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Short Communication

Supramolecular Behavior of the Amphiphilic Drug (2*R*)-2-Ethylchromane-2-Carboxylic Acid Arginine Salt (a Novel PPARα/γ Dual Agonist)

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Purpose. This study was conducted to evaluate the aggregation properties of an amphiphilic drug.
Methods. Aggregation of the drug was studied by various methods including phase-contrast and polarized microscopy, spectrophotometry, surface tensiometry, atomic force microscopy, and dynamic light scattering. Lymph-cannulated rats were used to assess fractions of drug that were absorbed into lymphatics.
Results. During the pharmaceutical development of an α/γ dual PPAR agonist, a derivative of a chromane-2-carboxylic acid (compound **1**), it was discovered that the compound was able to form various aggregates in aqueous media from pH 6.5 to 7.1, whereas aggregating predominantly into micelles at higher pH values. Critical micelle concentrations seemed to be quite low, about 0.25 mM (0.17 mg/mL) in deionized water as determined by spectrophotometric (dye) and surface tensiometry (du Nuoy) methods. Aggregation of compound **1** into large supramolecular aggregates was visualized via phase-contrast microscopy and atomic force microscopy. The observed aggregates ranged from 250 nm to greater than 10 μm in size. Formation of liquid crystalline phases was observed by polarized microscopy as the material was gradually hydrated with water. Lymph studies in rats indicated that up to 6.9% of the orally administered dose of compound **1** in pH 6.5 buffer appeared in lymph, suggesting that supramolecular aggregation may also occur *in vivo* leading to partitioning between the portal and the lymph routes.
Conclusions. The aforementioned supramolecular aggregation was found to have a profound effect on the pharmaceutical development of the drug and potentially on *in vivo* absorption of the drug.

KEY WORDS: AFM; aggregation; liposomes; lymphatic absorption; microscopy; self-assembly; ufasomes.

INTRODUCTION

The self-assembly of amphiphilic drugs is very important for pharmaceutical drug development (1–3). Thibert *et al.* (4) characterized the association behavior of a leukotriene D_4 receptor antagonist while emphasizing the importance of a thorough understanding of the self-assembly of a compound for successful pharmaceutical development of liquid dosage forms. Enever *et al.* (5) showed that the self-assembly of flupenthixol into micelles significantly influenced its rate of oxidation in solution, whereas the rate of degradation of penicillin was also significantly affected by self-assembly (6). Vadas *et al.* (7) reported about the influence of amphiphilicity of another amphiphilic leukotriene D_4 receptor antagonist on its solid-state properties, demonstrating the importance of understanding the surface-active properties of the drug for development of solid dosage forms. Other examples of amphiphilic drugs include corticosteroids (8), brequinar sodium (9), prostaglandin F_2 (10), disodium chromoglycate (11), tricyclic antidepressants (12), and analgesics (13).

Self-association of amphiphilic compounds in aqueous solutions is the result of the presence of both hydrophilic and hydrophobic portions in the same molecule (14). In an effort to minimize highly unfavorable interactions of the hydrophobic portion of the compound with water molecules, the molecules of the amphiphile tend to self-associate, leading to formation of various supramolecular aggregates such as micelles, liposomes, and other phases. The presence of the planar aromatic system in the leukotriene D_4 antagonist reported by Vadas *et al.* (7) was suggested to facilitate self-association through stacking of the aromatic groups in the solid state, leading to formation of various thermotropic and lyotropic mesomorphic phases. Formation of micelles from MK-0476, as reported by Thibert *et al.* (4), seemed to be attributable to the presence of the large hydrophobic region comprising several aromatic rings. In another case, a vertical stacking self-association of rigid aromatic portions seemed to be responsible for the formation of micelles of brequinar sodium in aqueous solution (9). Mukerjee (15) provided details on the forces of self-association and detailed descrip-

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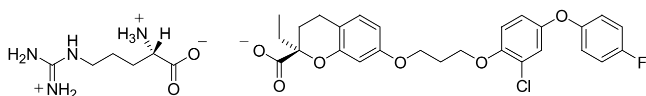

Fig. 1. Structure of compound **1** (shown as the arginine salt).

tions of various classes of hydrophobic groups that led to aggregation. In addition, an excellent review by Ringsdorf *et al.* (16) summarizes a variety of supramolecular aggregates that could be formed in solution and in the solid state.

(2*R*)-7-{3-[2-Chloro-4-(4-fluorophenoxy)phenoxy]propoxy}-2-ethylchromane-2-carboxylic acid (compound **1**) was identified as a potent and selective PPARα/γ dual agonist that exhibited significant antihyperglycemic and hypolipidemic activities in various animal models (17). Compounds with PPARα/γ activity are of great interest for the treatment of type 2 diabetes and dyslipidemia. During the pharmaceutical development of compound **1** (refers to the arginine salt unless stated otherwise) (Fig. 1), it was discovered that the compound was able to form a variety of supramolecular structures in aqueous media from pH 6.5 to 7.1 but aggregated predominantly into micelles at higher pH values. Such behavior proved challenging for the preparation of aqueous and solid formulations of compound **1** and thus warranted a detailed physicochemical investigation of its behavior. The self-assembly of compound **1** was characterized by various techniques including phase-contrast and polarized microscopy, atomic force microscopy (AFM), light scattering, spectrophotometry, and surface tensiometry. Considering similarities in behavior with medium- and long-chain fatty acids that can partition between the lymph system and the portal blood, a potential *in vivo* formation of the observed lamellar aggregates reminiscent of "ufasomes" under physiological conditions sparked additional interest regarding the possibility of partial lymphatic absorption of compound **1**.

MATERIALS AND METHODS

Compound **1** was obtained from the Process Research Department (Merck Research Laboratories, Rahway, NJ, USA) as a crystalline arginine salt and used without further purification (purity > 98.5%, no single impurity > 0.5%). Pinacyanol chloride was purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were reagent grade. Degassed distilled deionized water was used for all experiments.

Critical micelle concentrations (CMC) were measured using a spectrophotometric method and the du Nuoy tensiometry method. Surface tension measurements were performed using a Model 21 Fisher Tensiomat (Fisher Scientific, Pittsburgh, PA, USA) utilizing the du Nuoy method. A 6-cm platinum ring was raised and lowered manually into solutions of the drug in deinonized water with concentrations ranging from 0.008 to 1.0 mg/mL. As the ring was released from the surface of the fluid, a corresponding surface tension measurement (in millinewtons per meter) was recorded. After three readings at a specific concentration, the ring was rinsed several times with 10% HCl, methanol, and deionized water followed by flame-drying. All experiments were performed at ambient temperature (23°C). The spectrophotometric method for CMC determination is based on addition of the dye (pinacyanol chloride), which changes its absorbance upon micellization of an anionic surfactant. The absorbance is then measured at 610 nm, where a sharp increase in absorbance is detected near the CMC (18–20). Changes in absorbance are so pronounced that the CMC can easily be determined by the naked eye. Below the CMC, solutions of the dye appear pink, and (in contrast) turn bright blue above the CMC. The spectrophotometric method was performed by recording absorbance measurements at 610 nm on an Ocean Optics Model DT 1000 UV spectrophotometer (Dunedin, FL, USA). The solutions of drug concentration from 0.008 to 1.0 mg/mL in deionized water were prepared by addition of 10 μL of a 6-mM solution of a dye (pinacyanol chloride prepared in methanol) to 6 mL of the respective compound **1** solutions of varying concentration in aqueous media. These solutions were vortexed for 10 s and equilibrated for 20 min at ambient temperature (23°C) in the dark before absorbance was measured at 610 nm.

Phase-contrast microscopy was performed using an Axioplan 2 microscope (Zeiss, Thornwood, NY, USA). Samples were prepared by smearing compound **1** on a Falcon culture slide followed by the addition of a drop of 10–100 mM phosphate buffer (pH 6.5–7.2). The samples were allowed to equilibrate at ambient temperature for 30–60 min prior to microscopic observation.

Dynamic light-scattering experiments were performed on a dynamic light-scattering instrument (Stabilite 206, Spectra Physics, San Francisco, CA, USA) at 250-mW laser power using 10 s accumulation time per run. The data collection was based on the average of five runs.

AFM measurements were performed using a Dimension 3100 microscope (Digital Instruments/Veeco Inc., Santa Barbara, CA, USA). Samples for imaging were prepared in the following manner: a 2-mM solution of compound **1** in water was protonated with 0.5 molar equivalents of HCl. A drop of this solution was deposited on a mica surface and allowed to dry under ambient conditions for 1 h following observation by AFM.

Polarized microscopy was performed with an Eclipse ME 600 microscope (Nikon, Tokyo, Japan). A large excess of solid was allowed to gradually hydrate with water for 24 h prior to observation under crossed polars.

An equilibrium titration curve was obtained by titration of 2 mL of a 2-mM (above CMC; see Results) aqueous solution of compound **1** in deionized water (containing 10 mol % excess of NaOH) with 0.01 N HCl.

Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee. Mesenteric lymph-cannulated, male Sprague–Dawley rats, weighing 350–500 g, were purchased from Charles River Laboratories (Durham, NC, USA). Animals received 5 mL/kg of a 10-mg/mL solution (specific activity of $[^{14}C]$ compound **1** was 456.7 μCi/mg) in 50 mM phosphate buffer (pH 6.5; oral gavage). Lymph was collected at 0.5- to 1-h intervals from the mesenteric cannula, up to 6 h after the dose was administered. Radioactivity in the collected samples was determined by scintillation counting and represented compound **1** equivalents absorbed from this formulation.

RESULTS

Aqueous solutions of compound **1** at pH > 7.1 appeared as optically clear solutions and foamed readily upon

agitation. The latter property is often an indication of surface activity and the potential for self-assembly into various aggregates. The CMC was 0.17 ± 0.02 mg/mL in unbuffered water, decreasing to 0.05 ± 0.01 mg/mL in pH 7.5–8.9 buffers at 0.1 M ionic strength (adjusted with NaCl). Both spectrophotometric and tensiometry methods used for CMC determinations produced identical results, and the representative plots of dependence of the absorption of the dye on the CMC and respective changes in the surface tension by the du Nuoy method are shown in Fig. 2. The decrease in CMCs with increasing ionic strength can be attributed to shielding of the negatively charged carboxylates, which reduce the electrostatic repulsion between the head groups and allow molecules to self-assemble at lower concentrations (14). Solution pH (7.5–8.9) and buffer type (borate, phosphate) did not seem to have a significant effect on the CMC at constant ionic strength.

The apparent pK_a of compound **1**, determined by titration of 2 mL of a 2-mM [based on the procedure of Morigaki *et al.* (21)] aqueous solution of compound **1** in deionized water (containing 10 mol % excess of NaOH) with 0.01 N HCl, was found to be 7.1—almost two units above the "monomeric" pK_a of 5.3 (Fig. 3). Such shifts in pK_a were previously observed and were coined "bilayer effects," being attributed to the formation of lamellar complexes composed of mixtures of ionized and neutral forms (22).

The self-assembly of compound **1** seemed to be more intriguing as the pH was lowered to 6.5–7.1, where aqueous solutions of compound **1** appeared hazy. Investigation of these solutions under a regular bright-field microscope did not conclusively identify the nature of these aggregates. Thus, we used a phase-contrast microscope because of its ability to enhance the contrast of weakly dense structures, which has proven to be an invaluable tool for observation of objects such as liposomes, cells, and tissues (23). When a small amount of compound **1** was smeared on a microscope slide, followed by addition of a small drop of a 10–100 mM phosphate buffer, a large number of various supramolecular structures spontaneously formed, as shown in Fig. 4A–C. The observed phases included large spherical objects reminiscent of liposomes and other floppy, irregular morphologies that could be seen as long interconnected threads spreading through the bulk media, sometimes reaching several hundred microns in length. A cascade of unilamellar liposomes formed

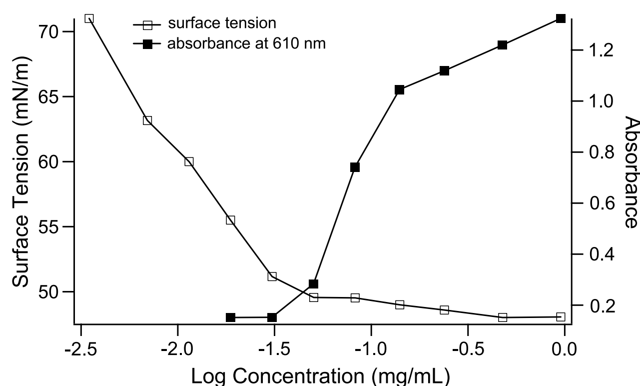

Fig. 2. Determination of CMC of compound **1** in borate buffer (pH 8.9) at 0.1 M ionic strength by using spectrophotometry and surface tensiometry (du Nuoy method).

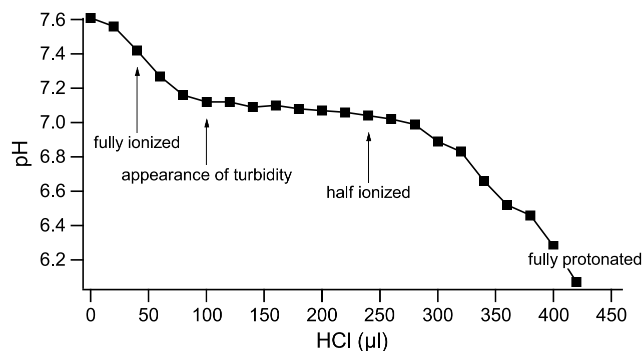

Fig. 3. Equilibrium titration curve of compound **1** in water. pH was measured after equilibration of solutions for 24 h at 25°C. See text for experimental details.

in 100 mM pH 6.5 phosphate buffer is depicted in Fig. 4A; the cascade is reminiscent of an "onion"-like phase. The appearance of an analogous phase has been previously documented in the literature for a sodium bis(2-ethylhexyl)sulfosuccinate) (AOT)/brine system (24). It seemed that vesicular aggregates formed in 100 mM pH 6.5 phosphate buffer emanated from large structures reminiscent of balls of wool that gradually underwent swelling (Fig. 4B). Smaller vesicular aggregates (depicted in Fig. 4C) formed in 10 mM pH 6.7 phosphate buffer with sizes on the order of 2–5 µm. Formation of large aggregates were also observed by AFM when compound **1**, half-protonated with HCl, was deposited on a mica surface (25,26). A large number of spherical aggregates with diameters ranging from ca. 250 nm to 2 µm were observed (Fig. 4D). The large size of the aggregates observed by phase-contrast microscopy and AFM is consistent with the data obtained by dynamic light scattering and is in agreement with vesicular/lamellar nature of aggregates, which usually range from 30 nm to >10 µm (27). In contrast, micellar aggregates are typically up to 5 nm in diameter and are difficult to detect via microscopic techniques. Therefore, the large size of the observed aggregates favors the lamellar nature of the observed aggregates. Based on microscopic techniques and dynamic light-scattering techniques, lamellar aggregates formed up to 5 wt.% concentration. It is likely that the composition, the structure, and the ratio of the observed aggregates are changing with further increase in concentration, a topic that will be a subject of future investigation.

Formation of liquid crystalline phases was also observed via polarized microscopy as the solid crystalline material was gradually hydrated with water. Characteristic birefringent textures of lamellar phases were also evident following observation under crossed polars as the material was gradually hydrated with water (Fig. 5) (28).

Potential aggregation of compound **1** under physiological conditions from pH 6.5 to 7.4 may have important *in vivo* ramifications. Approximately 2% of the orally administered dose of compound **1** in 50 mM pH 6.5 phosphate buffer was recovered in mesenteric lymph after 6 h of lymph collection. The oral bioavailability of compound **1** was relatively poor in male rats because of incomplete absorption and hepatic metabolism. Based on the assessment of fraction of dose absorbed in male rats, lymphatic absorption represented between 3.8 and 6.9% of oral absorption of compound **1**. The importance of lymphatic absorption is that the drug

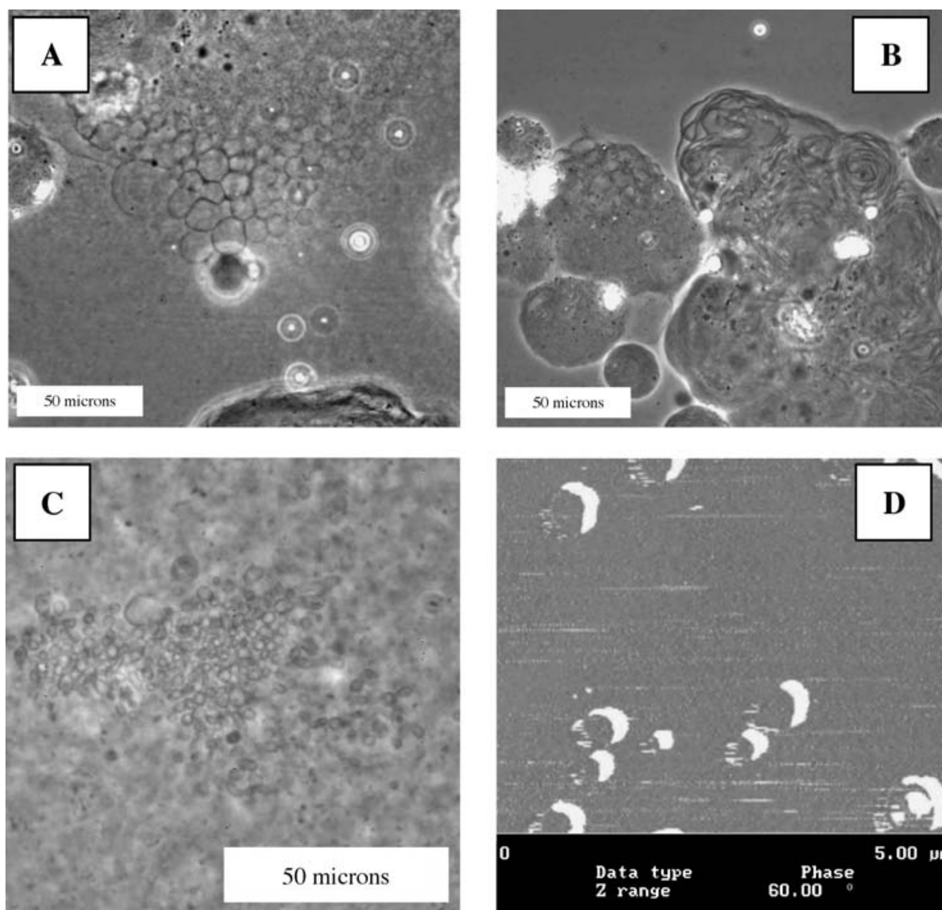

Fig. 4. Phase-contrast microscopy (A–C) and atomic force microscopy (D) images of aggregates of compound **1** at pH 6.5–7.1.

initially bypasses hepatic first-pass metabolism, leading to potentially improved systemic exposures.

DISCUSSION

The observed critical micelle concentration for compound **1** seemed to be very low. This observed CMC, which was on the order of 0.25 mM (water), is almost 3 orders of magnitude lower than the CMC of sodium octanoate (100 mM) (29). As the latter was reported to form vesicles at pH 6.5, the significantly lower CMC of compound **1** suggested a high propensity for compound **1** to conform into higher-order lamellar aggregates (30). Indeed, as the solution pH was lowered from 6.5 to 7.1, various larger ufasome-like aggregates were observed by multiple techniques.

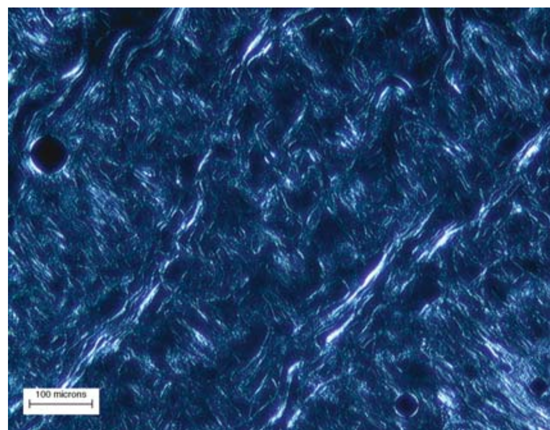

Fig. 5. Polarized microscopy image of the formation of the liquid–crystalline phase upon slow hydration of compound **1** with water.

At this point, before we proceed into the detailed discussion of the nature of the observed supramolecular aggregation, a brief historical insight should be presented in regard to the behavior of analogous long-chain fatty acids. In the early 1970s, Gebicki and Hicks (31,32) reported the first instance of the formation of supramolecular aggregates (liposomes) from single-chain fatty acids. They coined these supramolecular aggregates "ufasomes" because of the presence of unsaturated fatty acids in their structures. Hargreaves and Deamer (33) later reported formation of similar structures from other single-chain carboxylate surfactants. These discoveries sparked interest in the academic world of the likelihood of the presence of fatty acids under prebiotic conditions and suggested that ufasomes, because of their structural similarities to cell membranes, may have played an important role in the formation of the first cell membranes necessary for the creation of the first living cells (29,34). Luisi *et al.* (21,35,36) later reported on the ability of fatty acid vesicles to undergo autopoietic self-reproduction, reinforc-

ing the supposed role of these structures in the prebiotic world.

On the basis of similarity in behavior with long-chain fatty acids capable of forming lamellar structures in aqueous solutions, the supramolecular aggregates of compound **1** are stabilized by hydrogen bonds between the protonated and ionized carboxyl groups (22). The importance of hydrogen bonding was previously demonstrated by Fukuda *et al.* (37), who showed that a mixture of single-chain amphiphiles carrying opposite charges were able to form vesicles. These compounds, if dissolved separately, form micelles in aqueous media. However, their mixture produced vesicles that were stabilized by electrostatic interactions of the polar groups. Likewise, equimolar addition of long-chain fatty alcohols (e.g., decanol) to micellar solutions of carboxylates also produced vesicles (33). Thus, partial neutralization of compound **1** may lead to association of the ionized and protonated species into dimers and other higher-order molecular aggregates. Such aggregates were previously proven to exist, given that the strength of the hydrogen bonding in aqueous solutions of salts of carboxylic acids has been estimated to be 5 kcal/mol (38). These structures may further self-assemble into other higher-order aggregates, although the exact ratio of ionized to unionized species may vary (22). Kunitake *et al.* (39) previously emphasized the importance of the presence of at least two benzene rings for aggregation into stable bilayer assemblies. Note that compound **1** lacks a long hydrocarbon tail that has been considered to be one of the necessary prerequisites for the formation of stable bilayers. Perhaps the presence of three aromatic rings as well as the elongated form of compound **1** may facilitate its supramolecular aggregation.

Thus, it seems that because of a much higher apparent pK_a (= 7.1) of compound **1**, a large amount of the protonated form of compound **1** exists at pH 6.5–7.1, well above the pK_a of 5.3 determined in methanol/water solutions. Cistola *et al.* (22) have extensively studied the pH-dependent phase behavior of medium- and long-chain fatty acids. They observed a variety of phases including free fatty acid, lamellar structures composed of the protonated and ionized forms of the fatty acids, as well as micellar and aqueous phases. Such aggregations led to local decreases in pH at the surface of the aggregates (21,22,32,33). During the titration of aqueous solutions of compound **1**, turbidity occurs at pH 7.1, where compound **1** is only 15% protonated. For comparison, in the case of sodium dodecanoate, turbidity appears in the pH range ca. 7 to 8.6, depending on the concentration of the salt (33). Likewise, in the case of sodium methyldodecanoate, turbidity appeared at pH 8.8, where the compound is only 10% protonated (21). In comparison with other amphiphilic carboxylates, experimentally determined pH values in water at ionization midpoint (apparent pK_a) were found to be 8.0 for laurate, 6.8 for decanoate, and 8.0–8.5 for oleate, which are significantly higher than the "monomeric" pK_a of 4.8 (22). Based on similarities between the medium- and long-chain fatty acids, observed lamellar phases likely coexist with the free fatty acid form as well as the micellar phase and the ratio of various aggregates will be quite sensitive to drug concentration and pH (21,22). Upon titration of aqueous solutions of compound **1** above its CMC, the system would initially contain a mixture of monomers and micelles (pH > 7.1). Upon further decrease in pH, the system would contain a complex mixture of lamellar aggregates, micelles, and monomers, eventually leading to the appearance of the free acid form. Elucidation of the exact composition and the structure of the presented aggregates at higher concentrations (above 5 wt.%) warrants future investigation with the use of liquid nuclear magnetic resonance (^1H, ^{13}C, pulse-gradient spin–echo studies), X-ray diffraction, freeze-fracture electron microscopy, and other techniques.

As a result of a lower apparent pK_a, solutions of compound **1** prepared in unbuffered water were plagued by erratic and irreproducible behavior. The solutions would either remain clear or turn hazy (due to formation of large aggregates), which seemed to depend on multiple factors, including the type and acidity of glassware and location of water supply. Slight changes in pH induced by these factors could significantly affect the self-assembly of the drug and lead to multiple hurdles in the preparation of reproducible aqueous formulations at various sites. Indeed, changes in pH induced by absorption of atmospheric CO_2 leading to aggregation of long-chain carboxylates have been reported (29,38). Therefore, in an effort to ensure reproducible formulations, aqueous preparations of compound **1** had to be buffered to pH > 7.5.

Because the carboxyl group of compound **1** can be significantly protonated above the "monomeric" pK_a, there may be a significant amount of lamellar structures under the physiological conditions of pH 6.5–7.4. The potential formation of colloidal aggregates of compound **1** in the intestinal lumen, where the pH ranges from 5 to 8 prior to absorption, may also influence the absorption of the drug. Most reports from the literature speculate that colloidal aggregates are not likely absorbed intact, but this hypothesis has not been confirmed or proven otherwise (40). However, it is known that liposomes, because of their structural similarities to cell membranes, may undergo endocytosis by the cell membrane, being encapsulated as a whole entity (16). M cells, which are often described as lymphoepithelial cells serving to transport materials from the gut lumen to the lymph duct, may also adsorb colloidal structures intact (41). The fate of the drug as soon as it passes through the epithelial membrane is potentially intriguing. There is some evidence in the literature that suggest lamellar structures may be formed intracellularly where the pH ranges from 6.5 to 7.4 (22). In this case, formation of such structures will lead to delayed absorption, preferentially into the lymph system. It was previously reported that colloidal-sized particles are preferentially absorbed into the lymph system (42,43). Furthermore, long-chain carboxylic acids are predominantly transported via the intestinal lymph, whereas medium-chain fatty acids (which exhibit similar self-assembly behavior to compound **1**) partition between the lymph and the portal blood (40). Comparison of the colloidal behavior of compound **1** and sodium decanoate (medium-chain fatty acid) reveals obvious similarities. Indeed, the pK_a of compound **1** (7.1) is very similar to the apparent pK_a of sodium decanoate (6.8) (22). Such resemblance of the colloidal behavior of compound **1** to medium-chain fatty acids suggests a possibility of similar *in vivo* behavior. Indeed, the lymph recoveries of sodium decanoate were reported to be on the order of 20% (44).

Although much of the *in vivo* data discussion is speculative in nature, we do hope that these data (powered with subsequent research) will help to gain further understanding of the *in vivo* behavior of amphiphilic drugs and shed more light on the potential link between lymphatic absorption and *in vivo* formation of large supramolecular entities such as ufasome-like objects.

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