Relationship Between Drug Absorption Enhancing Activity and Membrane Perturbing Effects of Acylcarnitines

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Acylcarnitines with chain lengths of 2 to 18 carbon atoms were tested for their effects on rat intestinal brush border membrane order (S) by fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH). These results were compared to the previously reported effectiveness of the acylcarnitines as absorption enhancers of the poorly absorbed antibiotic cefoxitin. Acylcarnitines with fatty acids less than 12 carbon units in length were ineffective in increasing drug absorption and perturbing brush border membrane order. Long-chain acylcarnitines (12–18 carbons) significantly increased the bioavailability of cefoxitin and decreased the lipid order of brush border membranes. The results suggest that, in order to promote drug absorption, the acylcarnitines must surpass a critical chain length (10 carbon units) to partition effectively into the membrane and, in addition, must perturb the lipid order beyond a threshold value (15–20%). Membrane perturbing capacity may serve as an indicator of the absorption enhancing potential of other aliphatic-type compounds.

KEY WORDS: acylcarnitines; fluorescence polarization; 1,6-diphenyl-1,3,5-hexatriene (DPH); rat intestine; brush border membranes; absorption enhancers; membrane lipid order.

INTRODUCTION

Several classes of absorption enhancers have been shown to alter the properties of isolated intestinal membranes by inducing a change in membrane lipid structure (1–5). Perturbation of membrane structure, whether naturally or artificially induced, can often be associated with a change in membrane permeability to drugs. For example, chlorpromazine, which produces a change at the membrane surface, was shown to increase the permeability of intestinal brush border membranes to β-lactam antibiotics (2). Likewise, salicylate was shown to perturb isolated epithelial cell membranes and increase the release of the impermeable dye fluorescein from isolated brush border membranes (3). However, a direct correlation between the potency of a particular compound for drug absorption enhancement and its potency for perturbing membrane order has not been demonstrated.

In vivo studies have shown that the long-chain acylcarnitines are effective enhancing agents of drug absorption in both the small intestine and rectum of the rat and dog (6). These compounds are active with a variety of drugs which possess different physical and chemical properties. In addition, significant drug absorption can be attained without observable histological alterations to the intestinal mucosa. This may be due to the fact that acylcarnitines are endogenous compounds found in both bile and intestine (7). Moreover, carnitine is an integral part of the mitochondrial membrane acyltransferase system which participates in the metabolism of long-chain fatty acids (8,9).

The purpose of this study was to determine the relationship between the absorption enhancing activity of the acylcarnitines and their ability to induce a change in membrane order. Brush border membranes from rat intestine labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH) were treated with a series of acylcarnitines with fatty acid chain lengths from 2 to 18 carbons and the lipid order parameter (S) was determined from the steady-state fluorescence anisotropy of DPH (10,11). These results were compared to the *in vivo* data reported previously on the absorption enhancing activity of these same acylcarnitines in the rat intestine (6).

MATERIALS AND METHODS

Reagents

All acylcarnitines were purchased as the mixed isomer chloride salt from Sigma Chemical Co. (St. Louis, MO). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Molecular Probes (Eugene, OR). Sodium cefoxitin (Mefoxin) was obtained from Merck Sharp & Dohme Research Laboratories. All other chemicals were reagent grade.

Animal Studies

In vivo studies were performed previously and the experimental methods are described elsewhere (6). Since the molar concentrations of the acylcarnitine solutions used in the *in vivo* studies were not identical, the percentage cefoxitin bioavailability was also reported per millimole acylcarnitine [Table I, potency index (PI)].

Brush Border Membrane Preparation

Brush border membranes (BBM) were isolated from rat small intestine using the Ca^{2+}-precipitation method of Kessler *et al.* (12). The final BBM pellets were resuspended in 5 ml PBS at a concentration of 2–3 mg protein/ml and were frozen in 1-ml aliquots at −20°C. Membranes were used within 1–2 weeks of preparation, wherein no significant differences were seen in response to acylcarnitine treatment and the morphology of the BBM remained identical to that described by Kessler *et al.* (12). The protein concentration of the BBM preparations was determined by the method of Lowry *et al.* (13).

Fluorescence Polarization

Fluorescence polarization of DPH was used to determine the effects of the acylcarnitines on the membrane order of BBM (14). BBM were used at a final protein concentration of 75–100 μg/ml and equilibrated to 37°C in an Aminco SLM 4800 fluorometer (Champaign, IL) equipped with a temperature regulated stir cell. DPH was added to a final concentration of 1 μM. The probe was allowed to equilibrate with

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Table I. *In Vivo* Absorption Enhancing Activity of Acylcarnitines in Rat Rectum[a]

Acylcarnitine	Percentage cefoxitin bioavailability	Potency index[b]
Control	2 ± 1.5	—
Acetyl (C_2)	6 ± 4.6[c,*]	0.29
Hexanoyl (C_6)	2 ± 2.0[c]	0.12
Octanoyl (C_8)	7 ± 4.0[c]	0.45
Lauroyl (C_{12})	51 ± 12.5[d]	3.88
Myristoyl (C_{14})	27 ± 3.0[e]	2.20
Palmitoyl (C_{16})	68 ± 4.2[f]	5.91
Stearoyl (C_{18})	52 ± 4.0[d]	4.83

[a] Bioavailability data from Ref. 6.
[b] Percentage bioavailability per millimole acylcarnitine.
* Superscripts c–f indicate statistically significant differences between groups at the $P < 0.01$ level, one-way ANOVA.

the BBM for 30 min before readings were taken. The steady-state fluorescence anisotropy (r_s) was determined using an excitation wavelength of 360 nm and an emission wavelength of 430 nm with a 385-nm cutoff filter. Calculations were performed with the aide of software provided by SLM Instruments, Inc. (Champaign, IL). Unbuffered, aqueous solutions of the acylcarnitines were used with the exception of stearoyl carnitine, which required warming to 45°C or the addition of 10% ethanol for dissolution. After the initial r_s value was recorded for the untreated BBM, an acylcarnitine solution was added to the cuvette in small increments to produce a concentration range of 10–250 μM. After each addition, the apparent r_s was recorded when no further changes were observed in the anisotropy readings (<3 min).

The lipid order parameter (S) was calculated from the steady-state anisotropy data by the following relationship:

$$S^2 = (\tfrac{4}{3}r_s - 0.1)/r_0$$

where r_s is the steady-state anisotropy and r_0 is the maximal fluorescence anisotropy value in the absence of any rotational motion. This relationship between r_s and S has been shown to be valid for biological membranes where $0.13 < r_s < 0.28$ (11,15). The value for r_0 used in this study was taken as 0.40 (11).

The fluorescent probe lifetime and lifetime heterogeneity was analyzed in the presence and absence of the acylcarnitines using two modulation frequencies (18 and 30 MHz) and methyl-POPOP as a reference probe (1.45 nsec) (16). Heterogeneity analysis was performed using software provided by SLM Instruments, Inc. The results showed that the compounds were not influencing the probe lifetime at the concentrations used in the experiments (data not shown).

RESULTS

In Vivo Enhancement Activity

Acylcarnitines that varied in their fatty acid chain length (C_2–C_{18}) were tested in a previous study for their ability to enhance cefoxitin absorption from the rat rectum (6). As shown in Table I, there was a dependence of cefoxitin bioavailability (F) on the length of the fatty acid chain of the acylcarnitine. Acylcarnitines with a fatty acid chain length less than 12 carbons were essentially ineffective enhancers of absorption from the rectum of rats, whereas acylcarnitines with a chain length of 12–18 carbon units significantly increased the bioavailability of sodium cefoxitin (from 2% to an average of 60%). Maximum enhancement of cefoxitin absorption from the rectum was obtained using palmitoylcarnitine (C_{16}), which had a PI of 5.9 (F = 68%), as compared to acetylcarnitine (C_2), with a PI of 0.29 (F = 6%).

Fluorescence Polarization

Fluorescence polarization of DPH was used to determine the effects of the acylcarnitine fatty acid chain length on the lipid order of brush border membranes prepared from rat intestine. The steady-state fluorescence anisotropy of DPH in biomembranes is representative of the degree of structural order (S) in the hydrophobic core of the lipid bilayer (11,15,16). A decrease in the steady-state fluorescence anisotropy of DPH signifies an increase in the probe rotational cone angle and, consequently, a reduction in the membrane order. In addition, to all acylcarnitines tested *in vivo*, the effect of decanoyl carnitine (C_{10}) was also examined in the fluorescence polarization studies.

The dependence of the order parameter of BBM on the fatty acid chain length of the acylcarnitines is shown graphically in Fig. 1. Little or no effect on S was seen for acylcarnitines with a chain length of 10 carbons or less, whereas acylcarnitines with a chain length of 12 or more carbons had a dose-dependent effect on reducing the membrane lipid order at the concentrations used in the study. The values of S for BBM treated with the C_{14}–C_{18} acylcarnitines reached a minimum (S_{min}) within the concentration range of the experiment (0–250 μM). This was typically followed by a slight increase in S prior to vesicle solubilization.

The S_{min} values, as well as the maximum change in S induced by each of the acylcarnitines, are listed in Table II. The greatest effect on lipid order was seen with myristoylcarnitine (32% decrease) followed by palmitoylcarnitine (24% decrease). Lesser effects were seen with the acylcar-

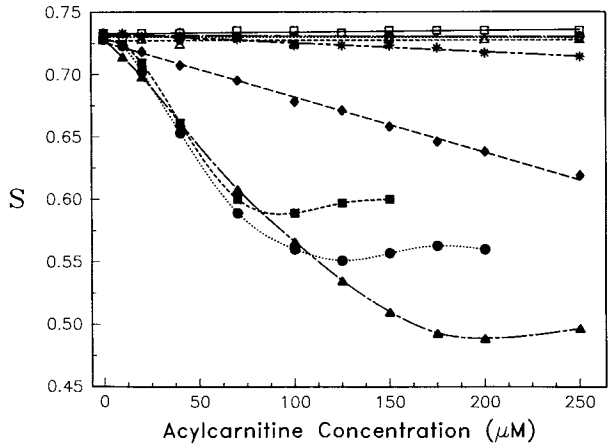

Fig. 1. Acylcarnitine effects on the lipid order (S) of rat intestinal brush border membranes *in vitro*. (□) Control; (◇) acetylcarnitine (C_2); (△) hexanoylcarnitine (C_6); (○) octanoylcarnitine (C_8); (*) decanoylcarnitine (C_{10}); (◆) lauroylcarnitine (C_{12}); (▲) myristoylcarnitine (C_{14}); (●) palmitoylcarnitine (C_{16}); (■) stearoyl carnitine (C_{18}).

Table II. *In Vitro* Effects of Acylcarnitines on Lipid Order (S) of Rat Intestinal Brush Border Membranes

Acylcarnitine	S_{min}[a]	Initial change[b] ($\times 10^{-4}$ $S/\mu M$)	ΔS	% change
Control[c]	0.731 ± 0.003	—	—	—
Acetyl (C_2)	0.728 ± 0.003	—	−0.002	00.3
Hexanoyl (C_6)	0.728 ± 0.002	—	−0.006	00.8
Octanoyl (C_8)	0.731 ± 0.002	—	−0.000	00.0
Decanoyl (C_{10})	0.714 ± 0.003	−00.7	−0.019*	02.6
Lauroyl (C_{12})	0.618 ± 0.002	−04.4	−0.109*	15.0
Myristoyl (C_{14})	0.489 ± 0.001	−15.0	−0.239*	32.8
Palmitoyl (C_{16})	0.552 ± 0.002	−18.4	−0.177*	24.3
Stearoyl (C_{18})	0.589 ± 0.002	−19.3	−0.139*	19.1

[a] Minimum value for S from Fig. 1.
[b] Slopes calculated between $S_{initial}$ and S_{min}.
[c] Membranes treated with buffer only.
* Statistically different from controls and each other at the $P < 0.01$ level, one-way ANOVA.

nitines containing a slightly shorter or longer chain length. Lauroyl carnitine (LCC) and stearoyl carnitine (SCC) had maximal effects of 15 and 19%, respectively.

The initial slopes of the concentration curves (calculated between $S_{initial}$ and S_{min}) increased with the fatty acid chain length of the acylcarnitine (Table II). The value of the initial slopes of the concentration curves increased with each addition of an ethylene group to the fatty acid chain beginning with a chain length of 10 carbons (0.7×10^{-4} $S/\mu M$, DCC) until a maximum was reached with a chain length of 16–18 carbons (18.4 and 19.3×10^{-4} $S/\mu M$ PCC and SCC, respectively). Lauroyl and myristoyl carnitine produced initial changes of 4.4 and 15.0×10^{-4} $S/\mu M$, respectively.

The effects of acylcarnitine chain length on the change induced in the order parameter (ΔS) and on *in vivo* potency (PI) are compared in Fig. 2. The marked increase in activity can be seen when the acylcarnitine chain length surpasses 10 carbon units for both the animal and the fluorescence polarization experiments. The data also illustrate a close relationship between the *in vivo* and the *in vitro* activity of the acylcarnitines with the exception of myristoyl carnitine (C_{14}).

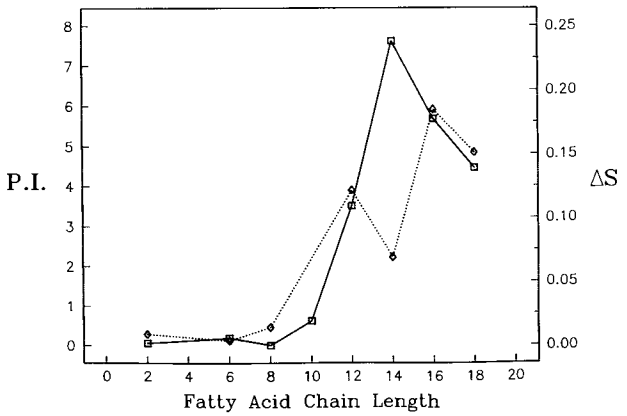

Fig. 2. Effect of acylcarnitine chain length on maximal change in lipid order (ΔS) *in vitro* and potency index (PI) *in vivo*. Potency index from Table I. Values for ΔS from Table II. See legend in Fig. 1 for key to chain length.

DISCUSSION

In Vivo/in Vitro Correlation

The ability of the acylcarnitines to enhance cefoxitin absorption *in vivo* correlated well with their capacity to decrease the lipid order of BBM *in vitro* with the exception of MCC (Fig. 2). In both cases a cutoff in activity was seen at a hydrocarbon chain length of 10 carbon units. In addition, the largest relative effects observed both *in vivo* and *in vitro* were attained with PCC (F = 68 and 24% ΔS, respectively).

Although the *in vitro* potency was different among the long-chain acylcarnitines (Fig. 1), this apparently had no bearing on their potency *in vivo* (Table I). Further increases in drug absorption did not appear to be dependent on increasing the fatty acid chain length of the acylcarnitine beyond 12 carbon units. This could be because F is a less sensitive indicator of potency since it is a combination of several processes or, perhaps, the limiting factor to the *in vivo* potency is the intrinsic permeability of the drug itself once a maximum effect has been obtained with the acylcarnitines.

The discrepancy between the MCC *in vivo* and *in vitro* data cannot be explained at this time. The previously reported *in vivo* potency of myristoyl carnitine was well below what might be predicted from the fluorescence anisotropy data. Subsequent *in vivo* experiments, however, have shown MCC to have comparable promoting activity to PCC (unpublished results).

Acylcarnitine Mechanism of Action

Perturbation of the lipid structure of biological membranes has long been correlated to changes in membrane activity and, in particular, changes in permeability (16). Several studies have linked a perturbation of intestinal BBM structure, induced by different classes of absorption enhancers, to a change in the permeability of the membranes to poorly absorbed drugs (1–5). These observations along with the results from this study suggest that increased drug absorption *in vivo* induced by enhancers may occur by a disruption of the membrane architecture.

It may be that the order of the lipid bilayer must be perturbed to a certain degree before a change in membrane permeability is observed. In this study, all long-chain acylcarnitines that significantly increased mucosal permeability to cefoxitin also reduced the order parameter by roughly 20% or more (Table II). Kutchai *et al.* observed that a 20% increase in the "fluidity" of the membrane lipids was necessary before a significant effect could be seen in the normal transport processes of membranes (17).

Considerable effects both *in vivo* and *in vitro* were observed beginning with LCC (C_{12}). Other studies have observed that surfactants with saturated 12-carbon chains are particularly effective in terms of membrane penetration and alterations in membrane permeability (18,19). The lauryl chain appears to possess optimal physical properties of lipophilicity and size for disrupting lipid bilayers.

There appears to be a narrow range of activity where the effectiveness of the acylcarnitines is related to their lipophilicity (C_{12}–C_{16}). The activity of the acylcarnitines is reduced on either side of this range (Fig. 2). It appears that the acylcarnitines must possess a critical chain length in order to partition into the membrane and, in addition, must perturb the lipid order beyond a threshold value (15–20%) to increase membrane permeability effectively. If the acylcarnitine chain length is greater than a certain number of carbon atoms (C_{16}), then incorporation into the lipid bilayer of the BBM may become unfavorable as is suggested by a reduction in both *in vivo* absorption enhancement and ΔS with the 18-carbon acylcarnitines (SCC).

Absorption Enhancer Screening

This report has demonstrated that a relationship exists between the *in vivo* drug absorption promoting activity of an agent and its *in vitro* membrane perturbing ability. It is proposed, therefore, that fluorescence polarization may be a valuable tool in screening other potential absorption enhancers, especially those with similar physicochemical properties.

Fluorescence polarization has been used in other areas of investigation to relate membrane perturbation effects to the *in vivo* activity of drugs. For instance, the short-chain fatty acids pentanoate through octanoate were found to have an increasing effect on mouse synaptic membranes that coincided with an increased ability to protect against chemically induced seizures (20). This is further evidence that fluorescence polarization may have utility as an indicator of the *in vivo* potency of membrane active compounds.

Since this technique involves monitoring the fluorescence activity of a probe that is incorporated into the core of the membrane bilayer (11), it is likely that only those enhancers that can partition into the membrane would be amenable to testing with this technique. A better understanding of penetration enhancer effects on membrane order might be achieved using probes which incorporate nearer the polar head groups of the membrane. These fluorescent probes, such as trimethylammonium diphenylhexatriene (TMA-DPH) and anilino-naphthalene sulfonic acid (ANS), may demonstrate surface effects of compounds which might not be detected at the membrane core as monitored by DPH (5,8,21). Studies investigating the effects of other classes of penetration enhancers on membrane order using both surface and core probes should provide valuable information.

In conclusion, there appears to be a critical carbon chain length (C_{12}) for acylcarnitine activity. At shorter chain lengths there was not a significant effect on either penetration of cefoxitin or perturbation of membrane order. Above this critical chain length, significant effects were observed on both the lipid order parameter of BBM and drug absorption enhancement. A comparison of the effect of the acylcarnitines on membrane order and penetration enhancement suggests a good *in vivo/in vitro* correlation exists. These results indicate that the effect on membrane order may be a relative indicator of absorption promoter potency, and therefore, fluorescence polarization may serve as a screening method for novel absorption enhancing agents.

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