)	T_{max1} (h)	AUC_{12-48} (ng · h /ml)
200	45.3 ± 10.4	9.0 ± 3.5	372.0 ± 47.5	63.3 ± 16.9	4.5 ± 1.9	621.0 ± 161.5
600	56.0 ± 17.5	10.0 ± 4.0	441.0 ± 103.0	83.3 ± 5.0	6.0 ± 0.0	789.3 ± 81.1
1,000	69.6 ± 12.4	12.0 ± 0.0	539.5 ± 88.6	91.6 ± 20.6	6.0 ± 0.0	880.0 ± 142.0

[a]Data are presented as the mean ± SD ($n = 4$ in each dosing group; 2 males and 2 females combined). Abbreviations: T_{max}, time to reach the maximum observed plasma concentration; T_{max1}, time from administration of the second dose; C_{max}, maximum observed plasma concentration; AUC_{0-12}, area under the concentration-time curve from time zero to 12 h; AUC_{12-48}, area under the concentration-time curve from 12 h to 48 h; $t_{1/2}$, terminal elimination half-life; n, number of dogs.

TABLE 11 Day 7 toxicokinetic parameters[a]

Dose mg/day	Value after: First dose C_{max} (ng/ml)	First dose T_{max} (h)	First dose AUC_{0-12} (ng · h /ml)	Second dose C_{max} (ng/ml)	Second dose T_{max1} (h)	Second dose AUC_{12-48} (ng · h /ml)
200	66.5 ± 21.6	5.0 ± 1.2	691.3 ± 244.6	61.9 ± 21.0	3.8 ± 2.1	1,242.3 ± 679.2
600	105.1 ± 15.7	1.0 ± 2.0	992.3 ± 157.9	96.2 ± 16.2	4.8 ± 2.5	2,418.5 ± 223.4
1,000	99.7 ± 11.2	3.0 ± 6.0	952.5 ± 87.6	88.6 ± 5.7	0.5 ± 1.0	2,063.5 ± 73.9

[a]Data are presented as the mean ± SD ($n = 4$ in each dosing group; 2 males and 2 females combined). Abbreviations: T_{max}, time to reach the maximum observed plasma concentration; T_{max1}, time from administration of the second dose; C_{max}, maximum observed plasma concentration; AUC_{0-12}, area under the concentration-time curve from time zero to 12 h; AUC_{12-48}, area under the concentration-time curve from 12 h to 24 h; $t_{1/2}$, terminal elimination half-life; *n*, number of dogs.

based formulations have been developed to overcome some of the toxicity problems associated with the conventional formulation. There are several lipid-based parenteral formulations which have been marketed to treat fungal infections. These include the liposomal formulation AmBisome, the lipid complex formulation Abelcet, and a colloidal dispersion formulation, Amphocil (Amphotec) (18–20). More recently, an emulsion form of AmB (Amphomul) was developed and completed its phase III clinical trial in 2014 (39). The aim of this trial was to assess the safety and efficacy of the parenteral lipid emulsion formulation compared to those of AmBisome as a single-infusion treatment for VL (39). However, its use has been limited by dose-dependent nephrotoxicity and the need for parenteral administration (1, 2, 8, 9), which may be inaccessible to many; its expense; the difficulty with its administration to patients, requiring appropriate medical personal and sterile conditions; and the lack of formulation stability under nonrefrigerated conditions.

To overcome these challenges, the development of an oral formulation of AmpB that is cost-effective, easy to administer, and nontoxic yet that retains pharmacological activity and the ability to be stored at room temperature would be ideal (1, 2). However, to date, few oral formulations of AmpB have been developed (21–37).

In this study, we report that oral dosing of three novel formulations of AmpB following single and multiple doses for up to 14 days was well tolerated in dogs. The oral absorption of AmpB from the iCo-010 liquid formulation and the iCo-019 and iCo-022 capsule formulations were similar, with no significant differences between the formulation groups for C_{max}, T_{max}, and $AUC_{0-7last}$. The tissue distribution of AmpB following dosing with iCo-019 and iCo-022 was similar, with the highest levels being found in gastrointestinal tissues, followed by the kidney, liver, and mesenteric lymph

TABLE 12 Tissue levels of amphotericin B in male and female beagle dogs[a]

Dose (mg/day) and tissue	Tissue concn (ng/g)
200	
Kidney cortex	75.4 ± 44.5
Kidney medulla	205.9 ± 105.9
Spleen	10.3 ± 7.2
Liver	32.8 ± 16.1
Mesenteric lymph node	30.2 ± 17.9
600	
Kidney cortex	291.0 ± 5.4
Kidney medulla	564.8 ± 97.3
Spleen	19.5 ± 3.3
Liver	107.8 ± 3.0
Mesenteric lymph node	68.0 ± 31.9
1,000	
Kidney cortex	224.0 ± 51.4
Kidney medulla	562.8 ± 123.5
Spleen	22.9 ± 2.5
Liver	96.5 ± 12.4
Mesenteric lymph node	84.0 ± 2.0

[a]Data are presented as the mean ± SD ($n = 4$ in each dosing group; 2 males and 2 females combined).

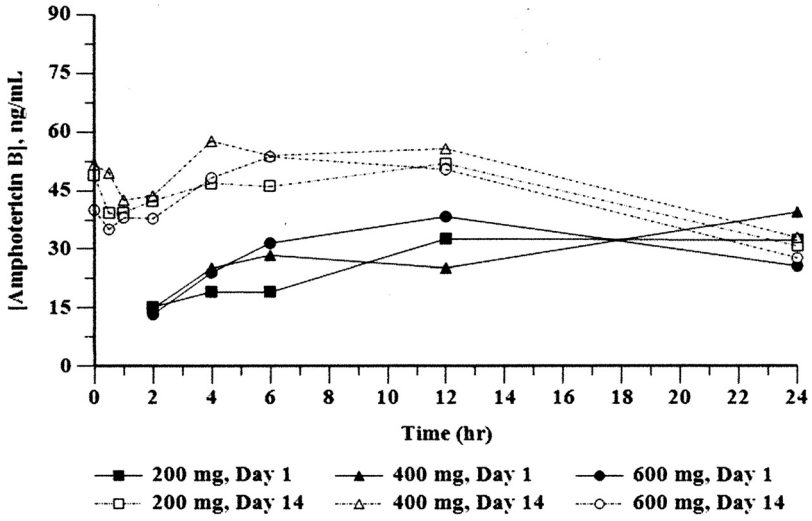

FIG 2 Plasma levels of amphotericin B following dosing on day 1 and day 14 in male dogs.

node; lower levels were observed in the lung, spleen, and testis, and very low levels were observed in regions of the brain and heart.

Studies comparing our oral formulations of amphotericin B to parenteral formulations of amphotericin B have been completed and have demonstrated the similar efficacy of the different formulations (11–13). Amphotericin B is both amphiphilic and amphoteric, which contributes to its unusual pharmacokinetic and biodistribution profile. We have previously reviewed the specific pharmacokinetic and pharmacodynamic properties of amphotericin B (1, 2, 8, 9) which lead to its feature of accumulating in tissues upon multiple dosing. The total tissue exposure, i.e., AUC, and the tissue depot effect, rather than C_{max} or absolute bioavailability, are the parameters critical for its pharmacological efficacy, with the absolute bioavailability estimated to be approximately 2 to 3% for the oral AmpB formulations tested in this study. This pharmacokinetic profile and the tissue distribution are different from those of parenteral AmpB formulations, such as liposomal AmpB. Thus, C_{max} alone is not the most important parameter for assessing the potential for efficacy at the level of infected tissues. The data presented here demonstrate a prolonged half-life and an AUC consistent with

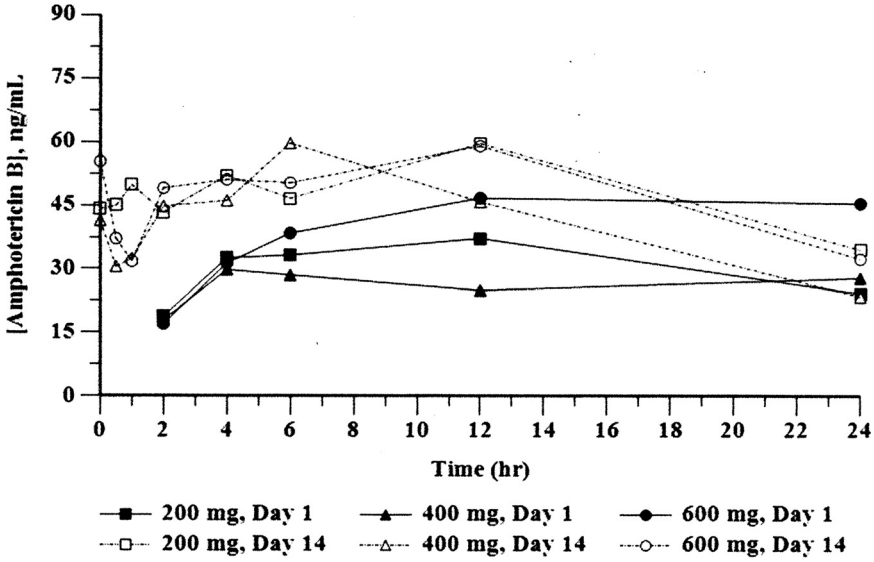

FIG 3 Plasma levels of amphotericin B following dosing on day 1 and day 14 in female dogs.

TABLE 13 C_{max}, T_{max}, and $AUC_{0-Tlast}$ following dosing with iCo-019 at doses of 200, 400, and 600 mg/day on days 1 and 14 in male and female beagle dogs[a]

Dose level (mg/day) and dosing day	C_{max} (ng/ml)	T_{max} (h)	$AUC_{0-Tlast}$ (ng · h/ml)
200 ($n = 6$)			
Day 1	38.8 ± 5.1	13.7 ± 8.6	672.5 ± 107.3
Day 14	58.9 ± 15.2	9.7 ± 3.7	1,114.5 ± 272.7[b]
400 ($n = 6$)			
Day 1	35.0 ± 14.0	10.7 ± 7.3	580.2 ± 337.3
Day 14	59.5 ± 16.9	5.0 ± 1.1	1,085.5 ± 234.4
600 ($n = 10$)			
Day 1	45.3 ± 11.7	13.2 ± 6.2	823.6 ± 244.8
Day 14	58.7 ± 11.2	8.4 ± 4.0	1,103.1 ± 213.1

[a]Data are presented as the mean ± SD ($n = 6$ for the 200- and 400-mg dosing groups [3 males and 3 females combined]; $n = 10$ for the 600-mg dosing group [5 males and 5 females combined]). Abbreviations: T_{max}, time to reach the maximum observed concentration; C_{max}, maximum observed plasma concentration; $AUC_{0-Tlast}$, area under the concentration-time curve from time zero to the time of the last quantifiable concentration; $t_{1/2}$, terminal elimination half-life; *n*, number of dogs.
[b]$P < 0.05$ versus day 1.

extended tissue exposure. The AUC calculated for the subjects treated with our oral AmpB formulations was also greater than that achieved by an alternative oral AmpB formulation, cochleate AmpB, tested in recent human clinical trials. A phase I human trial was recently completed for iCo-019 and iCo-022 and showed a pharmacokinetic profile similar to that presented here, along with excellent safety and tolerability results (42). Therefore, efficacy can be expected to be achievable at the doses used in the present study. A limitation of this investigation is that we were not able to do efficacy studies in beagles. Future studies highlighting the efficacy/toxicity ratio of our formulations will be completed.

Taken together, these data suggest that we have developed a novel oral AmpB formulation that is safe and tolerable following the administration of single and multiple doses to beagle dogs. In addition, the prolonged plasma half-life and increased AUC observed in both the single-dose and multiple-dose studies suggests that iCo-019 has a long circulation time, which may result in the ability of the formulation to increase and sustain amphotericin B tissue concentrations within infected tissues without the associated gastrointestinal, liver, and kidney toxicity. These characteristics make this formulation a suitable candidate for future efficacy studies.

MATERIALS AND METHODS

Experimental design. (i) Formulation comparison study. The formulation comparison study was designed to determine the pharmacokinetics of AmpB following a single oral dose (100 mg) in a liquid formulation (the iCo-010 formulation) and from two capsule formulations (the iCo-19 and iCo-022 formulations) of AmpB and the tissue distribution of AmpB 24 h following 3 days of administration of a single oral dose per day of the capsule formulations of AmpB in beagle dogs (Table 1).

The liquid formulation of AmpB (iCo-010) was administered to three male dogs, and the capsule formulations of AmpB (iCo-019 and iCo-022) were administered to each of two groups of six male dogs. Blood was collected for pharmacokinetic evaluation on days 1, 2, and 3 (up to 72 h postdosing). Dogs receiving the capsule formulations further received a single oral dose of 100 mg once daily for three more days, and on the 4th day, blood samples were taken at 24 h postdosing and the dogs were humanely sacrificed with the removal of the following organs, from which tissue samples were taken for analysis of the AmpB content: brain (cerebrum, cerebellum, medulla), heart, kidney (cortex and medulla), liver, lung, spleen, testes, mesenteric lymph node, and gastrointestinal tract sections (duodenum, jejunum, ileum, and colon). A sample of the intestinal contents was also collected. Amphotericin B plasma and tissue concentrations were determined by a validated liquid chromatography-mass spectrometry (LC-MS) assay, and pharmacokinetic noncompartmental analysis was completed using WinNonlin software (Certara, Inc.). The tolerability of the formulations within each dog was assessed by measuring changes in body weight and food and water intake and by monitoring for any signs of gastrointestinal disturbances, such as vomiting or diarrhea.

(ii) Multiple-dose studies. *(a) Seven-day treatment studies.* The 7-day treatment study evaluated the toxicity and toxicokinetics of AmpB following 7 days of twice-daily repeated oral dosing of a novel capsule formulation of AmpB in beagle dogs (41, 43, 44) and determined the AmpB levels in several

tissues of interest following repeated exposure to three dose levels of AmpB in a novel capsule formulation.

Three groups of beagle dogs were administered the test item at the following dose levels: 200, 600, and 1,000 mg/day. The dose was administered twice per day (roughly 12 h apart) via capsules, with each capsule containing 100 mg AmpB. The dogs weighed between 7.2 and 8.6 kg for males and 5.0 to 6.6 kg for females at the start of treatment. Over the 7-day dosing period (using the body weights at the start and end of dosing), this equated to doses of 22.7 to 35.7 mg/kg, 73.2 to 103.4 mg/kg, and 120.5 to 161.3 mg/kg for dose levels of 200, 600, and 1,000 mg/day, respectively. Each test item group consisted of 2 male dogs and 2 female dogs. A control group (2 males and 2 females) was included and was administered capsules containing only the excipient ingredients used in the test item formulation.

Clinical pathology and toxicokinetic evaluations were performed prior to necropsy. At necropsy, a comprehensive list of organs was collected, weighed, and preserved. The liver and kidneys were examined histologically. Tissue levels of AmpB were measured in the kidney (cortex and medulla), spleen, liver, and mesenteric lymph nodes.

(b) Fourteen-day treatment studies. The 14-day treatment study assessed the toxicity and toxicokinetics of AmpB when administered orally to dogs daily in a capsule formulation for a 14-day period. This study also assessed the progression or regression of any effects following a 14-day treatment-free recovery period in animals in the vehicle control and high-dose groups.

Twenty-one male dogs and 21 female dogs (Ridglan Farms Inc., Mt. Horeb, WI, USA) ages 7 to 9 months were acclimated for 14 days prior to the start of treatment. During the acclimation period, the dogs underwent physical examinations, fecal flotation analysis, ophthalmological examinations, electrocardiogram (ECG) and blood pressure measurements, clinical pathology evaluations, and measurement of body weights and food consumption. Following the prestudy evaluations, the dogs were randomized to three test groups and two control groups.

Three groups of dogs were dosed with AmpB supplied in gelatin capsules at dose levels of 200, 400, and 600 mg/day for 14 days. The dogs administered the test item weighed between 7.3 and 10.0 kg for males and 6.4 and 7.5 kg for females at the start of treatment. Over the 14-day dosing period, this equated to doses of 24.4 to 82.2 mg/kg/day for males and 29.4 to 93.8 mg/kg/day for females (determined using day 1 body weights for each group). A control group of dogs was included in the study and was dosed with empty gelatin capsules that were the same type of capsules used in preparing the test item for dosing. A vehicle-control group of dogs was also included in the study and was dosed with capsules containing only the excipient ingredients used in the test item.

During the treatment period, all animals were observed twice daily. Animals also underwent detailed physical examinations on a weekly basis. Body weights were recorded weekly, and food consumption was recorded daily. Ophthalmoscopy was performed at the end of the treatment period (day 13). ECGs and blood pressure were measured for all animals on day 13. Blood was collected from all animals for clinical pathology evaluations at the end of the treatment period and prior to necropsy for recovery animals. Urine was collected before the study and at the end of the treatment and recovery periods. Blood samples were collected on days 1 and 14 before dosing and at 0.5, 1, 2, 4, 6, 12, and 24 h postdosing for the evaluation of amphotericin B levels in plasma. At the end of the treatment period, the animals were euthanized and submitted for gross necropsy, and the major organs were weighed. Recovery animals were euthanized and submitted for gross necropsy, and the major organs were weighed at the end of the 14-day recovery period. Histopathological examinations were performed on a comprehensive range of tissues from all animals.

Amphotericin B oral formulation. Amphotericin B was formulated into hard-shell capsules which contained 100 mg of AmpB incorporated with a proprietary blend of mono- and diglycerides, in addition to D-alpha-tocopheryl poly(ethylene glycol) succinate (vitamin E–TPGS). The physical chemical properties of each of these components have been discussed previously (10–13, 45).

Animal husbandry. Beagle dogs from Nucro-Technics' animal colony were used for this study (species, *Canis familiaris*; strain, beagle [Hsd Rdg: Dobe Harlan]; source, Ridglan Farms Inc., Mt. Horeb, WI, USA; body weight, 10.0 to 12.0 kg at the start of dosing; age, 8 to 9 months at the start of dosing). Each animal was uniquely identified by the supplier using a tattoo on the inner surface of one ear. Animals were housed individually in tandem dog cages (0.91 square meters). The animal number and group number appeared on a card attached to the outside of each cage. The animal room environment was controlled (targeted ranges were a temperature of 18 to 29°C, s relative humidity of 30 to 70%, and greater than 15 air changes/hour) and monitored. The photocycle was 12 h of light and 12 h of dark.

Diet/water. Teklad certified lab dog diet (catalog number 8727C) was offered to the dogs once a day during a 4- to 6-h feeding period throughout the study period. Municipal water provided through automatic valves was available *ad libitum*.

Oral administration. For oral administration of the test item in capsules, the capsules were placed on the back of the tongue, followed by the administration of approximately 10 ml of tap water. The oral cavity was examined to ensure that the capsule was swallowed. For oral administration of the test item as a liquid, the test item was administered to dogs via a gastric tube, followed by the administration of approximately 10 ml of tap water.

Mortality. Mortality checks were performed and documented twice daily during the study period.

Clinical evaluations. Cage-side clinical evaluations were conducted twice. In addition, the animals were closely monitored for 1, 2, and 4 h after each dosing. Detailed clinical examinations were conducted pretreatment. Elements of observation included the reaction to treatment, such as changes in skin, fur, eyes, and mucous membranes. Respiratory, circulatory, autonomic, central nervous system, and somatomotor activity and behavior patterns, along with any other signs of ill health, were also monitored.

Clinical signs were recorded once a day during the morning observation period. If the observations made in the afternoon differed from those made in the morning, they were also recorded. Additional observations were recorded as they were observed. Animals judged to be abnormal were examined by a veterinarian or a technician working under the supervision of a veterinarian.

Body weights. Body weights were recorded at pretreatment and on days 1, 4, and 7.

Blood sampling for pharmacokinetics. Blood samples were collected from all animals following the first dose and also at 24 h following the last dose of the repeated-dose phase.

For the purpose of collection of the samples indicated above, each animal was bled from the jugular vein. Each blood sample (approximately 2 ml) was collected into a Vacutainer tube containing an anticoagulant (K_2EDTA). The time of collection (the actual time, in conjunction with the day and time of dosing) was recorded for each sample.

Following collection, the blood was placed in a refrigerated centrifuge for 20 min at 2,000 rpm in order to separate the plasma. The recovered plasma was stored in duplicate vials and frozen (at -80 ± 10°C) pending analysis. For each step, in the preparation of plasma, the samples were, as much as possible, protected from ambient light.

Following the final blood collection time for group 3, each animal was returned to the Nucro-Technics' colony.

On day 7, 24 h following the last of four repeated doses and after taking the last blood sample, dogs from groups 1 and 2 were euthanized using an overdose of sodium pentobarbital, administered intravenously, followed by exsanguination. As quickly as possible following exsanguination, duplicate samples (approximately 1 g, with exception of the mesenteric lymph node) of the following tissues and intestinal contents were collected by necropsy and snap-frozen for determination of the distribution of AmpB: brain (cerebrum, cerebellum, medulla), heart, kidney (cortex and medulla), liver, lung, spleen, testes, mesenteric lymph node, gastrointestinal tract tissues (duodenum, jejunum, ileum, and colon), and intestinal contents.

Pharmacokinetic analysis. Plasma concentration-time data were analyzed by the noncompartmental method, using validated Phoenix WinNonlin (version 6.3) software (Pharsight Corp.), to obtain the pharmacokinetic parameters.

The following pharmacokinetic parameters were calculated: $AUC_{0-Tlast}$, area under the plasma concentration-time curve from time zero to the time of the last quantifiable concentration (time T_{last}), calculated using the linear trapezoidal rule; $AUC_{0-\infty}$, the area under the plasma concentration curve from time zero extrapolated to infinity, where $AUC_{0-\infty}$ was calculated as $AUC_{0-Tlast} + (C_{last}/k_{el})$, where C_{last} is the last quantifiable concentration and k_{el} is the elimination rate constant; C_{max}, the maximum observed plasma concentration; T_{max}, the time of the maximum observed plasma concentration determined from the nominal time of blood sampling; k_{el}, the elimination rate constant, where k_{el} was estimated using linear regression on the terminal phase of the semilogarithmic concentration-time curve and a minimum of three data points were used for the calculation of k_{el} (no weighting was applied to the regression line); $t_{1/2}$, terminal elimination half-life, calculated from $\ln(2)/k_{el}$; MRT-Last, the mean residence time to the last time point measured, calculated from the ratio of the area under the first moment curve (AUCM) from sampling time zero to the last sampling time to $AUC_{0-Tlast}$ ($AUCM_{0-Tlast}/AUC_{0-Tlast}$); and MRTobs, the observed mean residence time, calculated from the ratio of the area under the first moment curve from sampling time zero to infinity to the AUC from time zero to infinity ($AUCM_{0-\infty}/AUC_{0-\infty}$).

In cases where the extrapolation of the $AUC_{0-Tlast}$ to $AUC_{0-\infty}$ was >30%, the derived pharmacokinetic parameters were deemed unreliable. In cases where the correlation coefficient (R^2) of the terminal phase was <0.9 and the extrapolation of $AUC_{0-Tlast}$ was >30%, the derived pharmacokinetic parameters were not reported.

Sampling times. Sampling times were as follows: on day 1, prior to dosing (within 15 min before dosing) and at 2, 4, 6, 8, 10, and 12 h postdosing; on day 2, prior to dosing (24 h after the first dosing, predosing); on day 5 and day 7, prior to dosing; on day 10, prior to dosing and at 2, 4, 6, 8, 10, and 12 h postdosing; on day 11, 24 and 36 h after the last dose; on day 12, 48 h after the last dose; and on day 15 and day 20.

Analytical analysis. Amphotericin B plasma concentrations were determined by a validated assay using liquid chromatography-tandem mass spectrometry (LC-MS/MS); the assay limit of quantitation was 0.5 ng/ml to 501 ng/ml. Representative plasma, tissue, and intestinal content sample analysis was performed at Nucro-Technics' Bioanalytical Laboratory using a qualified LC-MS/MS method for the determination of amphotericin B, as previously described (13).

Safety analysis. Dogs were monitored for gastrointestinal disturbances (i.e., nausea and diarrhea) and changes in several hematological, coagulation, and biochemical markers, including assessment of kidney and liver function.

Data collection and statistical analysis. In-life data, necropsy, organ weight, and pathology data were collected using Ascentos software (version 1.3.0; PDS Inc.). Numerical data collected during the course of the study were subjected to calculation of the group average. Plasma concentration-time data were analyzed by the noncompartmental method, using validated Phoenix WinNonlin software (version 6.3; Pharsight Corp.), to obtain the toxicokinetic parameters. Data transformation and preparation of graphics were performed using Microsoft Excel software, and tables were generated using Microsoft Word software (Microsoft Corporation). The experimental data were plotted and analyzed using one-way analysis of variance with Tukey's *post hoc* test in GraphPad Prism software (version 8.0; San Diego, CA, USA). Data are presented as the mean with standard deviation (SD). Significance was set at a P value of ≤ 0.05.

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P.H. is chief medical officer of iCo Therapeutics Inc. and holds stock options within the company. K.M.W. is director of research at iCo Therapeutics Inc. and holds stock options within the company. E.K.W. declares no conflict of interest.

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