Development, Characterization, and Pharmacokinetic Evaluation of a CRV431 Loaded Self-Microemulsifying Drug Delivery System

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ABSTRACT - PURPOSE: The objective of this study was to develop a self-microemulsifying drug delivery system (SMEDDS) formulation for the oral delivery of CRV431, a non-immunosuppressive analogue of cyclosporine A. Relative to cyclosporine A**,** CRV431 is poorly soluble in lipid solvents and thusly presents a challenge for the development of a formulation of sufficient oral bioavailability for clinical use. **METHODS:** The solubility of CRV431, a cyclosporine derivative, was determined in a range of commonly used surfactants, oils and co-solvents. A pseudo-ternary phase diagram was constructed from the most soluble excipients and prototype formulations, SERIES 1 and SERIES 2 were developed. The pharmacokinetics, following single oral doses of 1 and 3 mg/kg of CRV431 SMEDDS, was studied in healthy human volunteers using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). **RESULTS:** The maximum drug load for the SERIES 1 formulations was less than 40 mg/ml. Manipulation of the excipient ratios allowed for the development of SERIES 2 formulations, which had higher drug loading capacity and stability for CRV431 compared to SERIES 1. Further improvements allowed for the development of an optimized SMEDDS formulation containing up to 90 mg/ml CRV431 and which generated a microemulsion mean particle size of 25 nm when dispersed into aqueous media. The pharmacokinetics of the optimized CRV431 SMEDDS displayed excellent total body exposure and dose-proportional effects in humans, and high drug levels in the liver of rats. **CONCLUSIONS:** The developed SMEDDS formulation should allow for effective clinical development of CRV431, targeted to the treatment of liver diseases including hepatitis B (HBV), fibrosis, and hepatocellular carcinoma.

INTRODUCTION

CRV431 is a small molecule cyclophilin inhibitor under clinical development for the treatment of liver diseases including fibrosis and hepatocellular carcinoma. In preclinical studies CRV431 has shown anti-viral activity against a number of viruses including hepatitis B, hepatitis C, and HIV (1). CRV431 is a derivative of cyclosporine A (CsA), a neutral cyclic peptide consisting of eleven amino acids, wherein amino acids 1 and 3 have been chemically modified (Figure 1). These chemical modifications, respectively, remove immunosuppressive activity and enhance the binding to cyclophilin (1). The cytochrome P450-mediated Phase I in vitro metabolism of CRV431 was previously studied using selective chemical inhibition, recombinant human enzymes and liver microsomes (2). These studies revealed that the major enzymes involved are cytochromes P450 3A4 and 3A5, which produce various hydroxylated and demethylated metabolites. Importantly, all of the species identified in human microsomes were correspondingly identified in monkey and/or rat microsomes.

CsA, as a result of its particular structure, high molecular weight (1,203Da), high lipophilicity, high polar surface area, and low permeability has poor biopharmaceutical properties, and is classified as Class IV (2-4) according to the Biopharmaceutics Classification System (BCS) (5). Over the years, various oral drug carriers have emerged, such as solid dispersion (2, 6-7), nano-suspensions (8), liposomes (9) lipid nanoparticles (10), poly(lactic-co-glycolic acid (PLGA) nanoparticles (11), and self-microemulsifying drug-delivery systems (SMEDDS) (12). Only the SMEDDS of cyclosporine (Neoral®) has been successfully marketed owing mostly to its significant oral absorption, large-scale manufacturing capability, and low cost.

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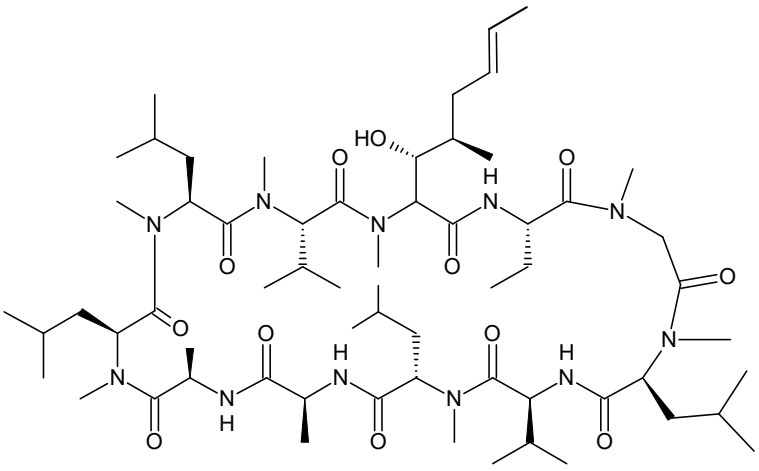
CsA has low aqueous solubility (less than 30 µg/ml), good solubility in organic solvents (greater than 200 mg/ml) such as ethanol, methanol, and DMSO, and lipophilic solvents and oils (around 100 mg/ml) (13). Owing to their poor water solubility, CsA and cyclosporine-based analogues have often been formulated as SMEDDS. In general, SMEDDS are isotropic mixtures of drug, oil, surfactants, and co-solvents, which form an oil-in-water microemulsion (less than 100 nm in diameter) upon contact with aqueous medium under gentle agitation (14-16). The oil phase and co-solvent being the primary driver of drug solubility while the surfactant being the primary driver of microemulsion formation due to its amphoteric properties. The commercial CsA SMEDDS (Neoral®) contains a surfactant and oil combination with several co-solvents which disperse quickly in water with a particle size of approximately 30 nm (17). Neoral® is highly solubilizing for CsA (> 100 mg/ml) and results in good blood exposure (18). Relative to cyclosporine, CRV431 has an increased aqueous solubility (150 µg/ml). While this may enhance pharmacological efficacy it is, however, insufficient for direct oral tableting. Additionally, CRV431 is significantly less soluble in lipophilic excipients classically used for SMEDDS development and consequently represents a challenge to develop a clinical product with good solubility, stability and blood exposure. In this manuscript, we discuss the development of a SMEDDS formulation, which optimizes the solubility of CRV431 and demonstrates significant blood exposure in humans following a single oral dose in healthy subjects.

MATERIALS and METHODS

Drugs and reagents

CRV431 was synthesized in-house to a purity of 97.3 % by modification of CsA and stored at 5°C. CsA was obtained from *IVAX* (Opava, Czech Republic). Lauroglycol 90, Labrasol, Labrafil M 2125, Labrafil 1944, Pecol, Labrafac WL, Capryol 90, Maisine CC, and Transcutol were purchased from *Gattefosse* (Montreal, Canada). Propylene glycol, filtered water, acetonitrile, methanol, scintillation vials and 12x75 ml borosilicate test tubes were purchased from *Fischer Scientific* (Pittsburgh, USA). Anhydrous ethanol was purchased from *Commercial Alcohols* (Toronto, Canada). Vitamin E, Tween 80, Tween 40, Tween 20, castor oil, dimethyl sulfoxide, Chremophor EL and Chremophor RH 40 were purchased from *Sigma-Aldrich* (St. Louis, USA). Span 80 was purchased from *EMD Millipore Corporation* (Burlington, USA). PEG 400 was purchased from *BDH Incorporated* (Mississauga, Canada).

Cyclosporine A



CRV431

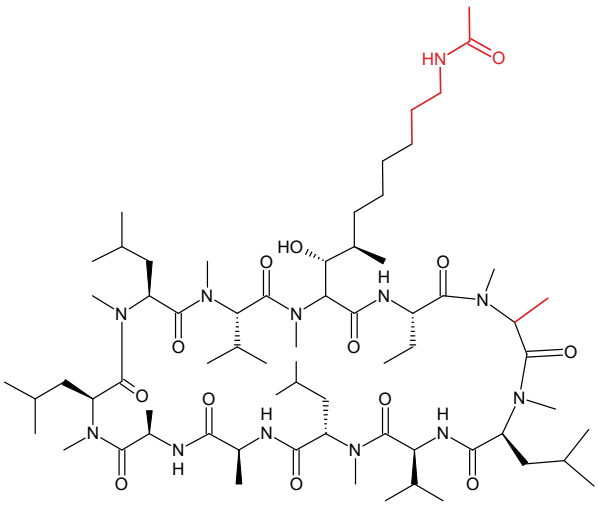


Figure 1. Chemical structures of Cyclosporine A and CRV431 indicating the modification positions (in red)

Physicochemical properties

To determine the aqueous solubilities of CRV431 and CsA, drug stocks at 20 mM in DMSO were diluted 30-fold to achieve a final concentration of 666 µM in phosphate buffered saline (PBS, pH 7.4), placed on a shaker overnight, and centrifuged at 3,300 rpm for 5 min to pellet insoluble drug. The supernatant was collected, diluted 1:1 with methanol, and drug concentrations measured by HPLC (Agilent 1100 series, UV detection) relative to standard curves. The polar surface area and log P were calculated using Molinspiration Cheminformatics (V2018.10, USA). Plasma protein binding was determined by equilibrium dialysis assays. Briefly, drug plasma samples were prepared to achieve a final concentration of 5 µM in human blood plasma by 500-fold dilution of 2.5 mM DMSO-drug stocks. Plasma solutions (300 µl) and PBS, pH 7.4 (500 µl), were added to adjacent chambers of Rapid Equilibrium Dialysis units (*Thermo Scientific*; 8K MWCO). Plates were sealed with plastic film and shaken on an orbital shaker at 225 rpm at 37°C for 4.5 hr. Plasma and PBS samples were collected, and drugs extracted with a zinc sulfate/methanol precipitation method. After centrifugation at 3,300 rpm for 10 min, soluble drugs in methanol supernatants were analyzed by Liquid Chromatography (LC) Electrospray Ionization (ESI) Mass Spectrometry (LC-ESI-MS) (Agilent 1100 series, Santa Clara, USA) and quantified relative to standard curves.

Excipient Solubility

The solubility of CRV431 was measured in groups of surfactants, oils, and co-solvents commonly utilized for the commercial preparation of SMEDDS formulations suitable for use in water dispersions and softgel capsules. CRV431 (50 mg) was added to the bottom of a 75x125 mm glass test tube and brought up to the 1 ml mark with excipient. The sample was placed on a rocker and visually assessed for solubility after overnight mixing. If solubility was not achieved, excipient was added in 0.5 ml increments, with subsequent overnight mixing, until a clear solution was reached. Visual assessment was considered sufficient to allow for solubility differentiation among the excipients, and relative to CsA.

Construction of Pseudo-Ternary Phase Diagrams

For construction of a pseudo-ternary phase diagram, mixtures containing different compositions of Cremophor RH 40 (surfactant), Maisine CC (oil) and co-solvents (Vitamin E, propylene glycol, Transcutol and ethanol) were evaluated to assess the phase boundary between microemulsion formation (clear to the eye) and non-microemulsion formations (cloudy to the eye) for the various excipient ratios.

CRV431 SMEDDS Development and Optimization

CRV431 (50-100 mg) was added to the bottom of a 75x125 mm glass test tube. Excipients were separately combined and mixed well prior to adding to CRV431 and brought up to the 1 ml mark. Several series of SMEDDS formulations were prepared and assessed/optimized for drug solubility, stability (by LC-ESI-MS), and microemulsion formation and stability (visual inspection) in aqueous media.

LC-ESI-MS Quantitation of CRV431 in SMEDDS Formulations

For all SMEDDS formulation analysis 10 µl was removed and added to 10 ml of methanol in a scintillation vial. The sample was vortexed for 10 seconds to mix and 1 ml of this solution was added to 9 ml of methanol in another scintillation vial (total 10,000-fold dilution). A 5 µg/ml CRV431 standard in methanol was also prepared. Samples (1 ml) were transferred to injection vials and analyzed by LC-ESI-MS on an Agilent HP 1100 LC-MS. Samples were placed in an autosampler maintained at 5°C. Samples and the CRV431 standard were injected (1 µl) onto a Zorbax SB-C18 reverse phase HPLC column (1.8 µm Rapid Resolution HT Cartridge, 4.6 x 30 mm) maintained at 75°C using an acetonitrile-water gradient system containing 0.02% glacial acetic acid and 20 µM sodium acetate (Table 1). The sodium-adduct of CRV431 (1326 m/z) was analyzed by mass spectrometry (MS) using electrospray ionization in positive ion mode. The ESI-MS was optimized with N_2 gas temperature set at 350°C and drying gas at 12/L min. The fragmentor and capillary voltages were set at 260 and 4000 volts, respectively. The nebulizer pressure was set at 40 psig. CRV431 elution time was typically observed at 6.0 minutes. The concentration of the formulations was calculated by peak area comparison with the one-point standard (5 µg/ml).

Table1. Gradient conditions for elution of CRV431.

Time (min)	dH_20* (%)	ACN*	Flow Rate (mL/min)
0.0	55	45	1.0
6.0	25	75	1.0
8.1	0	100	1.0
10.0	0	100	1.0
10.1	55	45	1.0

*also contains 0.02% Glacial acetic acid + 20 µM Sodium Acetate

Characterization of Optimized SMEDDS Formulation

Dispersion Study

Approximately 5 mL of the selected media (water, 0.1 N HCL, or phosphate buffer (pH 6.8) was placed in a vial and four (4) drops (around 40 mg) of the optimized SMEDDS was added. The mixture was observed initially, after inverting, and after one hour of adding the optimized SMEDDS.

Particle size

The mean particle diameter of the optimized SMEDDS was measured at 25°C by dynamic light scattering (Zetasizer Nano, Malvern Instruments, Malvern, UK) at an angle of 173°. Each sample was measured in triplicate. Values were expressed as a mean ± standard deviation. Mean value was 25 nm.

Pharmacokinetics of optimized CRV431 SMEDDS in humans

CRV431 SMEDDS was orally administered to 6 healthy fasted human volunteers as a single dose of either 1 or 3 mg/kg (75 mg or 225 mg). The study was conducted at a clinical research facility for investigational medicines (Celerion, Arizona, USA) as part of a clinical Phase 1 single-ascending-dose (SAD) study and, as such, followed all FDA ethical guidelines. Patients were selected according to standardized inclusion/exclusion criteria for a Phase I SAD study. Healthy volunteers 18-55 years of age with no evidence of ongoing disease (as determined by the study investigator), or any use of nicotine (30 days prior to screening), or drugs of abuse (within the preceding 2 years), or use of chronic prescription medication (within 30 days), or acute prescription medication (within 14 days), or systemic over-the-counter medications including vitamins and herbal/natural supplements (within 7 days prior to the study dose) were enrolled in the study. The SMEDDS was dispensed (approximately 3 ml) into a 100 ml Gibco clear bottle and a 15-fold excess of filtered water (HPLC grade) was added. The mixture was swirled gently for 1-2 minutes until fully dispersed. The SMEDDS in-water dose was placed in the refrigerator and orally administered within 2 hours. Whole blood samples (0.5 ml) were drawn by venipuncture into K2-EDTA blood collection tubes at 0.5, 1, 2, 4, 6, 8, 12, 24, 48, and 72 hours. Samples were immediately frozen and analyzed by LC-ESI-MS within 5 days. Briefly, whole blood samples were thawed and extracted using a zinc sulfate/methanol precipitation method. Quantitative analysis was performed by a validated LC-ESI-MS method against a 7-point whole blood standard curve. Non-compartmental analysis was performed to calculate pharmacokinetic parameters (C_{max}, AUC, $t_{1/2}$, and T_{max}) using commercial software (WinNonlin Professional Edition, Version 7.0, Pharsight software).

Pharmacokinetics of CRV431 SMEDDS in rats

CRV431 SMEDDS (50 mg/ml) was administered to Sprague Dawley rats (n = 6) by oral gavage at 30 mg/kg/day for 7 days. At 12 hours post-dose on day 7, the animals were sacrificed and whole blood and livers were extracted and frozen. CRV431 was quantitated in whole blood using LC-ESI-MS (as for the human pharmacokinetic studies). For quantitation of CRV431 in liver, one gram samples of liver tissue were added to 9 ml of homogenizing solution (1% formic acid in water) and homogenized using a TissueLyser (Qiagen) at 30 Hz for 10 minutes. Aliquots were removed and analyzed by LC-ESI-MS as above.

RESULTS

Physicochemical properties of CRV431

The physicochemical properties of CRV431 and CsA illustrated in Table 2 indicate that CRV431 has a significantly higher aqueous solubility relative to CsA, which is reflected in a higher plasma free-

fraction and higher polar surface area (PSA). The Log P values of both drugs are very high and, consequently, neither drug is suitable for oral development as a dry tablet.

Excipient solubility

The solubility of CRV431 was measured in groups of surfactants, oils, and co-solvents commonly utilized for the commercial preparation of SMEDDS formulations suitable for use in water dispersions and softgel capsules for oral dosage forms.

Choosing excipients for SMEDDS development

Owing to the CRV431 solubility in Cremophor RH 40 and the use of this excipient in Neoral®, Cremophor RH 40 was chosen as the surfactant. For the oil phase, CRV431 was found to have the highest solubility in Vitamin E followed by Maisine CC. While for co-solvents the solubility of CRV431 was highest in ethanol, followed by transcutol, and propylene glycol. Accordingly, all of the aforementioned excipients were chosen for preliminary formulation prototype development.

Table 2. Physicochemical properties of CRV431 and CsA

COMPOUND	SOLUBILITY IN PBS	MOLINS LOG P	MOLINS PSA	PLASMA FREE FRACTION
CRV431	115.0 µM	3.98	308	7.6%
CSA	31.9 µM	3.61	278	0.14 %

Table 3. Excipient Solubility of CRV431 and CsA at 21°C

Category	Excipient	CRV431 Solubility (mg/ml)	Cyclosporine Solubility (mg/ml)
Surfactant	Tween 80	< 10	> 100
Surfactant	Tween 40	< 10	> 100
Surfactant	Tween 20	< 10	> 100
Surfactant	Lauroglycol 90	30	ND
Surfactant	Labrasol	< 10	ND
Surfactant	Cremophor RH 40	30	> 50
Surfactant	Cremophor EL	< 10	ND
Surfactant	Labrafil M2125	< 10	> 100
Surfactant	Labrafil 1944	< 10	ND
Surfactant	Span 80	< 10	ND
Surfactant	Capryol	25	ND
Oil	Pecol	< 10	ND
Oil	Miglyol 812	< 10	> 100
Oil	Maisine CC	< 20	100
Oil	Vitamin E	25	ND
Oil	Castor Oil	< 10	> 100
Oil	Labrafac WL 1349	< 10	> 100
Co-solvent	Propylene glycol	30	> 100
Co-solvent	Transcutol	40	ND
Co-solvent	PEG 400	< 10	> 100
Co-solvent	Ethanol	> 200	> 200
Co-solvent	Dimethyl sulfoxide	< 50	> 100

The solubility of CRV431 in the surfactants tested (Table 3) ranged between 10-30 mg/ml, in contrast to CsA, which was at least 50 mg/ml, and in most cases, > 100 mg/ml. Given the high lipophilicity of CRV431, this was a surprising finding. The CRV431 solubility was highest in Cremophor RH 40, Lauroglycol 90 and Capryol 90. The solubility of CRV431 in the oils tested was strikingly less (generally 10 mg/ml) than CsA (> 100 mg/ml), with the exception of Vitamin E (25 mg/ml). The solubility of CRV431 in various co-solvents was found to be higher (10-50 mg/ml) than the surfactants and oils tested, although the solubility of CsA was, again, generally greater.

Development of CRV431 SMEDDS

Preliminary formulation studies involved assessing the impact of various excipient ratios on SMEDDS miscibility and microemulsion formation, in the absence of drug. The study results (Table 4) indicate that Cremophor RH40/Maisine combination ratios less than 3 do not form microemulsions when dispersed in water. Microemulsion formation was assessed by visual inspection and must be clear to the eye to be considered for continued development. Water dispersions with Cremophor RH40/Maisine ratios less than 3 were all cloudy. The data is diagrammatically expressed in a pseudo-ternary phase diagram (Figure 2) wherein the phase boundary for microemulsion formation is clearly demarcated.

Development and Stability of CRV431 SMEDDS Formulations: SERIES 1 and 2

SERIES 1

Based on the preliminary SMEDDS microemulsion studies a prototype SMEDDS formulation (Table 5: formulation # 1) was prepared and assessed for CRV431 solubility and stability. The objective was to produce a miscible and stable SMEDDS formulation which solubilized CRV431 to at least 50 mg/ml, and formed a clear microemulsion in aqueous media. While formulation #1 initially solubilized CRV431 to 50 mg/ml and produced a clear microemulsion, upon subsequent storage, formation of CRV431 crystals adhering to the glass container became apparent within days and coincided with loss of CRV431 as quantitated by LCMS (Figure 3).

Further SMEDDS were developed (SERIES 1, Table 5) wherein the proportion of transcutol co-solvent was increased in an attempt to encourage further solubility and decreased crystal formation. All SERIES 1 formulations were able to develop microemulsions when dispersed in water (displayed within the blue circle of Figure 2 phase diagram). The stability results (Figure 3) however, show that all SERIES 1 SMEDDS are unstable and significant CRV431 crystals form upon storage at room temperature.

SERIES 2 SMEDDS

All SERIES 2 SMEDDS formed clear SMEDDS solutions (Table 6). These formulations are shown to fall within the microemulsion phase (**red** circled area) of the pseudo-ternary phase diagram (Figure 2).

Optimized CRV431 SMEDDS

To further enhance CRV431 solubility the ethanol content was increased. Table 7 demonstrates that when the ethanol weight ratio is increased to 2.4 the drug load of CRV431 can be increased to at least 90 mg/ml and is considered stable. A 100 mg/ml sample was prepared but did not fully solubilize CRV431 and was thus omitted from further testing. All sample preparations were stable when measured after 54 days of storage at room temperature. The CRV431 SMEDDS formulation containing Vitamin E/Maisine CC / Propylene Glycol / Transcutol/ Ethanol / Cremophor RH40 in the weight ratio of 1/1/5/5/2.4/4 is thusly considered the optimized CRV431 SMEDDS.

Table 4. Preliminary SMEDDS Studies: Excipient Compatibility and Microemulsion Formation as a function of excipient ratios

Cremophor RH40	Maisine CC	Vitamin E	Propylene glycol	Transcutol	Ethanol	SMEDDS (appearance)	Microemulsion formation
3	3	0	10	0	2	Biphasic	NO
3	5	0	8	0	2	Biphasic	NO
3	9	0	4	0	2	Miscible	NO
6.2	5	3	1.2	0	2	Miscible	NO
6.4	7.8	0	1.6	0	1.6	Miscible	NO
7.2	6	1	1.2	0	2	Miscible	NO
7	3	0	6	0	2	Miscible	NO
7	4.5	0	4.5	0	2	Miscible	NO
7	1	0	8	0	2	Miscible	YES
9	3	1	3	0	2	Miscible	YES
10	4.2	0	1.5	0	2	Miscible	NO
11	4	0.5	0	0	2	Miscible	YES
13	1	0	2	0	2	Miscible	YES

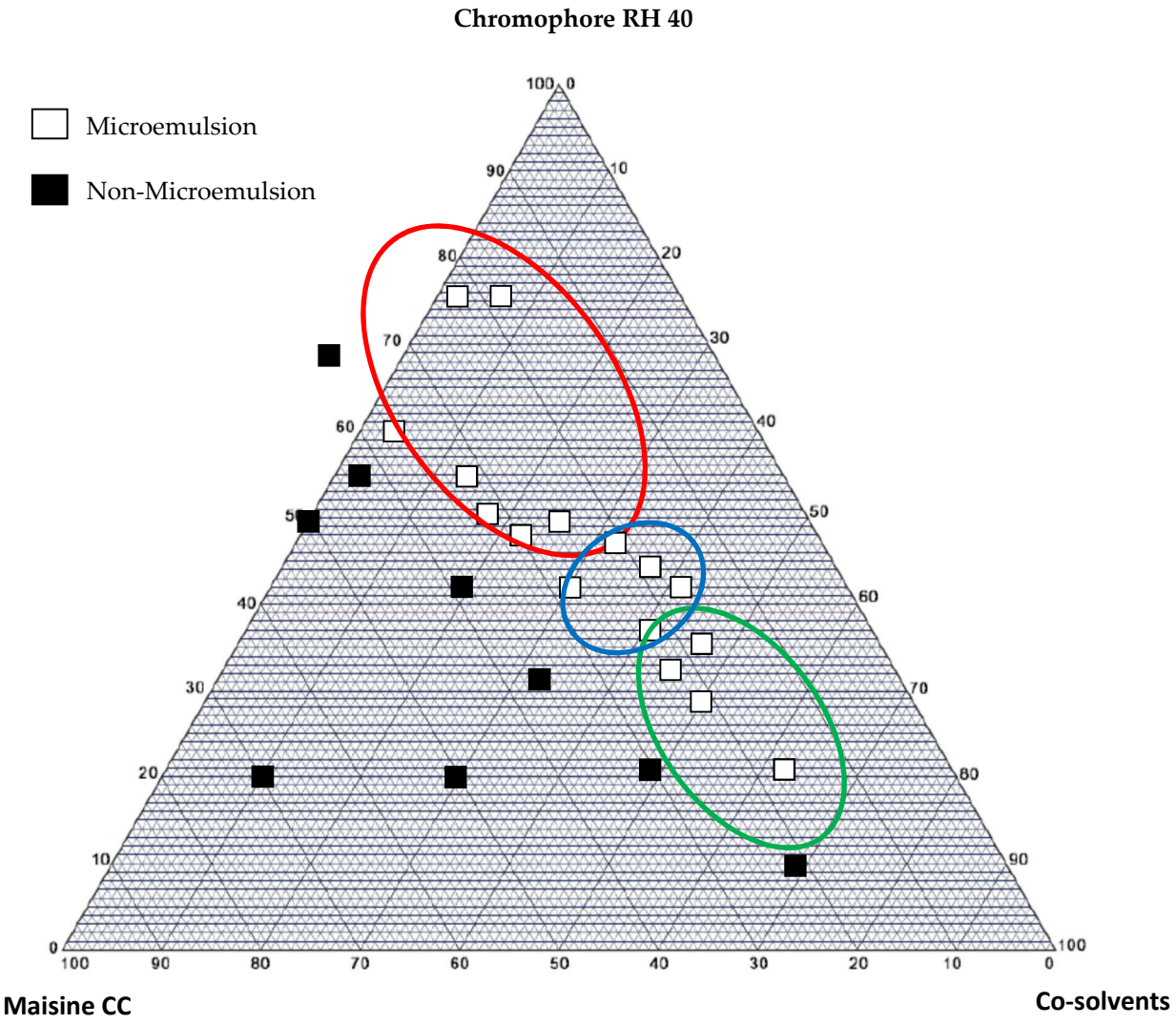


Figure 2. Pseudo-ternary phase diagram showing oil/water microemulsion region for ratios of Cremophore RH 40, Maisine CC, and co-solvents. SMEDDS within the red area represent the prototype formulations with high surfactant. SMEDDS within the blue area represent SERIES 1 formulations containing high co-solvent amounts. SMEDDS within the green area represent SERIES 2 formulations with low surfactant and high co-solvent amounts

Table 5. SERIES 1 excipients and ratios

Formulation #	Vitamin E	Maisine CC	Propylene glycol	Transcutol	Ethanol	Cremophor RH40
			Excipient Ratio by weight			
1	1	3	3	0	2	9
2	1	3	2	1	2	9
3	0.5	3	2	1.5	2	9
4	0.5	3	1.5	2	2	9
5	0.5	2	1.5	3	2	9
6	1	2	2	2	2	9
7	0.5	2	0.5	4	2	9
8	1	2	2.5	2.5	2	8

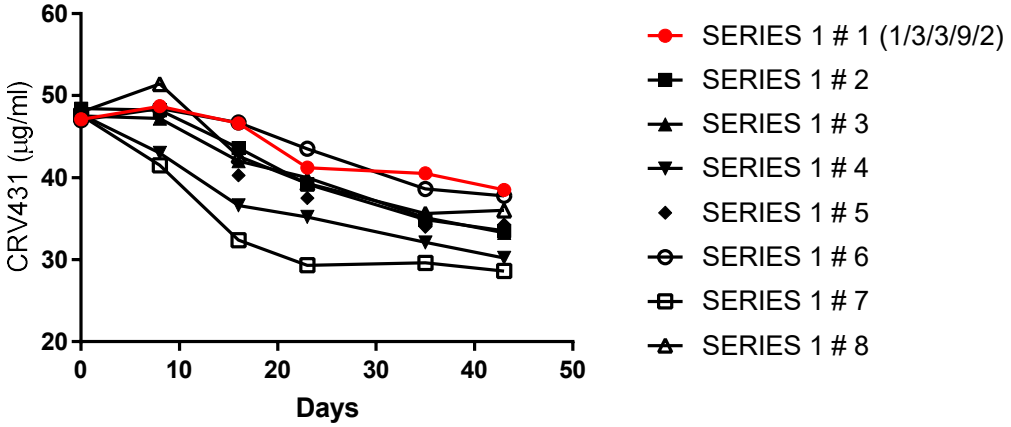


Figure 3. Stability of CRV431 SERIES 1 SMEDDS by LC-ESI-MS

Table 6. SERIES 2 SMEDDS. All formulations contained CRV431 at 50 mg/ml, except where indicated.

Formulation #	Vitamin E	Maisine CC	Propylene glycol	Transcutol	Ethanol	Cremophor RH40
1	1.5	2	2.25	2	2.25	8
2	1.5	2	2	2.25	2.25	8
3	1.5	2	2.75	2.5	2.25	7
4	1.5	2	2.5	2.75	2.25	7
5	1	2	3.5	3.5	2	6
6	1	1	5	5	2	4
6 (75 mg/ml)	1	1	5	5	2	4

All SMEDDS from SERIES 2 were shown to have much extended stability (Figure 4) relative to the original SERIES 1 SMEDDS prototype (1/3/3/9/2). SERIES 2 SMEDDS (#3-6) have no observable loss of drug after 77 days storage at room temperature.

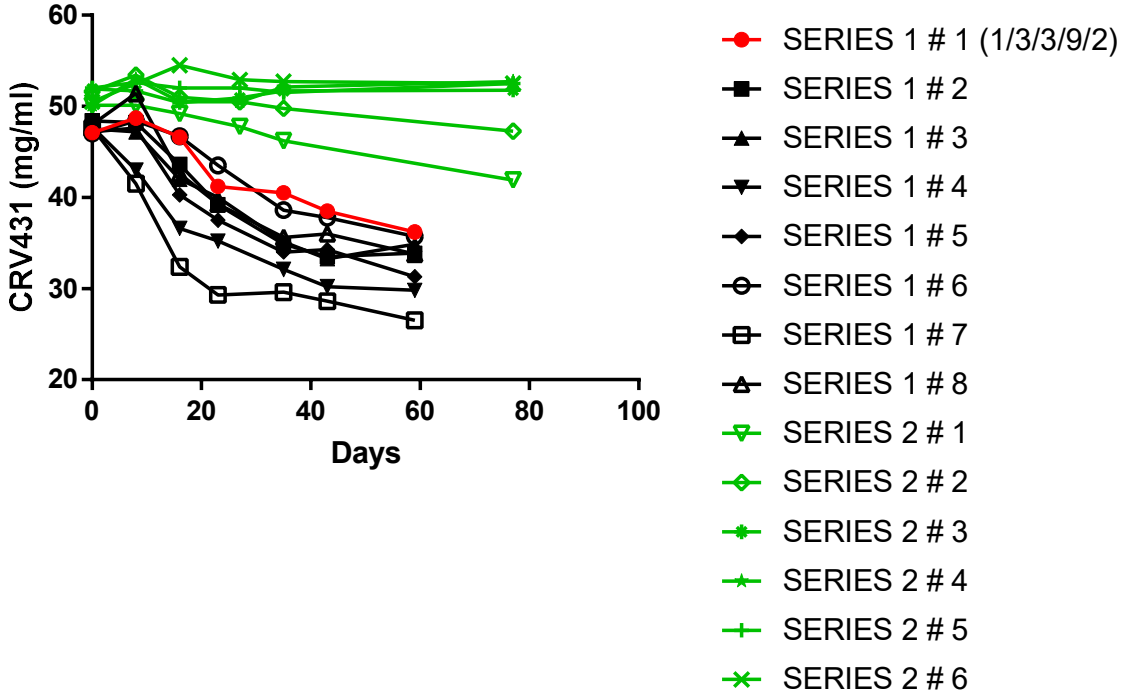


Figure 4. SERIES 2 SMEDDS stability compared with SERIES 1. Aliquots of SMEDDS formulations were removed at the indicated times and analyzed by LCMS.

Table 7. Solubility and stability of CRV431 in optimized SMEDDS.

SMEDDS Formulation: 1/1/5/5/2.4/4 (Vitamin E/Maisine CC/Propylene Glycol/Transcutol/Ethanol/ Cremophor RH40 (w/w/w/w/w/w)	
Prepared CRV431 Concentration (mg/ml)	Measured CRV431 Concentration on Day 54 (mg/ml)
75	75.2
80	78.6
90	90.0

Characterization of optimized CRV431 SMEDDS formulation

Microemulsion Formation and Stability of CRV431 SMEDDS

When CRV431 SMEDDS is dispersed in aqueous media, a visually clear solution is formed within less than one minute of gentle swirling and/or inversion. Water, 0.1 N HCL, and phosphate buffer microemulsion solutions remained clear for up to 1 hour at 37°C (Table 8).

Particle size

The mean particle diameter of the CRV431 SMEDDS formulation when dispersed in water was measured to be 24.6 ± 5.7 nm (mean ± standard deviation) (Figure 5). This size is consistent with the reported particle size of Neoral® aqueous dispersions (17).

Pharmacokinetics of CRV431 SMEDDS

The pharmacokinetics of CRV431 SMEDDS in humans demonstrated excellent exposure and approximate dose proportionality (Figure 6 and Table 9]. Relative to reported cyclosporine A (2.5 mg/kg) exposures in healthy human subjects (23), CRV431 C_{max} was slightly greater than Neoral®, and overall exposure (AUC) was approximately 14 times greater than Neoral®. In a rat study the level of CRV431 distributed in the liver (10 µg/g tissue) was found to be 6.5-fold greater than the whole blood fraction (Figure 7).

Table 8. Microemulsion stability in aqueous media at 37°C

Timepoint	Observations (Visual)		
	Water	0.1 N HCL	Phosphate Buffer (pH = 6.8)
Initial	Tiny droplets dispersed throughout	Tiny droplets dispersed throughout	Tiny droplets dispersed throughout
After swirling and inversion	Clear transparent solution	Clear transparent solution	Clear transparent solution
After 1 Hour	Clear transparent solution	Clear transparent solution	Clear transparent solution

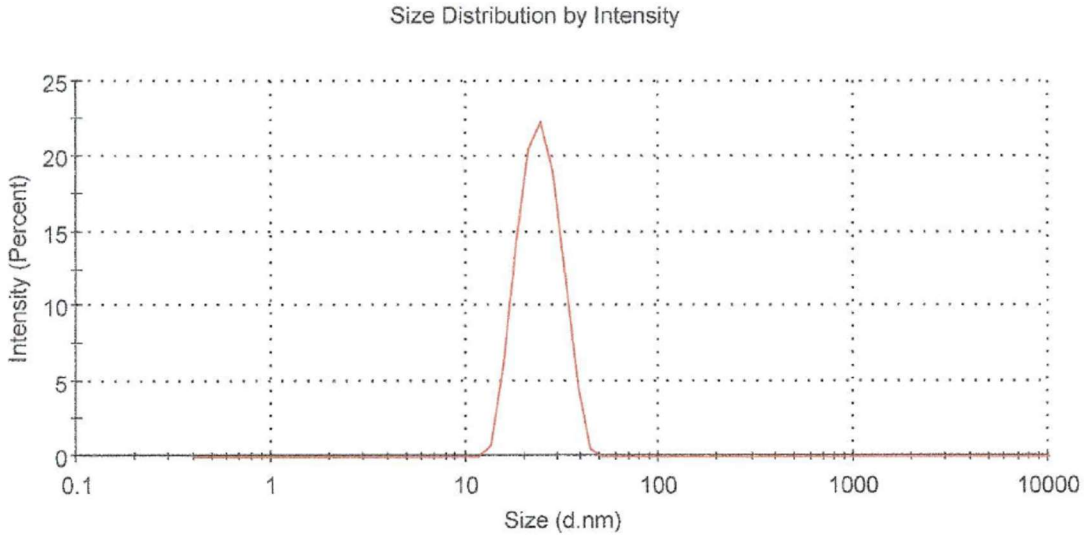


Figure 5. CRV431 SMEDDS Aqueous Dispersion. Representative particle diameter measurement.

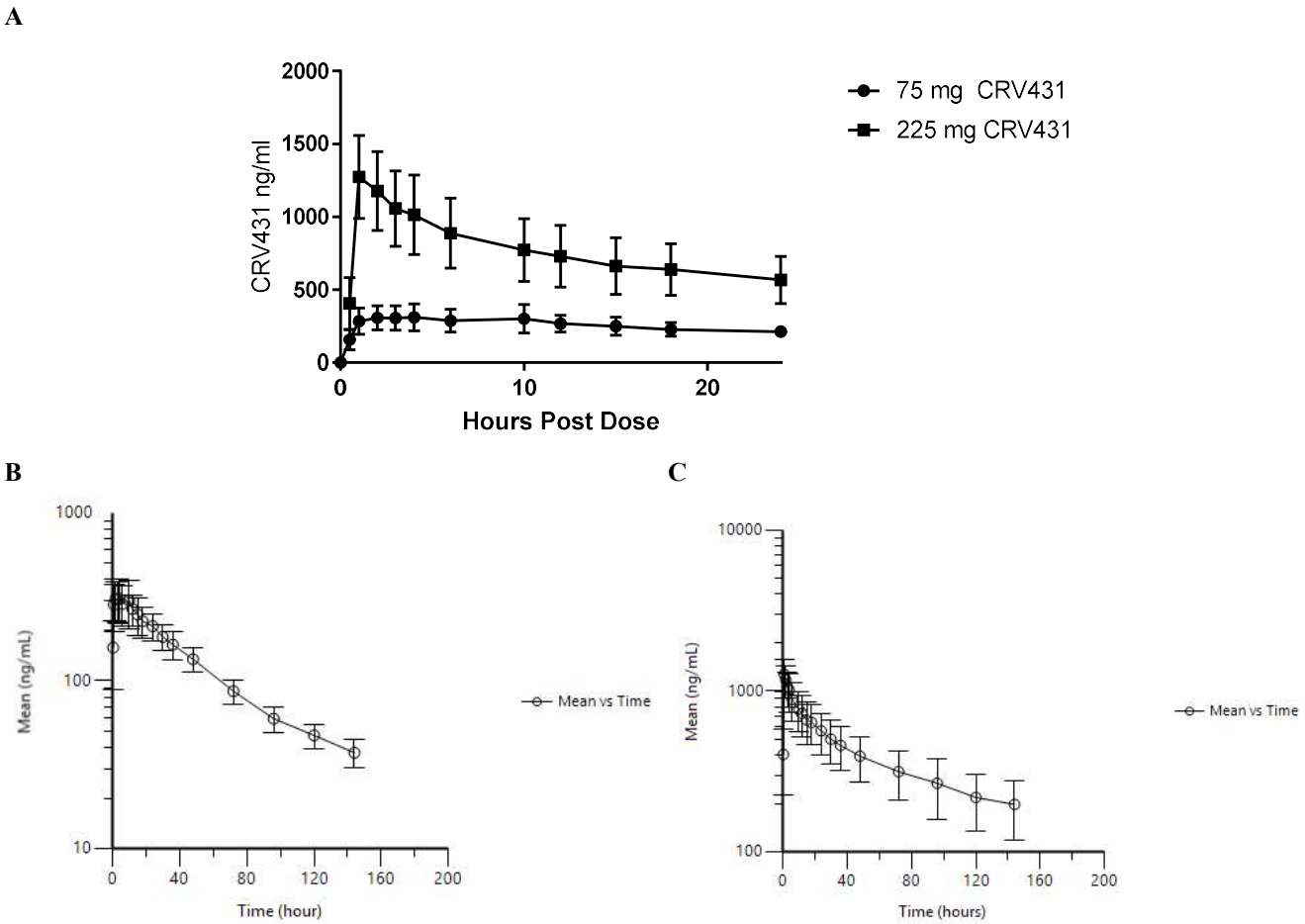


Figure 6: Pharmacokinetics of CRV431 in humans. Patients (n = 6) were given a single dose of CRV431 and whole blood aliquots analyzed by LC-ESI-MS at the indicated time points. Error bars represent mean ± standard deviation. (**A**) Linear scale. (**B**) Log scale for the 75 mg dose. (**C**) log scale for the 225 mg dose.

Table 9. Single-dose pharmacokinetics of CRV431 in 6 healthy human volunteers relative to cyclosporine A. Values represent mean ± standard deviation

Dosing Group	Dose (mg/kg)	AUC $_{0-\infty}$ (ng●hr/ml)	C_{max} (ng/ml)	T_{max} (Hours)	$t_{1/2}$ (Hours)
CRV431	1	20,916 ± 3,780	334 ± 106	4 ± 3.09	73.6 ± 15.2
CRV431	3	84,421 ± 32,373	1,368 ± 221	1.33 ± 0.52	97.4 ± 18.4
Cyclosporine A[A]	2.5	4,981 ± 1,584	944 ± 244	1.67 ± 0.48	6.3 – 20.4[B]

[A] reported in reference 23
[B] reported in reference 27

DISCUSSION

It is well known that the majority of the new synthesized chemical entities (approximately 60% of drugs) are poorly water soluble (19). Consequently, many of these substances have bioavailability problems after oral administration. The clinical success of these drugs depends heavily on finding approaches to enhance solubility and oral absorption. The importance of formulation development to successful drug development is particularly important in the case of CsA. CsA is a hydrophobic peptide with a unique structure consisting of 11 amino acid residues, seven of which are N-methylated. The extensive methylation and hydrophobic character of the amino acid residues together with their four intra-molecular hydrogen bonds, which confer a high rigidity to the cyclic structure, suggest that the drug has a very low aqueous solubility (20, 21). CsA also lacks ionizable functional groups, so manipulation of the pH will likely not enhance its solubility.

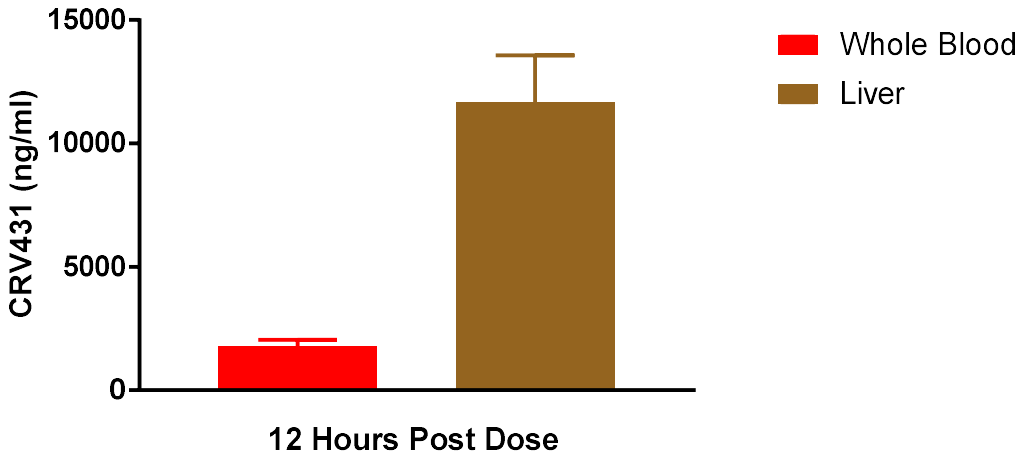


Figure 7. Whole blood and liver levels of CRV431 in Sprague Dawley rats. Sprague Dawley rats (n = 6) were dosed (30 mg/kg) with CRV431 SMEDDS (50 mg/ml) by oral gavage for 7 days and whole blood aliquots and liver homogenates were analyzed by LC-ESI-MS 12 hours after the final dose. Error bars represent mean ± standard deviation.

Early in development, the absence of adequate formulations for CsA placed scientists on the verge of ceasing development of this compound (22). The subsequent development of lipid based self-microemulsifying drug delivery systems (SMEDDS) formulations has allowed for the development of clinically viable formulations of many Class IV molecules. In the case of CsA and analogues it has allowed for the preparation of aqueous dispersions with at least 100-fold greater solubility relative to the native molecule. The commercial formulation of CsA (Neoral®) has a reported bioavailability in humans of 20-30 % (18, 23).

The aqueous solubility of CRV431 was found to be approximately 4-fold greater than CsA (115 vs 30 µg/ml), and this was also reflected in the calculated polar surface area (308 vs 273 angstroms). While the increase in water solubility might be expected to result in lower plasma protein binding (higher free faction), CRV431 is still considered sparingly soluble in water and would not be expected to have any appreciable clinical utility if formulated in a solid tablet. The calculated Log P for CRV431 (3.98) is still very high and, consequently, its solubility in lipid would be expected to be similar to cyclosporine. The actual solubility of CRV431 in various lipid surfactants and oils is, however, far less than cyclosporine (Table 3). The reason for this is not known, however, the implication for formulation development is substantial. Primarily, the intrinsic solubility of CRV431 in any given SMEDDS formulation would be expected to be significantly less than CsA, leading to a lower loading capacity. This represents a significant challenge. At minimum, for a microemulsion to form a SMEDDS formulation must, at least, contain an oil/surfactant mixture. Into this mixture various co-solvents can be added to enhance drug solubility. Common oil/surfactant pairings include Cremophor RH 40/Maisine or labrafrac (24), Tween/Miglyol or labrasol (25,26), labrafil/castor oil. In this study, the surfactant with the highest solubility for CRV431 included Cremophor RH 40, Lauroglycol 90 and Capryol 90. Both Lauroglycol 90 and Capryol 90 are water-insoluble surfactants and would not be expected to be useful in formation of oil-in-water microemulsions. Indeed, mixtures of either Lauroglycol or Capryol 90 with oil and co-solvent excipients were observed to form oil-droplet in water rather than miscible dispersions (data not shown). Therefore, owing to the CRV431 solubility in Cremophor RH 40 and the use of this excipient in Neoral®, Cremophor RH 40 was chosen as the surfactant. Based on the solubility of CRV431 in the oils and co-solvents tested, the excipients: Vitamin E; Maisine CC; ethanol; Transcutol; propylene glycol, along with Cremophor RH40 were chosen for SMEDDS prototype development. A pseudo-ternary phase diagram was constructed by plotting formulation percentages of Maisine CC (oil) vs Cremophor RH40 (surfactant) vs co-solvent mixtures in the absence of drug (Figure 2). These studies indicated that Cremophor RH40/Maisine combination ratios less than 3 did not form

microemulsions (white opaque by visual inspection). The phase boundary for microemulsion formation (clear in water dispersion) is clearly demarcated and represents approximately 40% of the total area of the phase diagram.

Based on the pseudo-ternary phase diagram a prototype SMEDDS formulation (Table 4: formulation # 1) was prepared containing Cremophor RH 40/Maisine CC/PG/Ethanol/Vitamin E in the ratio of 9/3/3/2/1 and containing 50 mg/ml CRV431. The SMEDDS formulation remained clear for several days after which transparent crystals became apparent and adhered to the bottom of the glass vessel. A series of SMEDDS were developed (SERIES 1, Table 5) wherein the proportion of transcutol co-solvent was increased in an attempt to encourage further solubility and decreased crystal formation. All SERIES 1 formulations formed microemulsions when dispersed in water; however, they were also unstable at room temperature. In general, as the level of transcutol increased there was an increase in formulation instability. This is likely less to do with the increase in transcutol and more to do with the reduction of the other co-solvents in which CRV431 is also moderately soluble. In any case, owing to instability, the SERIES 1 SMEDDS are not a viable direction to pursue for a clinical formulation.

Since the pseudo-ternary diagram (Figure 2) indicates that a microemulsion zone also exists at lower surfactant and oil ratios, the rationale behind the development of SERIES 2 CRV431 SMEDDS formulations was to increase the excipient co-solvents in which CRV431 is most soluble (vitamin E, propylene glycol, transcutol and ethanol) and minimize the surfactant and oil components in which CRV431 is least soluble, while maintaining the ability to form a clear microemulsion upon aqueous dispersion. All SERIES 2 SMEDDS have much extended stability (Figure 4) relative to the original prototype formulation (9/3/3/2/1). Several of the SMEDDS had no observable loss of drug after 77 days storage at room temperature and are, therefore, considered stable. The increased stability is rationalized as resulting from the increased solubility of CRV431 in these SMEDDS such that CRV431 is not at its limit of solubility as was the case for the SERIES 1 SMEDDS. Indeed, SMEDDS 1/1/5/5/2/4 (Vitamin E/ Maisine CC / propylene glycol / Transcutol / ethanol / Cremophor RH40 (w/w/w/w/w/w) was also easily prepared at 75 mg/ml. Since the formulation development objective was to optimize the CRV431 drug load, the weight ratio of ethanol was increased from 2 to 2.4. Considering that the clinical SMEDDS formulation will be manufactured and delivered to patients as a softgel capsule, further enhancement of ethanol was not considered since this amount of ethanol would be nearing softgel compatibility limits (personal communication with softgel manufacturer). The CRV431 SMEDDS (1/1/5/5/2.4/4) was found to have a solubility limit of 90-100 mg/ml and was considered stable. When the optimized CRV431 SMEDDS was dispersed in aqueous media, the particle size was measured to be 25 nm, which is consistent with the reported particle size (30 nm) of the Neoral® SMEDDS.

The pharmacokinetics of CRV431 SMEDDS (1/1/5/5/2.4/4) was studied in human healthy subjects at 1 and 3 mg/kg. Excellent exposures and dose proportionality was observed. Relative to reported cyclosporine A (2.5 mg/kg) exposures in healthy human subjects, CRV431 Cmax was slightly greater than Neoral®, and overall exposure (AUC) was approximately 14 times greater than Neoral®. This appears to be predominantly due to the large difference in half-life of CRV431, approximately 100 hours, relative to cyclosporine, which is reported to range from 6.3 hours in healthy subjects to 20.4 hours in severe liver disease (27). CsA and analogues are reported to be extensively distributed into the red blood cell fraction in whole blood distribution studies (18, 28, 29), most likely as a result of high affinity binding to cyclophilin. Indeed the inhibitor dissociation constant (Ki) for CRV431 is a least 10-fold more potent than CsA in inhibiting cyclophilin (1), and this is likely a major contributor to the extended half-life. Since the primary clinical focus of CRV431 is in the area of liver disease, the SMEEDS formulation must be capable of delivering sufficient drug to this organ. In rat repeat-dose studies we were able to demonstrate that CRV431 levels in the liver (10 μg/g tissue) were at least 6.5-fold greater than in whole blood. The ability to deliver high levels of CRV431 to its primary site of action in the liver will be important as this drug moves forward through clinical development.

DISCLOSURE

The authors are employed by ContraVir Pharmaceuticals.

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