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Research Article

Pre-Clinical and Phase I Clinical Study of Clopidogrel Lipid Suspension: Intravenously Injected Formulation Results in Faster Onset of Action and Dose-Dependent Inhibition of Platelet Aggregation

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Abstract

Clopidogrel Lipid Suspension was formulated in lipid based aqueous system and characterized for morphology and particle size using physicochemical techniques. Transmission Electron Microscopy (TEM) cryo imaging analysis revealed the presence of predominantly spherical unilamellar liposome particles with 25-110 nm size and the product was found to be stable at 2-8°C for 2 years. Repeated dose toxicity study of Clopidogrel Lipid Suspension showed no signs of toxicity in rats and minimal toxicity in mice. Pharmacokinetic studies indicated a rapid metabolism of Clopidogrel following intravenous administration of Clopidogrel Lipid Suspension compared to oral administration of Clopidogrel bisulfate. Intravenous administration of 5, 10, 20, and 40 mg/kg Clopidogrel Lipid Suspension showed a dose dependent effect on the platelet aggregation in mice. An oral dose of 20 mg/kg of Clopidogrel was required to produce similar effects by intravenous administration of only 5 mg/kg in mice with Clopidogrel Lipid Suspension. Phase I clinical safety studies were also conducted in 48 healthy human subjects with dose escalation from 25 mg to 75 mg. All subjects who experienced adverse events during this study recovered completely and no serious adverse events were reported.

INTRODUCTION

Clopidogrel is a potent antiaggregant and antithrombotic drug commonly used for the treatment of atherosclerosis in patients and for the secondary prevention of antherothrombotic events such as myocardial infarction, stroke and vascular death [1]. Clopidogrel is an inactive prodrug that requires metabolism by several cytochrome P450 enzymes including CYP2C19 isozymes to form the active thiol metabolite [2]. The thiol metabolite of drug binds irreversibly to the P2Y12 subtype of adenosine diphosphate receptor, which plays a major role in the platelet aggregation and cross-linking by the protein fibrin [3]. Clopidogrel, in combination with acetylsalicylic acid (aspirin) is indicated for the early and long-term secondary prevention of atherothrombotic events in patients with acute coronary syndromes-without ST segment elevation. It is also used as an alternative antiplatelet drug for patients who are intolerant to aspirin [4] and is administered in hospitals prior to diagnostic angiography in patients with acute coronary syndrome.

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Clopidogrel is available as its bisulfate salt for oral administration. Upon administration, approximately 85% of the prodrug is hydrolyzed by esterases to an inactive carboxylic acid derivative (Figure 1) and the remaining drug is partially metabolized to an active thiol-metabolite in liver [5]. Typically, oral formulation takes time to show effective blood levels of the active metabolite and its pharmacological response. The currently approved dosing regimen for Clopidogrel (Plavix®) stipulates a 300 mg loading dose followed by a 75 mg once a daily dose [6]. Clopidogrel in solid dosage form is considerably influenced by disintegration, dissolution and gastric emptying. The bioavailability through intestinal absorption of Clopidogrel is around 50%. Further, the P-glycoprotein (P-gp) transporter also limits the intestinal absorption of Clopidogrel, thereby controlling its anti-platelet activity. In clinical study, the platelet function was assessed in blood samples collected approximately 2.5 hours after oral administration of 300 mg of Clopidogrel and found an insufficient inhibition of ADP-induces aggregation in 40% patients [7].

The limitations of oral administration of Clopidogrel include use of high dose to reach effective drug level in blood, reaching effective drug level after several hours of dosing and bleeding complications. Hence, there is a need of Clopidogrel formulation for intravenous use especially for patients with immediate medication requirement and for whom oral route of administration is difficult.

Lipid-based formulations such as liposomes as drug delivery systems can offer several advantages over conventional dosage forms especially for parenteral administration. However, preparation of liposomes requires dissolving drug and the excipients in toxic organic solvents such as chloroform and ethanol and subsequently removal of solvent to form a dry film [8,9]. We have developed an aqueous based system for preparing lipid-based formulation to circumvent the use of organic solvents [10,11]. The current study describes the preparation of a well-characterized lipid-based formulation of Clopidogrel without the use of organic solvents for intravenous use. The intravenously administered drugs are mostly bio available and results in faster onset of desired effects in comparison to oral administration. This formulation is also beneficial for subjects who are non-responder to orally administered Clopidogrel. In such patients intravenously administering Clopidogrel Lipid Suspension can elicit a therapeutic response of Clopidogrel. In a hospital setting, intravenous formulations can be desirable because rapid onset of action is important in acute situations and also useful for many patients who are unable to take oral medications. The present study describes physicochemical characterization as well as the toxicity, pharmacokinetics, and the platelet aggregation inhibition of Clopidogrel Lipid Suspension in rodents. Safety studies were also conducted in healthy human subjects.

MATERIALS AND METHODS

Materials

Clopidogrel bisulfate was obtained from MSN Laboratories Limited, Medak, India. Soy phosphatidylcholine and Sodium Cholesteryl Sulfate were purchased from Lipoid, LLC, Newark and Genzyme Pharmaceuticals, MA respectively. Sodium Citrate, monobasic was purchased from Sigma-Aldrich, St. Louis, MO.

Preparation of Clopidogrel Lipid Suspension

Soy phosphatidylcholine, Sodium Cholesteryl Sulfate, and Clopidogrel bisulfate (mol ratio 8.4:0.1:0.5) were blended in sodium succinate buffer (pH 7.4) until the suspension was free from lumps and then passed through Emulsiflex C3 Homogenizer (Avestin, Inc. Ottawa, Canada) at 25,000 psi until less than 200 nm particle size wasachieved.. The resulting suspension was mixed with a solution of sucrose before it was filtered through 0.22 μm sterile PVDF Millipak100 filter under aseptic conditions. The final lipid-based suspension of Clopidogrel was dispensed in vials and lyophilized to yield a concentration of Clopidogrel (50 mg per vial).

Physicochemical characterization

Morphology: Clopidogrel Lipid Suspension was characterized for its morphology by Transmission Electron Microscopy (TEM) cryo imaging analysis. The lyophilized vial was reconstituted with 37 mL water for injection and further 1:10 dilution was made with 5% dextrose. Each sample was preserved in vitrified ice supported by holey carbon films on 400-mesh copper grids. The sample was prepared by applying 3 μL drop of sample suspension to a cleaned grid, blotting away with filter paper, and immediately proceeding with vitrification on liquid ethane. Grids were stored under nitrogen until transferred to electron microscopy for imaging. Electron microscopy was performed using an FEI Tecnai T12 electron microscope, operating at 120keV equipped with an FEI Eagle 4k x 4k CCD camera. Vitreous ice grids were transferred into the electron microscope using a cryostage that maintains the grids at a temperature below -170˚C. Images of each grid were acquired at multiple scales to assess the overall distribution of the specimen.

Particle Size Measurement: Clopidogrel Lipid Suspension lyophilized powder was reconstituted in Water for Injection and 0.9% sodium chloride solution separately and the particle size was measured using Nicomp Model 380/ZLS&S Potential/ sub-Micron Particle Sizer (Particle Sizing Systems, New Port Richly, Florida). The measurements were carried out at 23˚C at a scattering angle of 90˚

Percent Drug Association

Association of Clopidogrel with the lipids was determined using size exclusion chromatography. Briefly, 500 μL of reconstituted Clopidogrel Lipid Suspension was loaded on PD-10 Sephadex™ G-25M PD-10 column equilibrated with milliQ water. The column was then eluted with milliQ water and small fractions (~300 μL) were collected. Fractions containing the lipids were pooled and volume was measured. For control, 500 μL of reconstituted Clopidogrel Lipid Suspension was diluted to the measured volume with milliQ water. The eluted pooled fractions and control were analyzed by HPLC for the drug content.

HPLC method

The HPLC analysis of the drug product and the stability studies were carried out using Agilent 1100/1200 series HPLC system equipped with UV Detector, Ultron ES OVM (150 mm x 4.6 mm, 5 μ) column. A mixture of potassium dihydrogen orthophosphate buffer and acetonitrile (80:20) was used as mobile phase, column oven temperature was set at 30˚C and detection by UV was done at

220 nm wavelength. The related substances (known impurities) were also analyzed for their content at same wavelength. The concentration of soy phosphatidylcholine in the drug product was assayed on HPLC system at 205 nm wavelength using Astec Diol (250mm x 4.6mm, 5µ) and a mixture of ammonium acetate buffer (pH 4.1 ± 0.05) and methanol (100:900). Sodium cholesteryl sulfate concentration was determined at UV wavelength 210 nm using Peerless basic C8 (100 mm x 4.6 mm, 5µ) column a gradient of sodium dihydrogen orthophosphate dehydrate buffer (pH 6.0 ± 0.05) and methanol. The stability studies were performed for the lyophilized product for 2 years from the date of manufacturing and the stability of the reconstituted and diluted product was monitored up to 8 hours. The parameters monitored during stability studies include physical appearance, pH, particle size, drug content and the related substances.

Preparation of dosing solutions

The dosing solutions of required concentration (1 to 5 mg/mL) were prepared on the day of experiment by reconstituting lyophilized Clopidogrel Lipid Suspension powder with appropriate volumes of 0.9% sodium chloride solution for intravenous dose administration. Similarly, the dosing solution of Placebo containing inactive ingredients was prepared in n-saline. Further, a solution of Clopidogrel bisulfate (reference compound) at 2 or 4 mg Clopidogrel bisulfate/mL was prepared in sterile water for oral dose administration.

Animal toxicology

A multiple-dose study in ICR (CD-1) mice and Sprague-Dawley rats (Harlan Laboratories, Madison, WI) was conducted to test the safety and toxicity of Clopidogrel Lipid Suspension. Sets of 5 male (31-39 g) and 5 female (25-34 g) mice and sets of 5 male (250-290 g) and 5 female (230-260 g) rats were randomly selected for each of three test groups and one control group. Clopidogrel Lipid Suspension at dose levels of 20, 40 and 60 mg of Clopidogrel bisulfate/kg body weight was administered intravenously once daily for 5 consecutive days to three dose groups. The mice and rats in the respective control groups were similarly treated intravenously with n-saline at the equivalent volume relative to body weight. The animals were observed daily during the five days of the treatment period after daily intravenous dosing and at 15 and 30 min, and approximately at 2, 3, 4, and 6-hour post dose to detect clinical signs of toxicity. The animals were further observed periodically up to 2 weeks post dosing period. The guidelines for the Care and Use of Laboratory Animals and Standard Operating Procedures for animal well-being were approved prior to the execution of the study and were followed.

Platelet Aggregation Inhibition Study

The inhibitory effect of Clopidogrel bisulfate and Clopidogrel Lipid Suspension on the platelet aggregation was investigated using ICR (CD-1) mice. Clopidogrel bisulfate (the active ingredient of the marketed drug, Plavix®) at single dose levels of 20 and 40 mg Clopidogrel bisulfate/kg body weight was administered orally by gavage to each group (5 or 6 mice per group). The mice in the respective control group were similarly treated orally with the sterile water at the equivalent volume relative to the body weight.

Clopidogrel Lipid Suspension at single dose levels of 5, 10, 20 and 40 mg Clopidogrel bisulfate/kg body weight was administered intravenously to each group (5 or 6 mice per group). The mice in the respective control groups were similarly treated intravenously either with Placebo Lipid Suspension or n-saline intravenously at the equivalent volume relative to the body weight. All mice were anesthetized using isoflurane/propylene glycol (3:7, v/v) solution in an inhalation chamber after 30 minutes following the intravenous or oral dose administration. A cotton ball soaked with the isoflurane/propylene glycol (3:7, v/v) solution was placed close to the animal's nose in order to keep the animal in anesthetized state in the restrainer. The tail of the anesthetized mouse was cut (~0.5 cm distal tip of the tail) and placed in the pre-warmed (~37°C) phosphate buffered saline (PBS, pH 7.4) solution. The times when the bleeding started in the PBS solution and when the blood flow stopped were recorded. The mice were allowed to bleed to a maximum time of 15 min. The blood volume collected in the pre-weighed test tube containing PBS solution was also measured. The mice were observed for any deleterious effect right after the intravenous or oral dose administration up to 30 min before the bleeding time measurement started and subsequently during the day for clinical signs and behavior pattern as well as morbidity/mortality.

Pharmacokinetic Study

A rat study was conducted to compare the pharmacokinetic (PK) parameters of Clopidogrel carboxylic acid (a major circulating metabolite of Clopidogrel) following a single intravenous administration of Clopidogrel Lipid Suspension, and following a single oral administration of Clopidogrel bisulfate at the dose level of 20 mg/kg body weight. The Clopidogrel Lipid Suspension was administered intravenously to 9 rats and Clopidogrel bisulfate was administered orally by gavage to another group of 9 rats.

In order to minimize the body fluid loss, the blood was collected no more than three time points from each rat. The first two blood samples were collected from the tail vein and the third blood sample by cutting the jugular vein. The blood (0.5-1.0 mL) was collected in the lithium heparin tubes (Becton Dickinson, Franklin Lakes, NJ) at pre-dose and at 15 and 30 min, and at 2, 4, 6, 8 and 24 hours post dose administration. The rat plasma samples were obtained after centrifugation of blood samples at 3,000 x g for 10 minutes at 2-8° C. All plasma specimens were stored frozen at minus 20 ± 5° C until analysis.

Bioanalysis of Plasma Samples

For bioanalytical analysis, the Shimadzu HPLC system included Liquid Chromatography Pump (Low Pressure Gradient, Model LC-20AT), Auto Sampler (Model SIL-20AC), Diode-Array Detector (Model SPD-M20A), Column Oven (Model CTO-20A) and System Controller (Model CBM-20A). The EZStart software program (version 7.4) was used to control the HPLC system and to process the HPLC data. The reconstituted extracts of plasma samples were analyzed using a Zorbax Eclipse XD3-C8 column (4.6 mm i.d. x 150 mm, 5 micron particle size, column temperature set at 30°C) with UV detection at 240 nm. HPLC mobile phase A consisted of a solvent mixture containing 19% acetonitrile, 2% tetrahydrofuran and 79% 30 mM potassium phosphate buffer,

pH 3.0 (v/v). HPLC mobile phase B consisted of 100% methanol. The initial 6 min isocratic HPLC condition of 100% mobile phase A was followed by a subsequent 2 min linear gradient from 0 to 100% of mobile phase B at a flow rate of 0.9 ml/min. The internal standard (ticlopidine) and analyte (Clopidogrel carboxylic acid) were eluted at about 5 and 7 min, respectively.

A bioanalytical method reported by Souri et al. [12] with minor modifications was used to determine the amount of Clopidogrel carboxylic acid (CCA) present in rat plasma. A volume of 50 µL of internal standard (0.05 mg ticlopidine hydrochloride/mL) was added to 200 µL of rat plasma and mixed by hand shaking. A volume of 0.4 ml of 0.3 M phosphate buffer (pH 5) was added to the sample tube. The mixture was then extracted with 4 ml of chloroform by a slow vortex-action for 30 seconds, and then centrifuged at 3,000 g for 10 min. The organic layer (bottom) was withdrawn and transferred into another test tube. An amount of 1 g of anhydrous sodium sulfate was added to the organic extract and shaken by vortex-action. The sample tube containing sodium sulfate and the organic extract was centrifuged at 3,000 g for 10 min, supernatant transferred to another test tube and evaporated to dryness at ~40°C under the gentle stream of nitrogen. The dried content of the sample extract was reconstituted in 0.5 ml of HPLC mobile phase A. An aliquot of 50 µl of the reconstituted sample extract was analyzed by HPLC using the conditions described above.

Clinical Safety Studies

An open-label Phase I single dose escalating study was conducted to evaluate the safety of Clopidogrel Lipid Suspension for Injection relative to the oral Clopidogrel in healthy, adult, male human subjects under fasting condition. The study was carried out in compliance with the protocol and in adherence to good clinical practices as per Declaration of Helsinki (2004); ICH harmonized Tripartite Guidelines for Good Clinical Practice (1996).

A single intravenous dose of Test Product [Clopidogrel Lipid Suspension for Injection (Treatment Dose: 25 mg, 50 mg or 75 mg)] or single oral dose of Reference Product [Plavix® (Clopidogrel bisulfate) Tablets 300 mg (Treatment dose = 300 mg)] was administered to each subject.

RESULTS

Physicochemical characterization

Clopidogrel Lipid Suspension was prepared by high pressure homogenization of Clopidogrel, Soy Phosphatidylcholine, and Sodium Cholesteryl Sulfate Sodium Citrate buffer. Subsequently, the resulting homogenous suspension was lyophilized after mixing with aqueous sucrose solution. No organic solvent or detergents were used in the preparation of the formulation. The concentrations of drug and excipients, related substances in the drug product were assayed by HPLC. The drug assay was found to be 98.8% by HPLC analysis. The related substances include regioisomers of drug substance and acid impurity or metabolite potentially forms due to the hydrolysis of drug substance in basic or acid medium. The concentrations of drug, excipients, and particle size were checked before and after lyophilization. There was no difference in the results suggesting that the lyophilzation process had no negative effect on the drug product. The sterility test showed no evidence of microbial growth and free from bacterial endotoxin. The reconstituted Clopidogrel Lipid Suspension was found to be stable up to 8 h at room temperature. The diluted suspension with 5% dextrose was also found to be stable for 8 has confirmed by HPLC analysis. Percent drug association with lipids were determined by passing Clopidogrel Lipid Suspension through Sephadex™ G25 M PD-10 column and on analysis by HPLC revealed about 99% association with lipids.

Clopidogrel Lipid Suspension was characterized by Transmission Electron Microscopy (TEM) cryo imaging analysis which revealed the presence of predominantly spherical unilamellar liposomes having diameters typically in the range of 25-110 nm (Figure 2). The bilayer thicknesses of the liposomes were in the range of 4-5 nm thickness. The particle size distribution was measured using dynamic light scattering method. The pH after reconstitution of the formulation with water for injection was around 7.4 and the mean particle size was found to be 28.8 nm (Figure 3). The mean particle size after reconstitution with 0.9% sodium chloride was found to be 31.8 nm.

Animal toxicology

Multiple-dose studies were conducted to investigate the toxicity of Clopidogrel Lipid Suspension in mice and rats. The clinical signs such as changes in motor activity, skin/fur, eye and mucous membranes, respiratory system, central nervous system

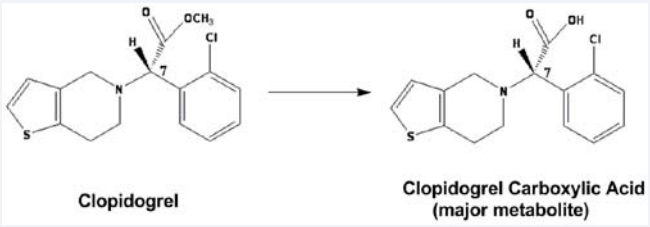

Figure 1 Metabolism of Clopidogrel to its Major Metabolite, Clopidogrel Carboxylic Acid.

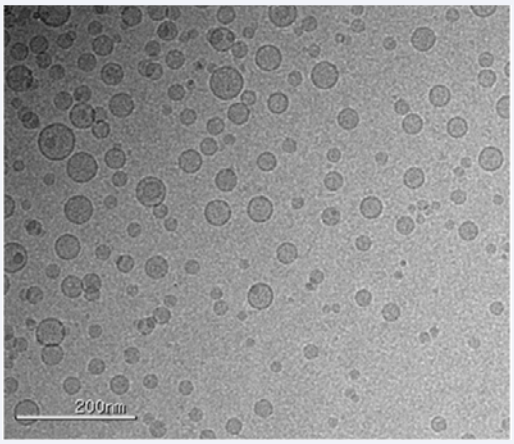

Figure 2 Transmission Electron Microscopy (TEM) cryo imaging analysis-Clopidogrel Lipid Suspension at a magnification of 52,000x showing unilamellar spherical liposome particles.

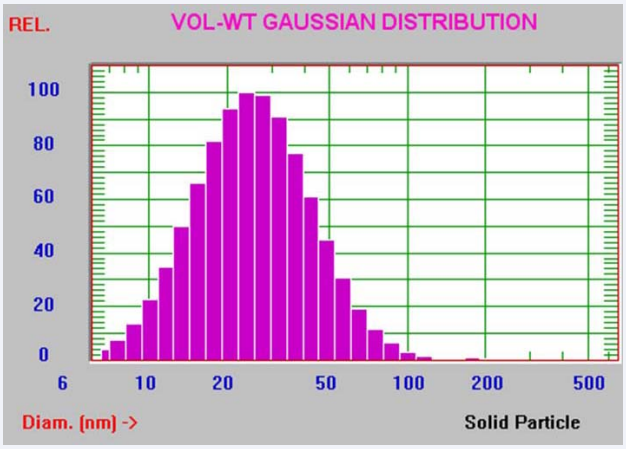

Figure 3 Particle Size Distribution of Clopidogrel Lipid Suspension: Mean Particle Size, 28.8 nm; distribution (<%) D25, 17.8 nm, D50, 25.2 nm, D75, 35.9 nm, D80, 39.2 nm, and D99, 85.7 nm. The measurements were carried out at 23°C at a scattering angle of 90°.

and behavior patterns were monitored. No apparent effect on the body weight in mice and rats from 3 dose groups (20, 40 or 60 mg/kg) in comparison to the respective control group was observed during the dosing and post dosing periods. Further, all the animals survived and no signs of toxicity were observed in rats from the three dose groups, and in mice from low dose group (20 mg/kg). Apparently some signs of toxicity such as, sudden immobility and heavy breathing were observed in a few mice from the 40 mg/kg group after the daily dose administration, but all of the mice survived during the dosing and post dosing periods. Signs of toxicity were observed in mice from high dose group (60 mg/kg) and 2 males and 3 females out of the 10 mice died after the dose administration during the 2-5 days of the dosing period. The results indicated that Clopidogrel Lipid Suspension was found to be safe in rats at all the tested dose levels and in mice at the low dose level of 20 mg/kg. Mice were found to be relatively more sensitive compared to rats at the mid and high dose levels.

Pharmacokinetic Study

The concentration of Clopidogrel carboxylic acid (CCA), the major circulating metabolite of Clopidogrel [1,13] was determined in rat plasma between 0-24 hours following the oral administration of Clopidogrel bisulfate and intravenous administration of Clopidogrel Lipid Suspension at the dose level of 20 mg Clopidogrel bisulfate/kg body weight. Pharmacokinetic parameters (Table 1) of Clopidogrel carboxylic acid were calculated using WinNonlin (Version 5.3, Pharsight Inc., USA). The maximum concentration (C_{max}) of CCA in rat plasma was38.0 µg/mL,that reached at 0.5 hour (T_{max}) following the intravenous administration of Clopidogrel Lipid Suspension (Figure 4). The C_{max} (20.4 µg/mL) was achieved at 2 hour (T_{max}) following oral administration of Clopidogrel bisulfate. The $AUC_{0\text{-}inf}$ and $AUC_{0\text{-}4h}$ values (µg.h/mL, Table 1) were 421 and 131 following intravenous administration of Clopidogrel Lipid Suspension in rats, respectively. The corresponding $AUC_{0\text{-}inf}$ and $AUC_{0\text{-}4h}$ values (µg.h/mL, Table 1) were 319 and 66.0 following oral administration of Clopidogrel bisulfate in rats, respectively. The results indicated a rapid metabolism of Clopidogrel following the intravenous administration of Clopidogrel Lipid Suspension compared to oral administration of Clopidogrel bisulfate (Table 1 and Figure 4). The minor active thiol-metabolite of Clopidogrel which is responsible for the platelet aggregation [1] was not analyzed in this study.

Platelet Aggregation Inhibition Study

The inhibition of platelet aggregation was measured by using the bleeding time and blood volume data (Figure 5a, 5b). Mice treated with water (oral administration) or placebo (IV administration) showed normal bleeding time (1-3 min) and blood volume (≤ 0.15 mL). Clopidogrel bisulfate administered orally at the dose level of 20 mg/kg resulted in an average bleeding time of 7.7 min and the average blood volume collected was 0.2 mL. Further, the average bleeding time (10.8 min) and the blood volume (0.35 mL) increased at the high oral dose level of 40 mg/kg body weight.

Similarly, mice were also treated intravenously with Clopidogrel Lipid Suspension at 5, 10, 20 and 40 mg/kg body

Table 1: Pharmacokinetics of Clopidogrel Carboxylic Acid in Rat Plasma following oral administration of Clopidogrel bisulfate and following Intravenous administration of Clopidogrel Lipid Suspension at the dose level of 20 mg/kg body weight.

Pharmacokinetic Parameters	Clopidogrel Bisulfate (PO)	Clopidogrel Lipid Suspension (IV)
T_{max} (h)	2.00	0.50
C_{max} (µg/mL)	20.4	38.0
$AUC_{0\text{-}inf}$ (µg.h/mL)	319	421
$AUC_{0\text{-}4h}$ (µg.h/mL)	66.0	131

PO: By mouth; IV: Intravenous; T_{max}: Time of the Maximum Measured Blood Concentration; C_{max}: Maximum Measured Blood Concentration; $AUC_{0\text{-}inf}$: The Area under the Blood Concentration Versus Time Curve from Time Zero to Infinity. $AUC_{0\text{-}4h}$: The Area under the Blood Concentration Versus Time Curve from Time Zero to 4 hours.

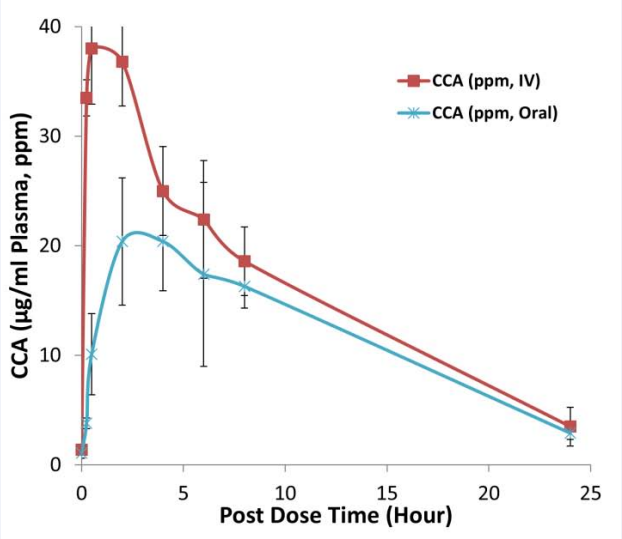

Figure 4 Clopidogrel Carboxylic Acid profiles in rat plasma following oral administration of Clopidogrel bisulfate and following intravenous administration of Clopidogrel Lipid Suspension at the dose level of 20 mg/kg body weight.

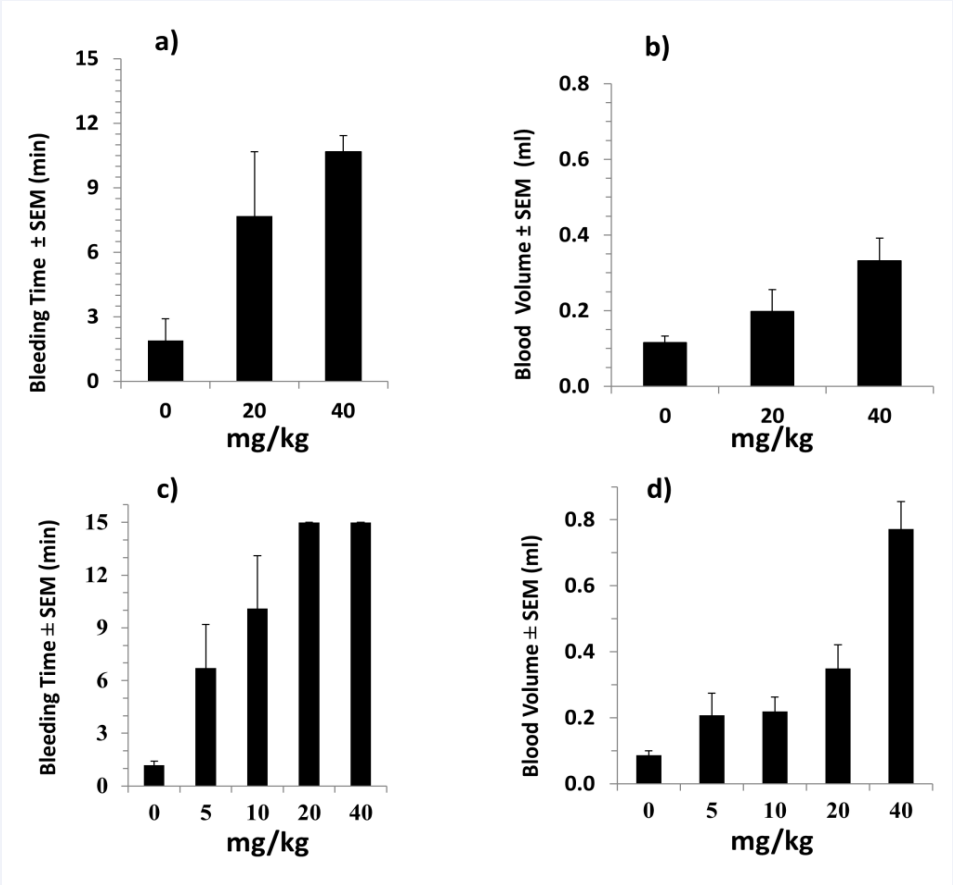

Figure 5 Bleeding Time (a) and Blood Volume (b) was monitored post dose 30 minutes following the administration of a single oral dose of Clopidogrel bisulfate to mice at 0, 20 and 40 mg/kg body weight. Single intravenous dose of Clopidogrel Lipid Suspension was administered to mice at 0, 5, 10, 20 and 40 mg/kg body weight. After 30 min, Bleeding Time (c) and Blood Volume (d) was monitored.

weight (Figure 5c, 5d). A dose dependent effect on bleeding time (6-15 min) and blood volume (0.2-0.8 mL) was observed at various dose levels. The observed results were similar between male and female mice. The treatment of mice intravenously at 5 mg/kg of Clopidogrel Lipid Suspension resulted in 6.7 min of bleeding time and 0.21 mL of blood volume. The average bleeding time of 10.1 min and the corresponding average blood volume of 0.22 mL were observed after administering the mice at the dose level of 10 mg/kg. At the dose level of 20 and 40 mg/kg body weight, all the mice continued to bleed for 15 min, the maximum time period allowed for bleeding. The corresponding average blood volume was 0.35 mL and 0.77 mL was recorded for the mice treated intravenously with Clopidogrel Lipid Suspension at 20 and 40 mg/kg treatment respectively. The results demonstrated that mice treated orally at 20 mg Clopidogrel bisulfate/kg body weight (average bleeding time 7.7 min and blood volume 0.20 mL) produced the similar effects to those mice which were treated intravenously with Clopidogrel Lipid Suspension at 5 mg Clopidogrel bisulfate/kg body weight (6.7 min bleeding time and 0.21 mL of blood volume).

Phase I Clinical Safety Study

There were 48 subjects dosed in the clinical study with 12 subjects in each cohort. All the dosed subjects completed the study. The safety assessment was performed on all subjects who received the study drug during the course of the study (Table 2).

Nine subjects (18.75%) out of 48 experienced a total of 13 Adverse Events (AEs) during the study. Oral administration of Plavix® (Clopidogrel bisulfate) tablets 300 mg (cohort 1) resulted in 4 AEs, 1 AE – headache was possibly related but, other 3 AEs (2 catheter site pain, 1 toothache) were unlikely related to the drug administration. After intravenous treatment with 25 mg Clopidogrel Lipid Suspension (cohort 2), 4 AEs were reported and all the 4 AEs were possibly related to drug administration (2 catheter site pain, 1 heart rate increased and 1 dizziness). In cohort 3 (50 mg dose of Clopidogrel Lipid Suspension), a total of 3 AEs were reported with 1 AE (abdominal pain) and other 2 AEs (1 oropharyngeal pain and 1 cough) were unlikely related to the drug administration. At the highest dose (75 mg) of Clopidogrel Lipid Suspension, only 2 AEs were reported in two subjects with blood in the urine and both were possibly related to drug administration. All subjects who experienced AEs during this study recovered completely with the exception of 2 subjects who were lost to follow-up and no serious adverse events (SAEs) were reported during the course of the trial.

DISCUSSION

A novel Clopidogrel Lipid Suspension for injection was

Table 2: Summary of Adverse Events after intravenous administration of Clopidogrel Lipid Suspension or oral administration of Clopidogrel bisulfate.

Adverse Event Summary	Cohort I (N=12)	Cohort II (N=12)	Cohort III (N=12	Cohort IV (N=12)	Total (N=48)
Number of subjects who received study treatment or underwent examination	12	12	12	12	48
Total number of AEs	4	4	3	2	13
Total number of serious AEs	0	0	0	0	0
Total number of AEs that caused subject discontinuation	0	0	0	0	0
Subjects with AEs	2 (16.67 %)	3 (25.00 %)	2 (16.67 %)	2 (16.67 %)	9 (18.75 %)
Subjects with serious AEs	0	0	0	0	0
Subjects withdrew or dismissed due to AEs	0	0	0	0	0
Subjects withdrew or dismissed due to emesis	0	0	0	0	0
Subjects withdrew or dismissed due to other reasons	0	0	0	0	0

Cohort I (Reference Treatment) = Plavix® (Clopidogrel Bisulfate) Tablet 300 mg
Cohort II (Test Treatment) = Clopidogrel Lipid Suspension for Injection 25 mg
Cohort III (Test Treatment) = Clopidogrel Lipid Suspension for Injection 50 mg
Cohort IV (Test Treatment) = Clopidogrel Lipid Suspension for Injection 75 mg

developed to improve rapid systemic availability of Clopidogrel in atherothrombotic patients. The primary rationale for the development of lipid-based formulation was to have an alternate route of administration of Clopidogrel to patients who are in need of immediate medication and for whom oral route of administration is rather difficult. The formulation was prepared in an aqueous media and no organic solvent was used at any step of the process. The lipids used in the formulation were naturally occurring lipids, which are categorized as generally recognized as Safe (GRAS) by US FDA. This formulation was well characterized by physicochemical methods to show that the particle size is small, homogenous and stable. The size and distribution of Clopidogrel Lipid Suspension after reconstitution with water for injection was measured using dynamic scattering method and the average size was found to be about 28 nm. Electron microscopy revealed the presence of spherical unilamellar liposome particles. The entrapment efficiency of the drug was determined by passing Clopidogrel Lipid Suspension through Sephadex™ G-25 PD-10 Column and on analysis found to be completely associated with lipids.

Clopidogrel has been reported to be safe in rodents. The acute oral LD_{50} in mice and rats exceeded 2,000 mg/kg and intravenous LD_{50} was 110 and 160 mg/kg in rats and mice, respectively [14]. Clopidogrel Lipid Suspension at the dose levels of 20, 40 and 60 mg/kg was administered intravenously daily for 5 consecutive days in both mice and rats. Signs of toxicity were observed only at mid and high dose levels in mice with 50% mortality during the 5-day of dosing period at the high dose. The observations were consistent with reported cardio-respiratory (rapid death after cyanosis, dyspnea, apnea) toxicity following intravenous dose [15]. The results of toxicity studies in rodent species are comparable with the reported toxicity and apparently naturally occurring lipids present in the formulation were not a factor for the toxicity observed in mice at higher dose levels. Safety studies conducted in human subject indicated that Clopidogrel Lipid Suspension up to 75 mg dose was well tolerated.

Clopidogrel is extensively hydrolyzed by esterases to an inactive carboxylic acid metabolite that accounts for 85% of Clopidogrel related compounds circulating in plasma with a very low level of the parent drug [13,16-17]. The minor but active thiol-metabolite which requires metabolic activation by hepatic Cytochrome P-450 pathway is responsible to exhibit antiplatelet activity and it is unstable and difficult to quantify [1,13]. An indirect approach for studying the pharmacokinetics (PK) of Clopidogrel is to estimate the major inactive metabolite, CCA [13]. The maximum concentration (C_{max}) of 20.4 µg/mL of CCA in rat plasma was attained at 3 hour (T_{max}) following the oral administration of Clopidogrel bisulfate and the $AUC_{0\text{-}inf}$ and $AUC_{0\text{-}24h}$ were 398 and 382.0 (µg.h/mL), respectively (Table 1 and Figure 4). A C_{max} of 38.0 µg/mL of CCA in rat plasma was attained at 0.5 hour (T_{max}) following the intravenous administration of Clopidogrel Lipid Suspension and the $AUC_{0\text{-}inf}$ and $AUC_{0\text{-}24h}$ were 398 and 382 (µg.h/mL), respectively. A significantly greater C_{max} and T_{max} values of CCA in the present study are in agreement with the reported rapid metabolism of Clopidogrel [1,18-19] following intravenous administration in comparison to oral administration. Considering greater quantity of CCA measured following intravenous administration of Clopidogrel Lipid Suspension, the active thiol metabolite is expected to be formed in higher quantities compared to the oral administration of Clopidogrel bisulfate and that should result into more effective platelet inhibition.

Savi et al. [3] reported 76% inhibition of platelet aggregation activity following intravenous administration of 25 mg/kg of Clopidogrel in rats. Recently, an intravenous formulation of Clopidogrel (PM103) was also tested in healthy male and female volunteers and found to be safe. PM103 produced dose-dependent inhibition of platelet aggregation and there were no serious adverse events [6]. In the present study, the results were found to be consistent and a dose dependent effect on the platelet aggregation was observed in the mice treated with Clopidogrel Lipid Suspension at 5, 10, 20 and 40 mg/kg of Clopidogrel (Figure 5). Almost 4 time's greater oral dose (20 mg/kg) of Clopidogrel was required to produce the similar effects observed by intravenously administration in mice (5 mg/kg) with Clopidogrel Lipid Suspension. This clearly indicated an early onset of suppression of platelet aggregation results by intravenous delivery compared to oral administration.

CONCLUSION

A newly developed, stable, well characterized lipid-based formulation of Clopidogrel was found to be safe. Intravenously administered Clopidogrel Lipid Suspension was more effective to inhibit platelet aggregation in comparison to the Clopidogrel bisulfate given orally. Phase I clinical study indicated that Clopidogrel Lipid Suspension given to healthy human via intravenous administration was safe and well tolerated under fasting conditions, and no significant safety issues emerged. Phase II study in patients are planned.

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