Section A-Research paper
ISSN 2063-5346

In vivo Evaluation of I.V Administered Lipid Suspension Injection of Fingolimod in Sprague Dawley Rats

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. DOI: 10.48047/ecb/2023.12.7.341

Abstract

The objective of this study was to determine the lymphocyte count of the test item Fingolimod Injectable lipid Suspension (7.0 mg/ml) after single dose intramuscular administration to Sprague Dawley rats. A total of 48 Sprague Dawley rats (24 males and 24 females) were randomly allocated to 4 groups. The total dose volume of placebo/test item was administered by intramuscular route and equal volume of the total dose volume was administered at two different sites (left and right thigh muscle). All the animals were observed once daily for clinical signs of toxicity and twice daily for mortality and morbidity throughout the experimental period. Body weights were recorded on treatment Day 1 (prior to dosing), days 7 and 14, and day 15 (fasting body weight). Site of injection was evaluated for erythema and oedema on the day of treatment at 1hr, 4hr, 6hr and 24hr post dosing. Ophthalmological examination was performed once during acclimatization for all the animals and during week 2 for Placebo control and high dose group animals (G1 and G4). Blood and urine samples were collected and analyzed on day 15 from all animals after overnight fasting. After blood collection, all the animals were euthanized under CO2 followed by exsanguination and subjected to gross pathological examination. The T/R ratio was calculated for the pharmacokinetic parameters of in cellular components, Cmax, Tmax, AUC_{0-t} and MRT cumulatively calculated. The blood concentration versus time data shown the similar exposure for both test and reference formulations. The T/R ratio of blood for Cmax, Tmax, AUC_{0-t} and MRT was found to be within the acceptable bioequivalence range which shows the equivalence of Test formulation with that of Reference formulation

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Keywords: Fingolimod -loaded lipid injection, Sprague-Dawley rats, AUC_{0-t} and MRT.

Introduction[1-5]

Long-acting formulations (LAFs) are used for pharmacotherapy as sustained-release medications over a period of several days, weeks, or even months. Compared to conventional preparations, LAFs have many distinguished advantages related to its long-lasting curative effect, as well as its reduced toxicity, dosage and frequency of administration. These outstanding features of LAFs have encouraged researchers to pursue their further development to fulfil the unmet need for long-term treatments of chronic diseases or other prevalent diseases that threaten human health.

Administering depot formulations parenterally is a recommended choice for drugs which undergo substantial first-pass metabolism or that are characterized by low oral bioavailability. The absorption, distribution, metabolism, and elimination of a drug strongly rely on the characteristics of the biological environment. Opting for a specific site of administration thus represents a strategy to modulate the drug release kinetics

Intravenous lipid suspension (ILS) has been used for parenteral nutrition with Food and Drug Administration (FDA) indications for caloric supplementation and essential fatty acid deficiency. Lipid suspension is also used as a carrier for lipid-soluble medications, most notably propofol. The benefits of lipid suspension for bupivacaine poisoning were first suggested in a rat model by Weinberg et al 1998. The subsequent study in dogs by Dr. Weinberg and the first report of use in humans by Rosenblatt et al. In 2006 have brough about a paradigm shift in the management of acute local aesthetic toxicity toward utilization of ILS. Intravenous lipid suspension (ILS) has recently received much attention in the treatment of acute local anaesthetic toxicity and a variety of other non-local anaesthetic poisonings.

The principles to produce long-acting therapeutics are based on maintaining the drug activity for longer periods of time and to improve their tolerance in the body. Toward this end, a diverse array of strategies has been developed to design LAPFs through endowing the therapeutic drugs with features that include slow and controlled release, delayed clearance, resistance to enzymes, increased stability, extended half-life. By rationally designing, LAPFs are safer and more efficient. Many LAPFs have been clinically approved, which has enabled the reduction of dosing frequency to daily, weekly, biweekly, or even monthly. Along with innovation of popular used approach for designing LAPFs and the emergence of novel strategies, LAPFs candidates have been scaled up to preclinical and clinical studies.

EXPERIMENTAL[6-10]

The objective of this study was to evaluate the tissue distribution of Fingolimod in tissues along with plasma and blood after single dose of lipid suspension in Sprague Dawley Rats.

In Vivo Pharmacokinetic Study in Sprague Dawley Rats[11-17]:

Experimental design:

The objective of this study was to investigate and compare the pharmacokinetics of Fingolimod intravenous bolus administration of test formulations and reference formulation in male Sprague-Dawley rats. The study was performed using parallel design (n=10/group)

In vivo study protocol

Subject selection

48 healthy male Sprague-Dawley rats weight range of 141.06 g to 167.26 g Kgs will be selected through physical examination.

Study Compliance

a. Environmental Conditions	:	Animals were housed under standard laboratory conditions, in an environmentally monitored air-conditioned room with adequate fresh air supply (12 to 15 air changes per hour), room temperature19.5°C to 23.8°C and relative humidity 47% to 65% with 12 hours fluorescent light and 12 hours dark cycle. The temperature and relative humidity were recorded once daily.
b. Housing	:	Two animals were housed in a standard polypropylene cage (size: L 430 x B 285 x H 150 mm) with stainless steel mesh top grill having facilities for holding pelleted food and drinking water in water bottle fitted with stainless steel sipper tube. Clean sterilized paddy husk was provided as bedding material.
c. Feed	:	Altromin Maintenance diet (pellet) for rats and mice 1324 manufactured by Altromin Spezial futter GmbH & Co. KG was provided ad libitum to the animals throughout the experimental period. The contaminant analysis test report for the feed is presented in Annexure 3.
d. Water	:	Water was provided ad libitum throughout the acclimatization and experimental period. Deep bore-well water passed through reverse osmosis unit was provided in plastic water bottles with stainless steel sipper tubes

Grouping

The animals were weighed and arranged in ascending order of their body weights. These body weight stratified animals were distributed to each group using Microsoft Excel Spreadsheet, such that body weight variation of animals selected for the study were not exceeding ± 20% (Male: +8.30 to -8.77% and Female: +5.25 to -11.61%) of the mean body weight of each sex. The grouping was done one day prior to the initiation of treatment. Body weight of the animals was analyzed statistically for mean body weight to rule out the statistically significant difference between groups within each sex.

Dose Selection and Justification

The human dose for proposed Fingolimod Extended-Release Injectable Suspension (7.0 mg/ml) injection formulation was 7 mg/14 days. Hence based on the conversion doses, selected for the study are 1, 5 and 10 mg/kg for low (G2), mid (G3) and high (G4) dose groups respectively which were 1-fold, 5-fold and 10-fold of HED respectively. In addition, concurrent control group (G1) was included which was administered with placebo.

Route of Administration and Justification for Selection of Route

The test item was administered through intramuscular route. Intramuscular route was selected as it is the intended route of administration in humans.

Dose Formulation

Ready-to-use dose formulations as provided by sponsor were used for administration. Prior to dose administration, placebo and test item were shaken well.

Administration of Placebo and Test Item

The placebo/test item was administered by intramuscular route and approximately equal volume of the total dose volume was administered to left and right thigh muscle. Site of administration was clipped one day prior to the treatment and as and when necessary. The administration site was marked with non-irritant marker and marking were maintained throughout the study period and remarked when necessary. The placebo control and test item were administered through intramuscular route to each rat as a single dose. The respective group animals were receiving respective formulations as per ‘study design’.

Safety Precautions

All necessary safety measures will be taken care during the conduct of study. When required, personnel involved in study must wear gloves, head cap and facemask in addition to protective clothing to ensure adequate personnel health and safety.

Invivo studies:

RESULTS

Table 1: Pharmacokinetic parameters of Fingolimod

Treatment (Route)	Matrix	Analyte	Tmax (h)	Cmax (ng/mL)	AUC last (ng.h/mL)	MRT last (h)	AUC ratio
TF_2 (IV)	Hemolysed Blood	Fingolimod	5.00 (4.00-16.0)	6.82 ± 4.26	240 ± 130	59.7 ± 25.6	NA
	Plasma	Fingolimod	10.00 (4.00-16.0)	0.39 ± 0.179	16.0 ± 5.06	97.7 ± 34.6	15.9

Table 2: Cellular components concentration-time data of Fingolimod following intravenous bolus administration of TF_1 formulation in male Sprague Dawley rats (Dose: 0.051 mg/kg; G3)

Rat No.	Plasma Concentrations (ng/mL)								
	Time (h)								
	4	**8**	**12**	**16**	**24**	**48**	**72**	**120**	**168**
1	2.64	0.942	1.58	0.813	0.747	0.423	0.268	0.179	0.312
2	1.08	0.843	0.672	0.576	0.537	0.468	0.216	0.66	0.402
3	1.12	1.04	1.29	1.45	0.873	0.185	0.597	0.579	0.237
4	1.67	0.639	1.52	3.09	0.81	0.588	0.648	0.152	0.152
5	10.3	3.57	3.21	3.81	3.60	1.28	0.333	0.186	0.218
6	3.3	0.194	2.86	0.552	0.423	0.36	0.247	0.143	0.149
7	7.92	2.98	5.49	3.6	2.98	1.61	0.69	0.0777	0.17
8	7.08	1.99	3.33	4.26	2.81	1.57	1.69	BLQ*	0.122
9	18.4	4.59	4.74	4.26	3.57	1.48	0.993	0.111	0.339
10	6.9	3.87	4.86	4.11	4.65	2.63	0.876	0.259	0.179
N	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**9**	**10**
Mean	**6.04**	**2.07**	**2.96**	**2.65**	**2.1**	**1.06**	**0.655**	**0.261**	**0.228**
SD	**5.4**	**1.57**	**1.68**	**1.61**	**1.58**	**0.78**	**0.453**	**0.211**	**0.0936**
CV%	**89**	**76**	**57**	**61**	**75**	**74**	**69**	**81**	**41**

BLQ: Below limit of quantification (LLOQ: 20 pg/mL) considered as zero for PK calculations; NA: Not applicable; * Considered as an outlier for PK calculations

Table 3: Plasma concentration-time data of Fingolimod free following intravenous bolus administration of TF_1 formulation in male Sprague Dawley rats (Dose: 0.051 mg/kg; G3)

Rat No.	Plasma Concentrations (ng/mL)								
	Time (h)								
	4	**8**	**12**	**16**	**24**	**48**	**72**	**120**	**168**
1	0.206	0.104	0.109	0.0776	0.0517	0.0362	0.0821	0.0359	0.0225
2	0.072	0.0927	0.0444	0.0979	0.0661	0.045	0.0502	0.0195	0.0269
3	0.147	0.0873	0.0717	0.0587	0.0752	0.0631	0.0546	0.047	0.0259
4	0.0874	0.0912	0.0758	0.129	0.0468	0.0446	0.0358	0.0436	0.0263
5	0.513	0.28	0.198	0.238	0.144	0.101	0.0376	0.0299	BLQ*

6	0.0664	0.0189	0.149	0.0366	0.0205	0.038	0.0486	0.032	0.0266
7	0.284	0.305	0.318	0.256	0.149	0.025	0.0292	BLQ*	BLQ*
8	0.586	0.306	0.226	0.167	0.117	0.0898	0.0688	BLQ	BLQ*
9	0.575	0.348	0.311	0.176	0.209	0.0783	0.055	0.0496	BLQ*
10	0.327	0.281	0.384	0.29	0.121	0.0945	0.0711	0.0442	0.0233
N	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**8**	**6**
Mean	**0.286**	**0.191**	**0.189**	**0.153**	**0.100**	**0.0616**	**0.0533**	**0.0377**	**0.0253**
SD	**0.207**	**0.122**	**0.119**	**0.0876**	**0.0579**	**0.0275**	**0.0169**	**0.0102**	**0.00187**
CV%	**72**	**64**	**63**	**57**	**58**	**45**	**32**	**27**	**7**

BLQ: Below limit of quantification (LLOQ: 20 pg/mL) considered as zero for PK calculations; NA: Not applicable; * Considered as an outlier for PK calculations

Table 4:Individual pharmacokinetic parameters of Fingolimod in cellular components following intravenous bolus administration of TF_1 in male Sprague Dawley rats (Dose: 0.051 mg/kg; G3)

Rat No.	**T_{max}[a] (h)**	**C_{max} (ng/mL)**	**AUC_{last} (ng.h/mL)**	**MRT_{last}**
1	4.00	2.64	106	69.7
2	4.00	1.08	104	104
3	16.0	1.45	117	90.0
4	16.0	3.09	119	64.7
5	4.00	10.3	264	24.5
6	4.00	3.30	228	76.0
7	4.00	7.92	248	27.2
8	4.00	7.08	336	51.2
9	4.00	18.4	503	57.3
10	4.00	6.90	313	42.8
N	**10**	**10**	**10**	**10**
Mean	**4.00 (4.00-**	**6.21**	**234**	**60.7**
SD	**NA**	**5.26**	**129**	**25.6**
CV%	**NA**	**85**	**55**	**42**

[a] T_{max} presented as median (min-max); NA:

Table 5: Individual pharmacokinetic parameters of Fingolimod free in plasma following intravenous bolus administration of TF_1 formulation in male Sprague Dawley rats (Dose: 0.051 mg/kg; G3)

Rat No.	T_{max}[a] (h)	C_{max} (ng/mL)	AUC_{last} (ng.h/mL)	MRT_{last}
1	4.00	0.206	12.7	93.8
2	16.0	0.0979	12.9	150
3	4.00	0.147	14.4	127
4	16.0	0.129	11.9	129
5	4.00	0.513	17.4	56.7
6	12.0	0.149	10.5	135
7	12.0	0.318	13.7	65.8
8	4.00	0.586	23.8	84.4
9	4.00	0.575	21.7	69.8
10	12.0	0.384	18.8	75.1
N	**10**	**10**	**10**	**10**
Mean	**8.00 (4.00-**	**0.31**	**15.8**	**98.7**
SD	**NA**	**0.193**	**4.46**	**33.6**
CV%	**NA**	**62**	**28**	**34**

[a] T_{max} presented as median (min-max); NA:

Figure 1: Plasma concentration-time profile of fingolimod and fingolimod phosphate following intravenous bolus administration of TF_1 formulation in male Sprague Dawley rats

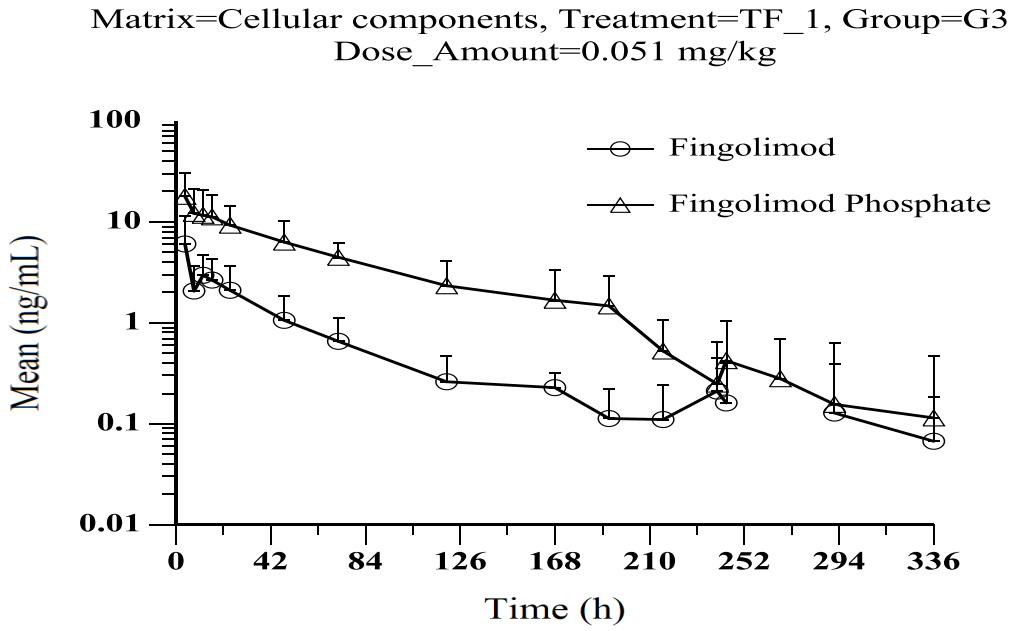

Table 6: Summary of haematology record

Group, Sex & Dose (mg/kg)	Statistical values	Mean Platelet Volume (MPV) (fL)	Reticulocyte Count (Retic) (%)	Neutrophils (Neut) (%)	Lymphocytes (Lymph) (%)	Monocytes (Mono) (%)	Eosinophils (Eos) (%)	Basophils (Baso) (%)	Absolute Reticulocyte Count (Retic) (10^9 cells/L)
G1, M & 0	Mean	6.23	4.76	21.27	71.98	3.73	0.93	0.68	360.42
	±SD	0.33	3.42	4.67	3.85	1.34	0.48	0.17	239.64
	n	6	6	6	6	6	6	6	6

CONCLUSION

Based on the results observed and under the experimental conditions employed, the single dose Fingolimod Extended-Release Injectable Suspension (7.0mg/ml) to Sprague Dawley rats did not result in any mortality and was well tolerated up to the high dose tested (10 mg/kg Body weight/day). Treatment resulted in decrease in WBC, lymphocytes and variations in few differential leukocyte counts, and histopathological decreased corticomedullary ratio in thymus in high dose males; and in spleen, decreased cellularity of white pulp in high dose rats was observe. From this it is concluded that Fingolimod in this sustained release dosage form is producing clinical action, by decreasing lymphocyte count in a gradual manner, which is evident to prove efficacy of the drug in the controlled release formulation

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