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Solid-State Behavior and Solubilization of Flash Nanoprecipitated Clofazimine Particles during the Dispersion and Digestion of Milk-Based Formulations

Malinda Salim,[†] Gisela Ramirez,[†] Andrew J. Clulow,[†] Yingyue Zhang,[‡] Kurt D. Ristroph,[‡] Jie Feng,[‡] Simon A. McManus,[‡] Adrian Hawley,[§] Robert K. Prud'homme,[‡] and Ben J. Boyd*[,†,∥]

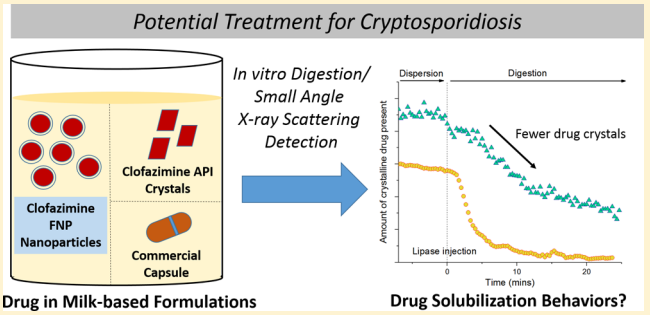
[†]Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences and [∥]ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, Monash Institute of Pharmaceutical Sciences, Monash University (Parkville Campus), 381 Royal Parade, Parkville, Victoria 3052, Australia

[‡]Department of Chemical and Biological Engineering, Princeton University, Princeton, New Jersey 08540, United States

[§]SAXS/WAXS Beamline, Australian Synchrotron, ANSTO, 800 Blackburn Rd, Clayton, Victoria 3169, Australia

Supporting Information

ABSTRACT: Clofazimine, a drug previously used to treat leprosy, has recently been identified as a potential new drug for the treatment for cryptosporidiosis: a diarrheal disease that contributes to 500 000 infant deaths a year in developing countries. Rapid dissolution and local availability of the drug in the small intestine is considered key to the treatment of the infection. However, the commercially available clofazimine formulation (Lamprene) is not well-suited to pediatric use, and therefore reformulation of clofazimine is desirable. Development of clofazimine nanoparticles through the process of flash nanoprecipitation (FNP) has been previously shown to provide fast and improved drug dissolution rates compared to clofazimine crystals and Lamprene. In this study, we investigate the effects of milk-based formulations (as possible pediatric-friendly vehicles) on the in vitro solubilization of clofazimine formulated as either lecithin- or zein/casein-stabilized nanoparticles. Milk and infant formula were used as the lipid vehicles, and time-resolved synchrotron X-ray scattering was used to monitor the presence of crystalline clofazimine in suspension during in vitro lipolysis under intestinal conditions. The study confirmed faster dissolution of clofazimine from all the FNP formulations after the digestion of infant formula was initiated, and a reduced quantity of fat was required to achieve similar levels of drug solubilization compared to the reference drug material and the commercial formulation. These attributes highlight not only the potential benefits of the FNP approach to prepare drug particles but also the fact that enhanced dissolution rates can be complemented by considering the amount of co-administered fat in lipid-based formulations to drive the solubilization of poorly soluble drugs.



KEYWORDS: *clofazimine, nanoparticles, milk, infant formula, drug solubilization, in vitro digestion, X-ray scattering*

INTRODUCTION

Clofazimine is a riminophenazine antibiotic that has been used for the treatment of multibacillary leprosy in a multidrug therapy recommended by the World Health Organization (WHO).[1] Its efficacy against *Mycobacterium tuberculosis* has also been evaluated because of the emergence of multidrug-resistant strains.[2] More recently, clofazimine was identified as a potential new drug for the treatment of cryptosporidiosis.[3] Cryptosporidiosis is an infection of the epithelial cells in the gastrointestinal and respiratory tracts by *Cryptosporidium* parasites[4] and is one of the leading causes of diarrhea in children in developing countries.[5] Children under the age of five, particularly those who are immunocompromised, are susceptible to *Cryptosporidium* infections where the diarrhea can cause severe dehydration and malnutrition that can lead to diarrhea deaths: more than 500 000 children die each year from diarrheal diseases,[6] and only one drug (nitazoxanide) has currently been approved as an oral suspension for systematic treatment of cryptosporidiosis.[7] An oral suspension is not favored for use in challenging climate conditions from a stability standpoint, and consequently there is therefore an urgent need to develop new solid-state drug formulations to achieve local and fast onset treatment with high efficacy for pediatric populations.

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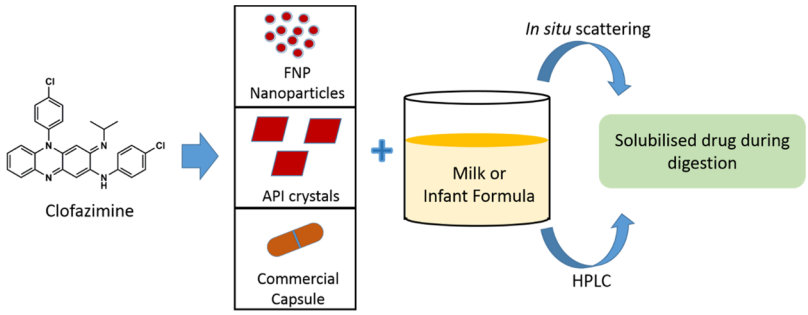

Figure 1. Schematic representation of the concept of this study on investigating the solubilization of clofazimine (in the form of NPs produced by the FNP process, clofazimine API crystals, and clofazimine in commercially available capsules) in milk or IF during in vitro digestion.

Clofazimine is a lipophilic compound (log P = 7.5[1]; chemical structure in Figure 1) that is categorized as either a class II or class IV drug in the biopharmaceutics classification system.[8] Hence, the dissolution rate of clofazimine in the gastrointestinal tract can become the rate-limiting step in oral absorption.[9] In the treatment of gut-resident cryptosporidiosis, rapid and complete dissolution of the drug is required for optimal treatment. A range of formulation approaches has been made to enhance the solubility and dissolution rate of clofazimine, including complexation,[10] formation of clofazimine salts,[11,12] encapsulation of clofazimine in polymeric or silica nanoparticles (NPs)[13,14] and liposomes,[15] incorporation of clofazimine in self-microemulsifying drug delivery systems,[16] and size reduction via high-pressure homogenization and milling.[17] More recently, the formation of clofazimine NPs using flash nanoprecipitation (FNP) followed by spray-drying to produce fine powders that can be readily reconstituted in aqueous solution for pediatric use has been reported.[18,19] FNP is a process of nanoprecipitation where the rapid, controlled mixing between an aqueous phase and an organic phase containing a drug and an amphiphilic stabilizer induces high supersaturation conditions that lead to precipitation and encapsulation of hydrophobic drugs.[20,21] The dissolution rates of clofazimine NPs in suspensions have been shown to be superior compared to Lamprene, the current commercial formulation based on micronized drug in an oil−wax matrix within 50 or 100 mg capsules.[18]

We have previously gained a new understanding of milk as a pediatric drug delivery system for poorly water-soluble drugs by considering the impact of digestion on the lipid species produced and their subsequent interaction with drugs during the digestion process.[22−25] Full cream milk typically contains 3.5−4.0% w/v fat, of which about 98% are triglycerides and could potentially serve as a lipid-based formulation for the delivery of poorly water-soluble drugs for pediatric acceptability.[25−27] Previous reports have shown that administration of clofazimine with fat-containing food can improve the oral bioavailability, although the results are highly variable.[28,29] Administration of clofazimine with a high-fat meal, for example, has been shown to improve the bioavailability of clofazimine compared to fasting.[29] Clofazimine should therefore be taken with food or milk, as recommended by WHO,[30] although the effects of milk on the solubilization of clofazimine are unclear and no bioavailability studies have been conducted.

Considering the poor aqueous solubility of clofazimine and its proposed use in pediatric treatment, the behavior of clofazimine formulations in the presence of digesting milk was studied. To further understand the behavior of clofazimine NPs in the gastrointestinal tract after oral administration with milk-based systems, in this work, the solubilization behavior of the different clofazimine formulations (NPs, reference drug, and the commercial formulation) during in vitro intestinal digestion was investigated using synchrotron small-angle X-ray scattering (SAXS). This approach enables the kinetics and the extent of drug solubilization to be determined and also enables potential identification of any intermediate solid-state forms of the drug which may form during digestion as clofazimine is known to exhibit polymorphism and solvatomorphism.[11,31] As differences in the molecular packing can give rise to different physical properties including melting point, dissolution, and solubility, these different crystalline forms can potentially affect the stability and oral bioavailability of clofazimine, and the characterization of the polymorphs and their transformations is of great importance. The influence of the types of lipids on the solubilization of clofazimine was probed using milk and a low-lactose infant formula (IF). The use of IF is particularly advantageous as it allows a wide range of fat contents to be explored by the use of varying amounts of IF powder dispersed in the system. High-performance liquid chromatography (HPLC) was used to quantify the phase distributions of clofazimine in milk and IF during and after digestion.

EXPERIMENTAL METHODS

Materials. Clofazimine active pharmaceutical ingredient (API) (≥98% purity), Trizma maleate reagent grade, sodium azide (≥99%, Fluka), and 4- bromophenylboronic acid (4-BPBA, >95% purity) were purchased from Sigma-Aldrich (St. Louis, Missouri). Lamprene capsules were sourced from Novartis Pharmaceuticals Corp., East Hanover, NJ. Calcium chloride dihydrate (>99% purity), sodium hydroxide pellets (minimum 97% purity), and salicylic acid were purchased from Ajax Finechem (New South Wales, Australia). Hydrochloric acid (36%) was purchased from LabServ (Ireland). Sodium chloride (>99% purity) was purchased from Chem Supply (South Australia, Australia). Lipase (USP-grade pancreatin extract) was purchased from Southern Biologicals (Victoria, Australia). Bovine milk (Pauls brand, 3.8% and 4.7 wt % fat) was purchased from local supermarkets (Victoria, Australia). IF powder (brand not disclosed as it is commercial-in-confidence) was generously provided by Medicines for Malaria Venture (Geneva, Switzerland). Nutritional information for the milk and the IF used in this study is summarized in Table S1 in the Supporting Information. Medium-chain triglycerides (MCTs, triglycerides of caprylic/capric acid, Labrafac WL 1349) were a generous gift from Gattefossé (Saint-Priest, France). Long-chain triglycerides (LCTs) containing high amounts of *sn*-2 palmitate (Infat CC) were a gift from Enzymotec Ltd. (Midgal HaEmek, Israel). Milk fat globular

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membrane (MFGM-enriched whey protein concentrate) was a generous gift from Arla Food Ingredients (Viby J, Denmark). Water was sourced from Milli-Q Millipore purification systems (Merck Millipore, Australia). Unless otherwise stated, all chemicals were used without further purification.

Preparation of Clofazimine Nanoparticles Using Flash Nanoprecipitation. The clofazimine NPs were prepared using zein (a hydrophobic protein)[32] and lecithin (mixtures of phospholipids) as the stabilizers, as they are generally recognized as safe ingredients. NPs were formed via FNP using a multi-inlet vortex mixer (MIVM) as previously described.[18,19,33] In brief, for the lecithin-stabilized formulation, clofazimine was dissolved at 50 mg/mL along with lecithin at 25 mg/mL in tetrahydrofuran and fed into an MIVM at 16 mL/min using a Harvard syringe pump apparatus. Three water streams, each at 36 mL/min, were also fed to the mixer. The resulting NPs were subsequently mixed with concentrated mannitol solution with an NP/mannitol weight ratio of 1:3 before drying using a B-290 mini spray-dryer (Büchi Corp., New Castle, DE) under conditions reported previously.[19] For the zein/casein formulation, four different streams were fed to the MIVM: (1) clofazimine at 6 mg/mL in acetone, (2) zein in 60%/40% ethanol/water v/v at 6 mg/mL, (3) citrate buffer at pH 7.4, and (4) sodium caseinate at 1 mg/mL in citrate buffer at pH 7.4.[18] These particles were also spray-dried. The NP powder samples were stored in a freezer at −20 °C after preparation. The schematic diagram and the mechanistic description for the formation of NPs are outlined in the Supporting Information, Figure S1. The size distributions of lecithin and zein/casein-stabilized NPs in water are shown in Figure S2 in the Supporting Information.

Preparation of Lipid Formulations and In Vitro Lipolysis Experiments. Dispersions of clofazimine API, Lamprene, and the zein/casein- and lecithin-stabilized clofazimine NPs were prepared by the addition of 50 mg clofazimine (and in the case of Lamprene and the NPs, the appropriate mass of formulation to match this mass of drug) to 2.5 mL of water and 0.25 mL of 1 M HCl. The amount of drug present in the digesting formulation was selected from preclinical results that validated the local concentration when completely dissolved. The drug solution was mixed with 17.5 mL of the medium for testing. The different media were digestion buffer alone (no fat), milk, IF of specific fat content (Table 1), dispersions of MCTs, LCTs, or 0.5 wt % methyl cellulose (MC)/0.5 wt % Tween 80 (T80) prepared in digestion buffer. The digestion buffer was 50 mM tris maleate containing 5 mM $CaCl_2{\cdot}2H_2O$, 6 mM NaN_3 and 150 mM NaCl, adjusted to pH 6.5 (see Table 1). The dispersions of MCTs and LCTs were prepared by mixing the triglycerides (0.76 g) with MFGM (0.11 g) as an emulsifier in 10 mL of digestion buffer prior to ultrasonication at 2 s on/off cycles and 25% amplitude with 3 and 4 min processing times (Misonix S-4000 ultrasonic liquid processor, NY, USA) for the MCTs and LCTs, respectively. The volume of the samples was subsequently adjusted to 20 mL with the digestion buffer.

Table 1. Summary of the Fat Content Used in the Formulations

formulation	fat content (w/v, %)	amount of fat in the formulation (g)	ratio of CFZ API equivalent to fat (mg/g)
tris buffer	0.0	0.00	N/A
milk	4.7	0.82	60.8
	3.8	0.67	74.6
IF	7.8	1.37	36.5
	3.8	0.67	74.6
	2.0	0.35	142.9
	1.0	0.18	277.8
	0.25	0.04	1250

For subsequent lipolysis experiments, the drug mixtures were added to a thermostatted glass vessel at 37 °C under constant magnetic stirring, and the pH of the samples was adjusted to 6.500 ± 0.005 prior to the injection of pancreatin solution (2.25 mL, with approximately 700 tributyrin unit/mL of digest) to initiate lipolysis. The pancreatin lipase solution was prepared from the pancreatin extract using the methods previously described.[34] The pH of the samples was maintained at 6.5 during digestion by the automatic dosing unit of the pH-stat apparatus (902 STAT titration system, Metrohm AG, Herisau, Switzerland) using 2 M NaOH. All of the lipolysis experiments reported herein were performed in the absence of bile salts (that could serve to accelerate lipid digestion and drug solubilization) with high enzymatic activity; it is important to recognize that the conditions in the small intestine of malnourished children with chronic diarrheal disease differ from that of healthy patients and that such conditions are not well characterized; so, they are used here as nominal reference points.[35]

Synchrotron SAXS Measurements. *Flow-through Measurements.* The pH-stat digestion apparatus was interfaced to the SAXS and wide-angle X-ray scattering (WAXS) beamline at the Australian Synchrotron (ANSTO, Clayton, Victoria), as has been described previously.[27] The samples from the digestion vessel (after pH adjustment to 6.5) were aspirated through a fixed quartz capillary (Charles Supper Co., Natick, MA, USA) mounted in the X-ray beam (wavelength = 0.954 Å, photon energy = 13 keV) using a peristaltic pump operating at approximately 10 mL/min. Two-dimensional SAXS images were acquired using a Pilatus 1 M detector with a 5 s acquisition time and a 15 s delay with a sample-to-detector distance of 562 mm (covering a q range of $0.04 < q < 1.82$ Å^{-1}), where $q = (4\pi/\lambda)\sin\theta$, with λ being the photon wavelength and 2θ the scattering angle. The raw data were reduced to scattering functions $I(q)$ versus q using the in-house developed software package Scatterbrain version 2.71.

Static Capillary Measurements. Diffraction patterns of the clofazimine API powder and the spray-dried clofazimine NPs (zein/casein- and lecithin-stabilized) were acquired using the SAXS/WAXS beamlines at the Australian Synchrotron (ANSTO, Clayton, Victoria). The powder samples were loaded into special glass microcapillaries of 1.5 mm outer diameter (Charles Supper Co., Natick, MA, USA) and placed in the X-ray beam (wavelength = 0.954 Å, photon energy = 13 keV). Acquisition times of 1 s were used, and the sample-to-detector distance was 560 mm to give a q range of $0.04 < q < 1.95$ Å^{-1}.

Hot-Stage Measurements. X-ray diffraction measurements of clofazimine across a range of temperatures were performed to confirm the identities of the drug polymorphs by correlation with the known temperature-dependent solid-state transformations.[31] Clofazimine API and the NP powder samples were placed on glass cover slips and covered with Kapton tape. The samples were secured in a Mettler Toledo FP82HT hot stage, with the sample window placed in the X-ray beam (wavelength = 0.954 Å, photon energy = 13 keV), and the temperature of the hot stage was increased at 10 °C/min until

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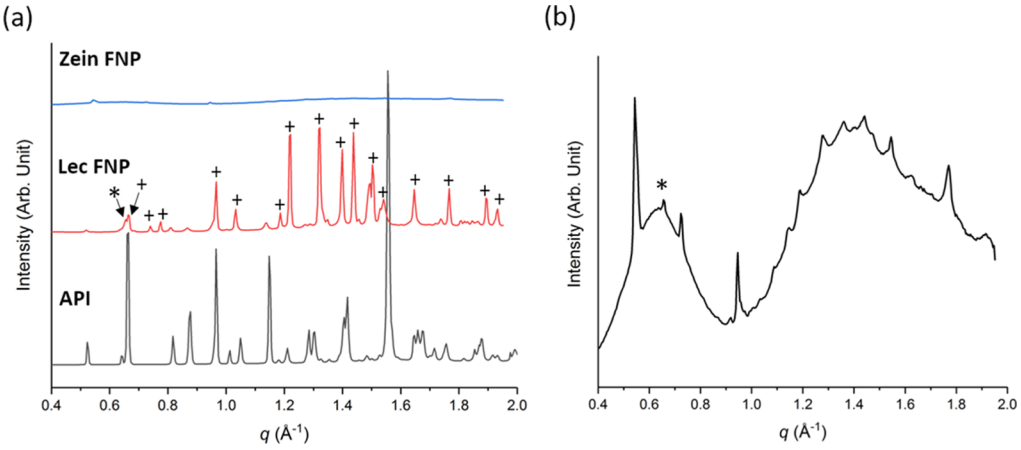

Figure 2. (a) X-ray scattering patterns for clofazimine API, lecithin-, and zein/casein-stabilized NPs, and (b) close-up view of the zein/casein NP powder pattern after background subtraction from an empty capillary. Positions of the mannitol peaks are indicated by "+" in panel (a), and the asterisk symbol "*" in panel (b) illustrates the F I peak of interest.

no diffracted X-rays from crystallites were detected. Acquisition times of 1 s were used with a 11 s delay between acquisitions while heating (corresponding to steps of 2 °C, one measurement every 12 s), and the sample-to-detector distance was 572 mm to give a q range of $0.04 < q < 1.93$ Å^{-1}.

Quantification and Distribution of Clofazimine in the Digested Formulations. Digested samples containing clofazimine in lipid-free (buffer only) and lipid-based formulations were collected before (0 min) and during digestion at predetermined time points (2, 5, 30, and 60 min). The enzymatic activity of the lipase was inhibited using 0.5 M 4-BPBA prepared in methanol, where 2 μL was added to 200 μL samples prior to ultracentrifugation at 434 900g for 1 h. The resultant lipid phase (lower density than the supernatant), aqueous supernatant phase, and the pellet phase (higher density than the supernatant) were collected separately, and the drug in each layer was extracted using methanol (500 μL of methanol for the pellet and lipid layers, and 400 μL in 100 μL of the aqueous supernatant layer). The methanol used for extraction contained salicylic acid as an internal standard for HPLC.[36] The samples were then vortexed and ultrasonicated in a water bath sonicator for approximately 40 min prior to centrifugation at 16 160g for 7 min. The supernatants were collected and diluted with the mobile phase for the HPLC measurements (26:74 v/v 0.25 M sodium acetate trihydrate, pH 3.3/methanol). The separation of clofazimine was performed using a 150 mm long reversed-phase C8 column (Phenomenex Luna, 5 μm particle size, 100 Å pore size, and 4.6 mm inner diameter) at 35 °C on a Shimadzu Nexera system based on isocratic elution with UV detection at 286 nm. The injection volume of the samples was 10 μL and the flow rate was 1 mL/min. The concentrations of clofazimine were determined based on the standard curves prepared from 0.01 to 40.0 μg/mL in the relevant digested media (buffer, IF, or milk).

RESULTS AND DISCUSSION

Solid-State Forms of Clofazimine and the Effects of Heating. Previous studies have shown that polymorphs of clofazimine can be identified as form (F) I, II, III, and IV, with the thermodynamic stability of the solids predicted to be in the order of F III > F II > F I at room and physiological temperatures,[31] whereas clofazimine F IV was only seen at high temperatures of approximately 91 °C.[31] The X-ray diffraction pattern of commercially available clofazimine sourced from Sigma-Aldrich (marked API in Figure 2a) is the F I polymorph, which is known to be arranged in a triclinic lattice with two molecules per unit cell.[37] The evaluation of the solid forms of clofazimine after the FNP process revealed the presence of different polymorphs with varying degrees of crystallinity observed depending on the types of stabilizers used (upper two profiles in Figure 2a). The X-ray diffraction pattern for lecithin-stabilized clofazimine NPs contained high-intensity well-defined Bragg peaks, including signals from both clofazimine and the mannitol excipient crystals (indicated by "+" in Figure 2a, with Figure S3 in the Supporting Information showing the X-ray diffractogram of mannitol[38]), whereas the zein/casein-stabilized NPs possessed less-intense diffraction peaks that were indicative of lower crystallinity. The lack of crystallinity of clofazimine in the zein/casein NPs was also reflected by the previously reported broadening of the melting temperature range and lower onset of melting temperature in the zein/casein-stabilized NPs when compared to clofazimine API.[18]

The analysis of the peak positions for lecithin- and zein/casein-stabilized NPs indicated that both the samples contained clofazimine in the F I polymorph. The scattering peak at q = 0.66 Å^{-1}, which was only present for the F I polymorph,[31] was clearly seen for lecithin NPs in Figure 2a and is clearer for zein/casein NPs in the expanded view in Figure 2b, labeled by an asterisk. However, peaks belonging to the F II polymorph were also observed in the zein/casein NP powder, with the characteristic peaks observed at q = 0.55 and 0.95 Å^{-1}. The diffraction peaks of the F II polymorph had higher intensities than the diffraction peaks of the F I polymorph, indicating that F II was the dominant polymorph for the zein/casein NPs. In the lecithin NP powder, peaks that did not belong to the F I polymorph also appeared but were not attributable to the other clofazimine polymorphs and could arise from the mannitol crystals in the excipients.[18,19] The positions of the peaks in the NP samples and the four polymorphs of clofazimine (data from the literature) are summarized in Table S2 in the Supporting Information, and the identifier codes for the Cambridge Structural Database were DAKXUI01 (F I), DAKXUI (F II), DAKXUI03 (F III), and DAKXUI02 (F IV).

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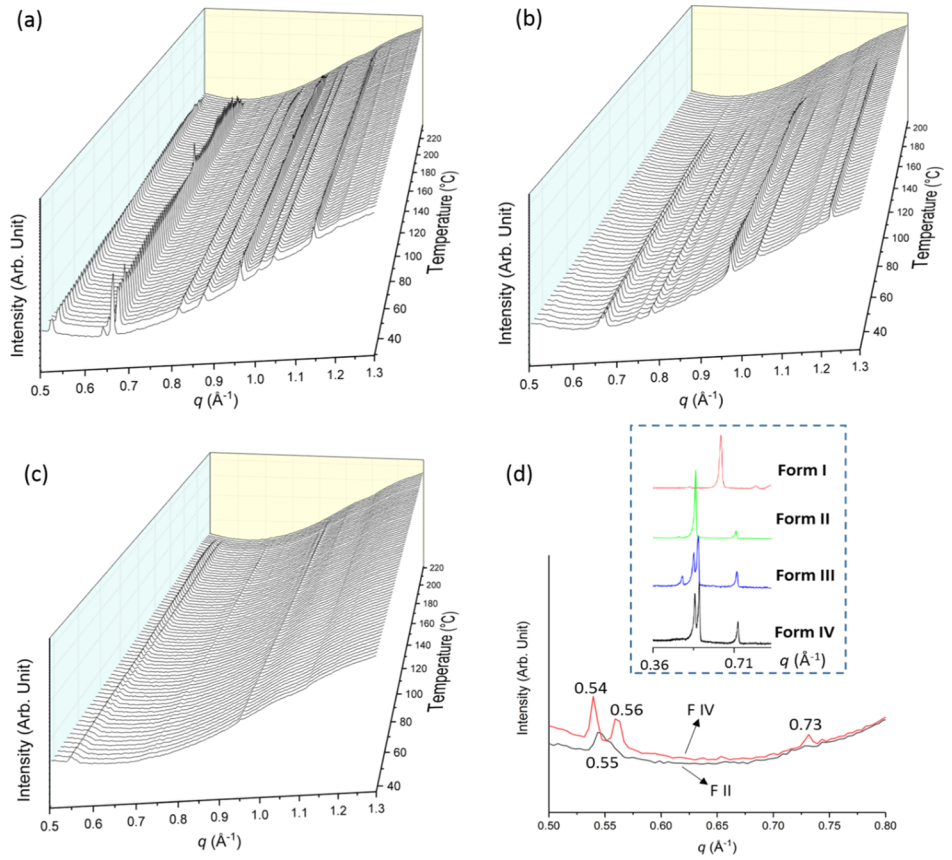

Figure 3. X-ray scattering patterns of (a) clofazimine API (b) lecithin NPs, and (c) zein/casein NPs as a function of increasing temperature. (d) Close-up view of zein/casein NPs at 40 °C (F II polymorph) and 180 °C (F IV polymorph) with reference to the clofazimine API powder X-ray diffractogram.[31]

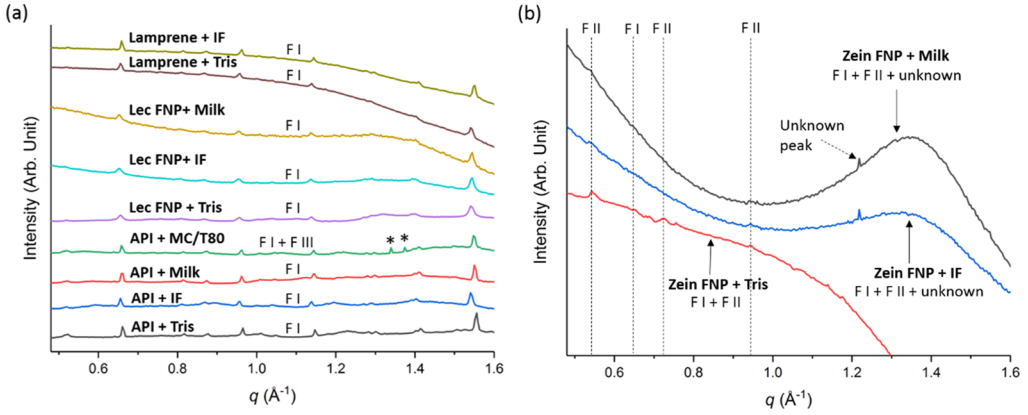

Figure 4. X-ray scattering patterns of (a) clofazimine API, lecithin FNP NPs, and Lamprene, as well as (b) zein/casein NPs after dispersion in tris and various media (milk and IF) after background subtraction of the tris buffer.

Previous reports have suggested that the transformations of clofazimine from one polymorphic form to another could also occur with changes in temperature, depending on the initial polymorphs present.[31] The clofazimine F IV polymorph was only seen after heating of the F II polymorph to ∼91 °C, whereas no changes in crystal structure occurred during the heating of the F I and F III polymorphs.[31] Therefore, no structural changes were anticipated during the heating of clofazimine API and the lecithin NP samples, but the polymorphic transformation from the F II to the F IV polymorph could take place in the zein/casein NP powders.

To test this hypothesis, clofazimine API and the NP samples were heated until no observable diffraction peaks were present, and the X-ray scattering profiles obtained during the heating cycle are shown in Figure 3. The diffraction peaks from the F I polymorph in clofazimine API disappeared after heating to approximately 228 °C, and no observable new peaks were observed during heating, indicating an absence of polymorphic transitions. In contrast, the diffraction peaks of the lecithin NPs disappeared when the sample was heated to between 164 and 172 °C because of the melting of the mannitol crystals,[19,38] with a weak residual peak observed at q = 0.66 Å^{-1}, characteristic of trace amounts of the F I polymorph. The

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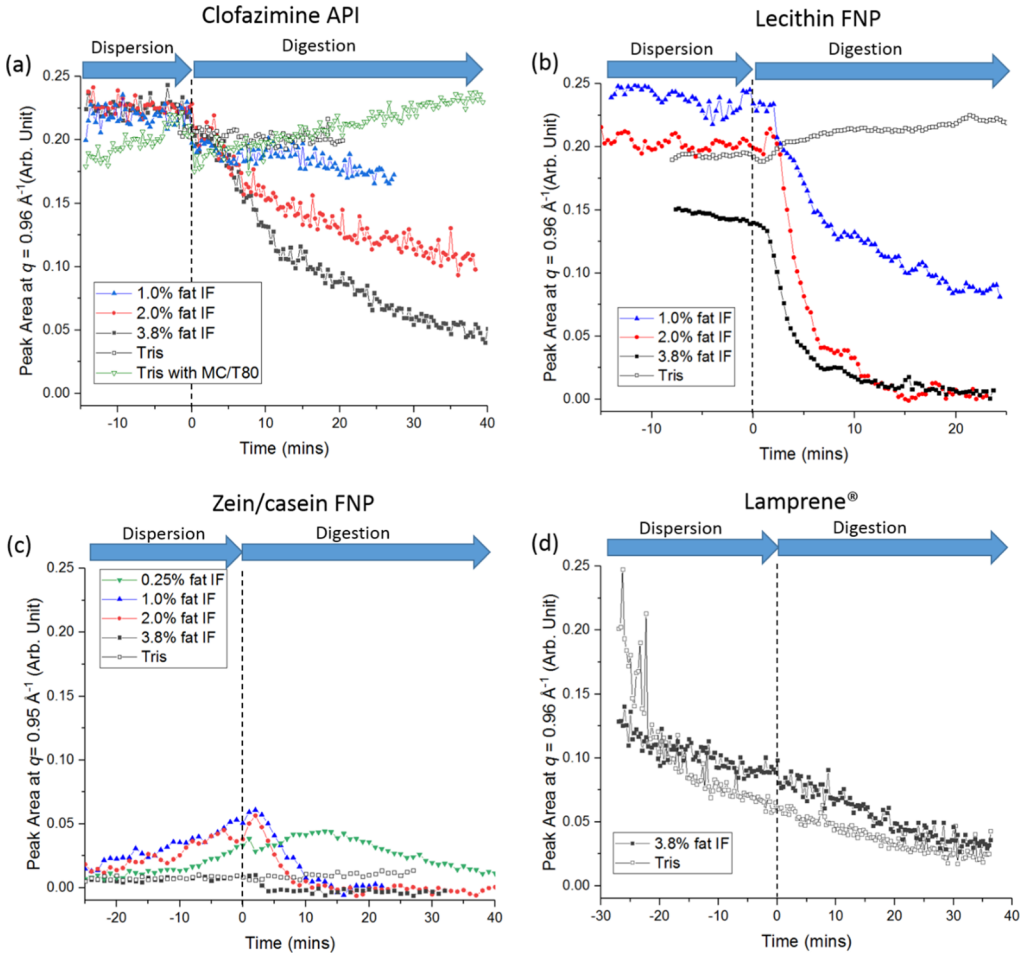

Figure 5. Area under the diffraction peaks for clofazimine in (a) clofazimine API, (b) lecithin NPs, (c) zein/casein NPs, and (d) Lamprene during dispersion (negative time scale) and digestion (positive time scale) in tris and IF from 0 to 3.8% fat. Peaks at q = 0.96 Å^{-1} and q = 0.95 Å^{-1} were representative of the F I and F II clofazimine polymorphs, respectively. Lipase was injected at time = 0 min.

results obtained were consistent with the melting endotherm from the differential scanning calorimetry (DSC) thermogram[19] and from the optical polarizing microscope images (Figure S4 in the Supporting Information) acquired during heating of the lecithin NP samples where two melting events (birefringent−isotropic transitions) were observed. In the case of zein/casein NPs, where a polymorphic transformation was expected upon heating, splitting of the peak at q = 0.55 Å^{-1} into two separate peaks at 0.54 and 0.56 Å^{-1} was observed as the temperature was increased to ∼89 °C, indicative of polymorphic transformations from the F II to the F IV polymorph. The diffraction peaks of the F IV polymorph subsequently disappeared completely at about 214 °C. This correlated well with the DSC profiles reported in the previous studies, which indicated the complete melting of clofazimine zein/casein NP samples at this temperature.[19]

Solid-State Forms of Clofazimine in Buffer, Infant Formula, and Milk during Dispersion. The propensity of one polymorphic form of a drug to transform into another as a solution-mediated transformation in the absence of digestion is of interest for pharmaceutical applications. This is because the physical properties of polymorphs are often significantly different, and this can impact their solubility and dissolution behavior in the gastrointestinal tract. Therefore, we investigated the polymorphic forms of clofazimine following dispersion of the different formulations in aqueous media with and without lipids present (milk/IF or tris buffer, respectively) prior to lipid digestion. The X-ray scattering patterns are summarized in Figure 4 and the polymorphic forms of clofazimine in the different formulations are annotated on the profiles.

The positions of the diffraction peaks for clofazimine on dispersion of Lamprene and lecithin NPs were comparable to those for clofazimine API, suggesting that the F I polymorph was present. However, additional peaks at q = 1.34 and 1.37 Å^{-1} (peaks with asterisks in Figure 4a) that were exclusive to the F III polymorph were observed when clofazimine API was dispersed in a solution containing MC/T80, a standard formulation used in pharmacokinetic studies in animal models. The results therefore suggest that these surfactants could potentially cause polymorphic changes of clofazimine, although these transformations were not seen when clofazimine was dispersed in milk and IF. Interestingly, comparisons between the diffraction peak positions for the lecithin FNP samples before (sample in the powder form, Figure 2) and after dispersion (Figure 4a) in buffer, IF, and milk showed the disappearance of several peaks, notably at q = 1.22, 1.32, 1.44, and 1.49 Å^{-1}, which was attributed to the solubilization of the mannitol excipient.

The scattering profiles for the zein/casein clofazimine NPs on dispersion in various media are shown separately in Figure 4b for clarity. No changes in the scattering of the F I or F II

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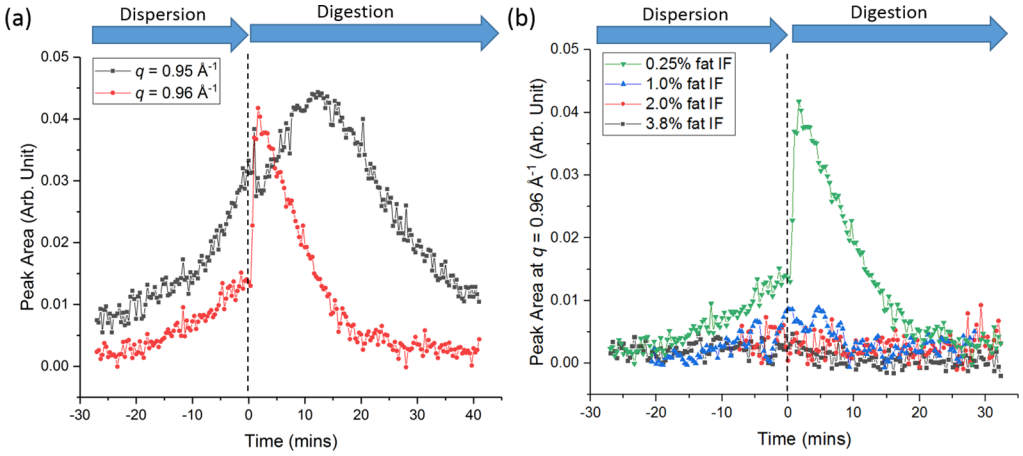

Figure 6. (a) Area under the diffraction peaks for clofazimine at q = 0.95 Å^{-1} (F II polymorph) and 0.96 Å^{-1} (F I polymorph) during dispersion (negative time scale) and digestion (positive time scale) of zein/casein-stabilized clofazimine FNP NPs in 0.25% fat IF. (b) Effects of fat content in IF on the formation of the F I polymorph (diffraction peak at 0.96 Å^{-1}) during dispersion and digestion.

polymorphs were apparent before and after dispersion of the zein/casein clofazimine NPs, although an additional peak at q = 1.22 Å^{-1} appeared when the zein/casein NPs were dispersed in milk and IF. The origin of this peak was unknown as it did not belong to the F I, F II, or F III polymorphs. The positions of the diffraction peaks for all systems are tabulated in Table S3 in the Supporting Information.

Solubilization of Clofazimine in Infant Formulas during Dispersion and Digestion. To understand the effects of fat content and the types of lipids on the solubilization of clofazimine in the API and the NPs as well as commercial Lamprene capsules, synchrotron X-ray scattering was used to monitor the changes in the intensity of the drug diffraction peaks over the course of dispersion and digestion. This technique has been shown previously to enable parallel determination of drug polymorphic transformations (digestion- and/or dispersion-induced) and drug solubilization.[25,39] Integration of the peaks at q = 0.96 Å^{-1} for the clofazimine F I polymorph and 0.95 Å^{-1} for the F II polymorph was performed to determine the peak areas, with a decrease in area representing drug solubilization and/or loss of crystallinity, whereas an increase in area represents drug precipitation in the crystalline state.[40] Figure 5 shows the effect of varying fat content using increasing amounts of IF powder on the solubilization of clofazimine during dispersion (negative time points) and digestion (positive time points), with the initiation of digestion by injection of lipase occurring at time = 0 min. The next two paragraphs discuss the dispersion and digestion behaviors separately for clarity.

Very little or no drug solubilization was evident during the dispersion phase for API or lecithin NP formulations (Figure 5a,b), although for lecithin, greater initial drug solubilization was evident when the starting fat content was higher (indicated by the lower initial peak area) because of drug dissolution into the native lipids in the media. This was not evident for the API, possibly because of the very poor solubility and compromised dissolution of the crystalline API compared to the rapidly dissolving NPs. The zein/casein particles provided comparably low initial diffraction peak areas, indicating significantly less crystalline drug being present at the start of the dispersion process, consistent with the reduced crystallinity seen in Figure 2. Interestingly, the peak areas for clofazimine with the zein/casein NPs steadily increased during dispersion, indicating drug crystallization or precipitation from the NPs which were mainly amorphous prior to dispersion but apparently crystallized upon contact with the dispersion media. However, the amount of crystallized clofazimine in zein/casein NPs was still relatively low compared to that of lecithin NPs. The increase in the amount of crystalline drug in suspension upon dispersion of the zein/casein clofazimine NPs was more pronounced in the IF samples compared to the lipid-free buffer, presumably because of the lipid particles and other IF components providing an increased capacity to dissolve the free drug. This can lead to supersaturation of the drug in solution, which provides a pathway for recrystallization to occur.

Digestion of the formulations generally resulted in decreases in diffraction peak areas because of solubilization, and it was generally observed that increasing the amount of fat present resulted in greater solubilization of clofazimine, although the extent of drug solubilization varied depending on the formulation. Complete disappearance of the clofazimine peak was observed at ≥2% and ≥1% fat for the formulated lecithin (Figure 5b) and zein/casein NPs (Figure 5c), respectively, after 20 min of digestion. In contrast, for the crystalline API (Figure 5a), the diffraction peak of clofazimine was still present after digestion at 3.8% fat despite having a similar initial degree of crystallinity to the lecithin NPs. The faster drug dissolution (into the digested lipid products) of the lecithin NPs could result from the particle size reduction through the FNP process and a synergistic solubilizing interaction of the digestion products with the additional lecithin stabilizer.[18]

In contrast to all of the other formulations, the commercially available Lamprene formulation displayed no significant solubilization of clofazimine attributable to the presence of added lipids during 30 min of in vitro digestion. A slow and progressive decrease in the clofazimine peak areas was seen in both the tris buffer and the IF media (Figure 5d) even prior to lipase injection, which could be caused by the slow dissolution of the micronized drug into the lipid excipients including beeswax and plant oils from the Lamprene formulation.[41]

In contrast to the lecithin NP system, where only the F I clofazimine polymorph was observed throughout the dispersion and digestion, clofazimine in the zein/casein NPs was predominantly the F II polymorph (indicated by the peak at q = 0.95 Å^{-1}) during the initial stages of dispersion, with the F I polymorph (indicated by the peak at q = 0.96 Å^{-1}) also

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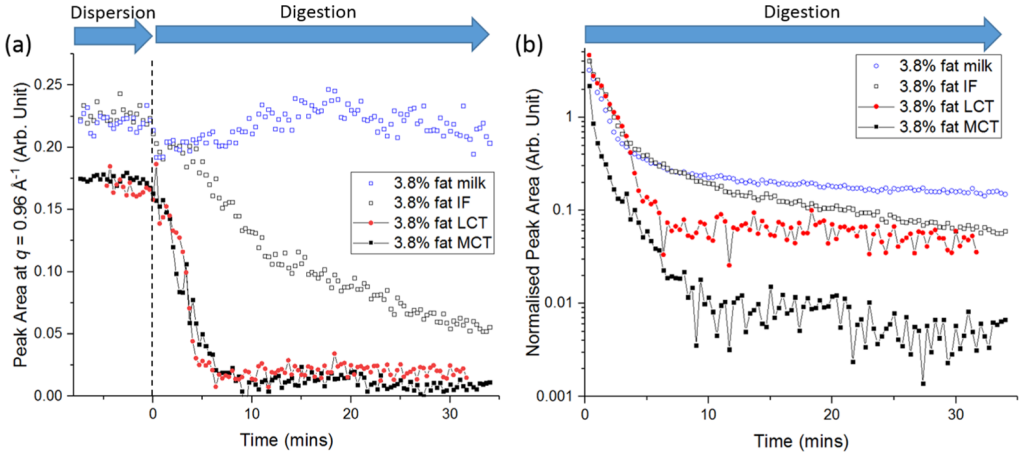

Figure 7. (a) Area under the diffraction peak for clofazimine at q = 0.96 Å^{-1} from clofazimine API during dispersion and digestion in milk, IF, LCTs, and MCTs containing 3.8% fat. (b) Corresponding clofazimine peak areas divided by the amount of titrated fatty acids (normalized peak area) during digestion in milk, IF, and LCTs, and MCTs.

appearing over time. Figure 6a shows the evolution of these diffraction peaks in 0.25% fat IF during dispersion and digestion. The results indicate that despite the growth in the diffraction peak area associated with both the F II and F I polymorphs (slightly faster for the least stable F I polymorph, which is in accordance with the Ostwald rule of stages),[42] the diffraction peak observed for the F I polymorph nearly disappears after 30 min of digestion, but the diffraction peak of the F II polymorph is still very evident after 40 min of digestion. This could suggest different solubilization behaviors of the different clofazimine polymorphs and that faster solubilization was observed for the less stable form. In addition, the growth of the F I polymorph at q = 0.96 Å^{-1} was also dependent on the fat content of the IF (Figure 6b). The larger peak area seen in IF with 0.25% fat compared to ≥1% fat was expected because of greater drug solubilization at higher fat contents.

Solubilization of Clofazimine in Milk and Medium- and Long-Chain Triglycerides during Dispersion and Digestion. In contrast to the observations with clofazimine in IF, with milk there was only a slight drop in the peak area on digestion after lipase injection, and this is possibly a dilution effect from adding the lipase solution, with no apparent decrease in diffraction peak area observed throughout the 35 min digestion period (Figure 7a). Similar profiles were obtained when the fat content of milk was increased from 3.8 to 4.7% although the overall peak areas in the high fat milk were lower presumably because of an initially greater solubility in undigested milk (see Figure S5 in the Supporting Information).

The reasons for the differences in the solubilization behavior of clofazimine in milk and the IF were unclear, although it was likely that differences in the types of lipids and other solubilizing excipients within the two systems may play an important role.[43,44] The notion that the solubilization of clofazimine may be affected by the types of lipids was also supported by studies performed by O'Reilly et al. where the authors observed fatty acid-dependent solubility of clofazimine in mixed micelles.[45] Consequently, the solubilization behavior of clofazimine API during digestion in MCT and LCT emulsified with MFGM isolate at the same drug-to-fat weight ratio (74.6 mg clofazimine API to 1 g of fat) was investigated. Figure 7a shows that clofazimine was solubilized to a greater extent in MCT and LCT emulsions compared to milk and IF. The amount of fatty acids released at certain time points and the extents of digestion varied between the different lipid emulsions even at the same fat content; therefore, comparisons were also made in Figure 7b based on the normalized peak area, that is, the peak area of clofazimine divided by the titrated fatty acids. The results show that the drop in normalized peak areas with digestion was consistent with the time-dependent data in panel (a), but clearer differentiation between the systems was apparent. Comparing the solubilization of clofazimine API by the lipids used to solubilize them gave an order of effectiveness of MCT > LCT > IF > milk during 30–35 min of digestion. Hence, our studies confirmed that the solubilization of clofazimine could be affected by not only the amount of fat present but also the lipid composition, with MCTs providing the most effective solubilization. The exact lipid composition of the IF has not been disclosed, but IFs are well-known to have lipid compositions different from bovine milk, and the results suggest that it is likely that the IF has more medium-chain lipids present than long-chain lipids when compared with bovine milk. To further elucidate the impact of fatty acid chain lengths on drug solubility, separate sets of experiments were performed to determine the solubility of clofazimine in MCT, LCT, and their corresponding fatty acids using caprylic acid and oleic acid as the representative lipids (see Supporting Information for the experimental methods). Despite unsuccessful attempts to determine the solubility of clofazimine in fatty acids because of the high viscosity of the samples, higher drug loadings in MCT were obtained compared with LCT (12.1 ± 0.5 and 6.8 ± 0.5 mg drug/g lipid, respectively).

Finally, although the diffraction studies enable understanding of the solid-state form of the drug and peak areas to estimate the proportions of the crystalline drug present, it is neither accepted as an analytical technique, nor can it provide clarity on the phase distribution of the noncrystallized drug. Therefore, to support the results from SAXS and to specifically elaborate on the apparent poor solubilization of clofazimine in milk when compared with IF during digestion, HPLC was used to quantify the amount of drug present in the lipid phase, the supernatant, and any precipitated pellets after ultracentrifugation. This method is typically used to determine the amount of excess nonsolubilized drugs or precipitated drugs not in a

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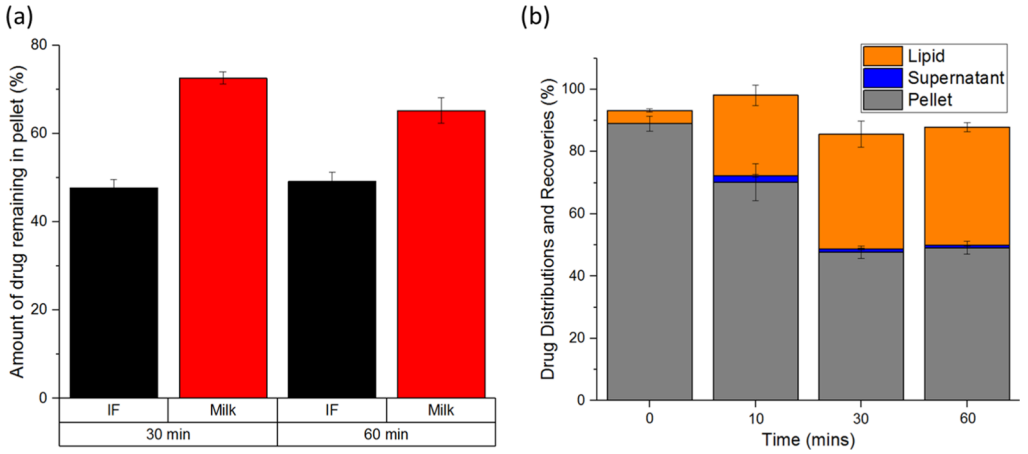

Figure 8. (a) Comparison between the amount of undissolved clofazimine recovered from the pellet phase in 3.8% IF and milk after 30 and 60 min in vitro digestion and (b) distributions of clofazimine with digestion time for 3.8% IF showing an increasing amount of drug partitioned into the upper lipid layer as digestion progressed.

solubilized state.[46] The digests were collected at predetermined time points during the digestion of milk and IF containing clofazimine API, and the lipolytic enzymes were inhibited prior to phase separation. The amount of clofazimine remaining in the pellet phase was higher for milk compared to IF at 30 min (and under prolonged digestion to 60 min), which supported the observations from SAXS that more crystalline drug was present after the digestion of milk than of IF (Figure 8a). The results also confirmed that clofazimine was increasingly partitioned into the upper lipid layer as digestion progressed because of the formation of digestion products, which self-assemble into liquid crystalline phases and reside at the upper layer after centrifugation[24] (Figure 8b). Furthermore, no drug solubilization was apparent when digestion was performed in the absence of lipids (Figure S6 in the Supporting Information), which is also in agreement with the SAXS results.

CONCLUSIONS

Clofazimine NPs prepared using the FNP process produced different solid-state forms of the API depending on the types of surface stabilizers used. Clofazimine in the lecithin NPs was comprised of the F I polymorph, whereas for the zein/casein NPs, the F II polymorph was the dominant form. The NPs exhibited lipid-dependent solubilization that required lipolysis of the coformulated fat. The amount of lipid required to solubilize the drug was lower for the zein/casein NPs than for the lecithin NPs, with clofazimine API requiring the most added fat to solubilize it. Rapid drug solubilization of the NP samples was observed during digestion, whereas solubilization of clofazimine from the commercially available Lamprene formulation was slower. The studies demonstrated that IF could be used as a lipid-based formulation to improve the solubilization of clofazimine in the small intestine as a reduced amount of residual undissolved drug crystals was apparent when compared with milk, based on the in vitro lipolysis experiments performed herein. The lipid composition in such systems appears to be important with MCT solubilizing clofazimine more efficiently than LCT or the other vehicles tested. The studies not only strongly support the suitability of clofazimine NPs for the treatment of cryptosporidiosis where fast onset of action by dissolution in the intestines is required, but also reinforce the potential benefits of coformulation with fat-containing excipients such as IF containing medium-chain fatty acids or simple triglyceride emulsions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.9b00276.

Schematic diagram and mechanistic description for the preparation of clofazimine NPs; size distributions of zein/casein and lecithin NPs in suspensions following redispersion of the spray-dried powders in water; optical polarizing microscopic images of clofazimine API and the NPs; nutritional information of milk and infant formula; X-ray scattering peak positions for clofazimine polymorphs, clofazimine API, and NPs in the powder form; X-ray scattering peak positions for clofazimine API and NPs in dispersions; and solubility of clofazimine in lipids (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: ben.boyd@monash.edu. Phone: +61 3 99039112. Fax: +61 3 99039583.

ORCID

Andrew J. Clulow: 0000-0003-2037-853X
Simon A. McManus: 0000-0003-1714-1259
Robert K. Prud'homme: 0000-0003-2858-0097
Ben J. Boyd: 0000-0001-5434-590X

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■ ABBREVIATIONS

FNP, flash nanoprecipitation; NPs, nanoparticles; IF, infant formula; MCT, medium-chain triglycerides; LCT, long-chain triglycerides; MC/T80, methyl cellulose/Tween 80; API, active pharmaceutical ingredient; SAXS, small-angle X-ray scattering; HPLC, high-performance liquid chromatography; DSC, differential scanning calorimetry

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