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Intestinal permeability enhancement of levothyroxine sodium by straight chain fatty acids studied in MDCK epithelial cell line

Dimple Pabla, Fatemeh Akhlaghi, Hossein Zia*

Biomedical and Pharmaceutical Sciences, University of Rhode Island, 125, Fogarty Hall, 41 Lower College Road, Kingston, RI 02881, USA

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ABSTRACT

Levothyroxine sodium (T4), administered orally, is used for the treatment of hypothyroidism. T4 is a narrow therapeutic index drug with highly variable bioavailability (40–80%). The purpose of the present study was to increase the transepithelial transport of T4 using straight chain fatty acids across Madin-Darby Canine kidney (MDCK) cell line. Capric acid (C10), lauric acid (C12) and oleic acid (C18) were studied in molar ratios of 1:0.5, 1:1, 1:2 and 1:3 (T4:fatty acid). Transport of the hydrophilic marker, Lucifer yellow, was also studied. All three fatty acids proved to significantly increase T4 transport and the order of enhancement was to the effect of C12 ≈ C18 > C10. This Increase in transport was accompanied by reductions in transepithelial electrical resistance (TEER) values, which indicates an opening of tight junctions. Cytotoxic effects of the fatty acids were evaluated by TEER measurements, lactate dehydrogenase release, percent viability and propidium iodide staining of the cells. At the lower molar concentrations of 1:1, the fatty acids did not show any toxicity. However, C12 and C18 when added, to T4:fatty acid molar ratio of 1:2 and 1:3, respectively showed severe toxicity with irreversible damage to the cells. Hence, addition of fatty acids to T4 formulations at low concentrations can significantly improve intestinal permeability of T4 without any toxicity potentially leading to improved bioavailability.

1. Introduction

Levothyroxine (T4), administered orally as a sodium salt, is the exclusive choice for the treatment of hypothyroidism and various forms of thyroid neoplasia (Singer et al., 1995). Levothyroxine has a narrow therapeutic index with severe adverse effects associated with hyper- and hypothyroidism including harmful cardiac and/or metabolic effects (Carr et al., 1988; Klein and Ojamaa, 2001). Careful dose titration and close clinical follow-up are, therefore, essential for the safe and effective use of T4.

The gastrointestinal bioavailability of T4 is known to be incomplete and fairly variable ranging from 40% to 80% (Benvenga et al., 1995; Stone et al., 1984), which leads to bioequivalence problems between available products (Klein and Danzi, 2003; Olveira et al., 1997; Shroff and Jones, 1980; Wartofsky, 2002). The available T4 formulations are also known to be unstable and unable to maintain potency throughout their shelf life, adding to the bioequivalence problem (FDA, 1997). Oral T4 is primarily absorbed in the jejuno-ileal segment of the small intestine (Hays, 1991). The most important factor affecting the oral absorption of a drug, besides dissolution, is the permeability of the drug across the gastrointestinal lining. Improving permeability may, therefore, potentially improve the bioavailability of a drug. Transport of hydrophilic drugs across the intestinal epithelium is confined mainly to paracellular pathways. However, the limited surface area and the tight junctions present between the adjacent cells restrict the transport of the drugs and are responsible for the low bioavailability of hydrophilic drugs across the paracellular route (Bohets et al., 2001).

T4 has been categorized into Biopharmaceutics Classification System (BCS) class III – a high solubility and low permeability drug (Lindenberg et al., 2004). Even though thyroid hormones are generally regarded as lipophilic, the highly polar zwitter-ionic nature of the alanine side chain prevents the passage of the molecule through the hydrophobic inner core of the lipid bilayer of the membrane (Fig. 1), resulting in low permeability values.

Medium chain fatty acids including capric acid (C10), lauric acid (C12), and unsaturated long chain fatty acids such as oleic acid (C18), have been shown to increase the permeability of a series of hydrophilic drugs by dilating the tight junctions and/or changing the cytoskeleton of the intestinal epithelial cells without pronounced cytotoxicity (Higaki et al., 2001; Lindmark et al., 1998, 1997; Constantinides et al., 1996; Kamm et al., 2000; Ogiso et al., 1991). One of the major advantages of these excipients

* Corresponding author. Tel.: +1 401 874 5022; fax: +1 401 874 5787.
E-mail addresses: hzia@uri.edu, dimple@mail.uri.edu (H. Zia).

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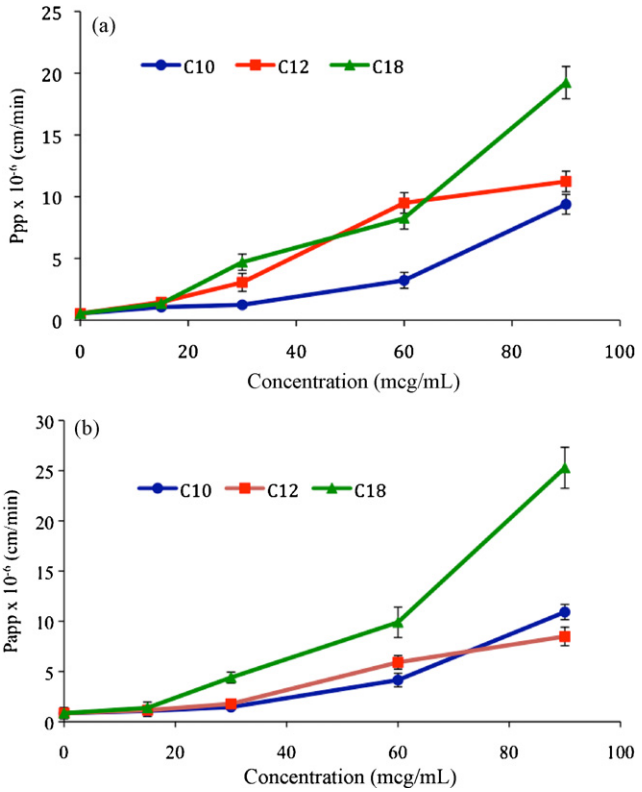

Fig. 1. Effect of concentration of fatty acids on apparent permeability coefficient (*Papp*) of (a) T4 and (b) LY; $n = 3$.

is the ease of incorporating into the conventional oral dosage forms without the need for complex or expensive formulation techniques.

In vitro epithelial cell models such as Caco-2 and MDCK are frequently used as simple and efficient methods for studying cell growth regulation, metabolism and transport mechanisms across the intestinal epithelia. For the present study, MDCK-II (Madin-Darby canine kidney) epithelial cell line was chosen to characterize the intestinal permeability of T4 in the presence of various fatty acids. MDCK cell line is derived from the kidney of a normal male cocker spaniel. MDCK cells differentiate into columnar epithelium and form tight junctions when cultured on semipermeable membranes and have been successfully used as a cellular barrier to assess the intestinal epithelial drug transport of numerous drugs (Cho et al., 1990, 1989; Irvine et al., 1999; Ranaldi et al., 1992, 1996).

Hence, the objective of this study was to evaluate permeability enhancement of T4 using straight chain fatty acids, C10, C12 and C18 by the use of MDCK cell monolayer model.

2. Materials and methods

2.1. Materials

Levothyroxine sodium, Lucifer yellow (LY), capric acid (C10), lauric acid (C12), oleic acid (C18) and ammonium hydroxide *TraceSelect* were purchased from Sigma Aldrich (St. Louis, MO, USA). MDCK-II cells were obtained from Dr. Borst Laboratory in The Netherlands Cancer Institute (Amsterdam, The Netherlands). Reagents for cell culture and transport studies including Fetal Bovine Serum (FBS), Hank's balanced salt solution (HBSS), phosphate buffered saline (PBS), nonessential amino acids (NEAA), HEPES, penicillin–streptomycin solution and trypsin–EDTA were purchased from Lonza Walkersville, Inc. (Walkersville, MD, USA). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Invitrogen Corporation (Carlsbad, CA, USA). The 24-well transwell plates, inserts for the plates (1.0 μm pore size, polyethylene track-etched membrane), sterilized cell culture flasks, serological pipettes, sterilizing 0.22 μm cellulose acetate filters were purchased from Fisher Scientific (Pittsburg, PA, USA).

2.2. Cell culture

The MDCK-II cells were cultured in DMEM supplemented with 10% FBS, 1% NEAA and 2% penicillin–streptomycin in a 37 °C/5% CO_2 humidified incubator. The cells were harvested at approximately 80–90% confluency using 0.25% trypsin–EDTA. The cells were then seeded onto transwell inserts at a density of 5×10^4 cells/cm^2 and grown for 5–6 days. A 100% confluent monolayer of cells on the inserts was used for transport assays. The formation of confluent monolayers and tight junctions was tested and confirmed by measuring transepithelial electrical resistance (TEER) across the cell monolayers and transport of LY, a hydrophilic marker primarily transported via paracellular pathway.

2.3. Measurement of TEER

The TEER test was performed using a Millicell ERS® voltommeter (Millipore, Bedford, MA, USA). The following equation was used to calculate the TEER values (Ω cm^2) across the cell monolayers:

$$\text{TEER} = (R - R_{\text{blank}})A$$

where R is the measured resistance across a cell monolayer, R_{blank} is the resistance of a blank well, and A is the surface area of the transwell filter (0.3 cm^2). TEER values between 200 and 300 Ω cm^2 were taken as indicative of formation of a cell monolayer with tight junctions.

TEER values were also measured at the end of each transport assay (2 h) to assess the toxic effects of fatty acids on the cell membrane's integrity. Also, at the end of each transport assay the cells were washed with HBSS three times and supplemented with complete media for 2 h. TEER measurements were repeated 2 h after treatment to evaluate the regeneration of cell monolayers from any possible toxic effects of fatty acids. The TEER values measured prior to transport assays were taken as the initial values expressed as 100%. All subsequent TEER values are expressed as a percent of the initial value.

2.4. Transport studies

Transport studies across the monolayer of MDCK-II cells were performed in 24-well transwell plates (0.8 mL) using a polyethylene track-etched membrane insert (0.3 mL volume and 0.3 cm^2 cell growth surface area). HBSS without Ca^{2+}/Mg^{2+} containing 1% DMSO and 10 mM HEPES (pH 6.4) was used as the donor (apical) solution whereas HBSS with Ca^{2+}/Mg^{2+}, 1% DMSO and 10 mM HEPES (pH 7.4) was used as the receiver (basolateral) solution. After confirming the formation of monolayer by TEER test, the cells were washed with HBSS three times and equilibrated in donor and receiver solutions for 30 min. Transport assays were conducted using 0.3 mL of test solutions prepared in the donor solution. T4 and LY, at concentrations of 30 and 100 μg/mL, respectively, were tested with and without fatty acids present. All the fatty acids were studied in ratios of T4:fatty acid of 1:0.5, 1:1, 1:2 and 1:3. Transport studies were carried out in a Barnstead lab-Line MaxQ 4000 incubated shaker (ThermoFisher Scientific, Fairlawn, NJ) at 37 °C and 100 rpm, to minimize the effects of "unstirred water layer". At time intervals of 0.5, 1, 1.5 and 2 h the inserts were moved to new wells

containing fresh receiver solution. All experiments were performed in replicates of three.

2.5. Drug analysis

Levothyroxine (T4) concentrations in the transport samples were analyzed using a validated and published ICP-MS assay (Pabla et al., 2008). Samples withdrawn at each time point from the transport studies were diluted appropriately with 0.5% ammonium hydroxide solution. An internal standard, antimony, was added to all the samples at a concentration of 10 ng/mL, prior to dilution. The diluted samples were then directly infused into a Thermo electron X7 ICP-MS instrument acquiring the data in peak jumping mode at m/z of 126.90 for iodide (obtained from the breakdown of T4) and 120.90 for antimony. T4 concentrations in the transport samples were extrapolated from calibration curves ranging from 0.3 to 100 ng/mL T4, run simultaneously with the unknown samples on the day of analysis.

2.6. Lactate dehydrogenase measurement

Lactate dehydrogenase (LDH) is a cytosolic enzyme that is released upon cell lysis. The presence of LDH in the apical compartment is indicative of cell damage. For LDH measurement, 50 μL of apical solutions at the end of transport assays was used. The LDH content was determined using an LDH kit – CytoTox96® non-radioactive cytotoxicity assay (Promega Corporation, Madison, WI). Control experiments were performed with a lysis solution provided with the assay kit to obtain maximum LDH release, set as 100%. The LDH measured in the apical solutions of cells treated with fatty acids were related to maximum LDH release caused by the lysis solution and expressed as % release.

2.7. Cell viability assessment

The effect on the viability of cells upon exposure of monolayers to fatty acids was studied using a CellTiter-Fluor™ Cell Viability Assay kit (Promega Corporation, Madison, WI). Cells were seeded in 96-well culture plates at density of 5×10^4 cells/cm^2 and cultivated under normal conditions for 5 days. The cells were then incubated with test solutions under similar conditions as the transport studies and cell viability was assessed at the end of 2 h. The fluorescent signal of the cells treated only with HBSS buffer was set as 100% viability.

2.8. Propidium iodide staining

Propidium iodide (PI) is a fluorescent agent that enters cells with damaged membranes and binds to nucleic acids producing a red signal in dead cells. It does not penetrate intact plasma membranes of living cells and therefore can be used to study cell viability and toxic effects of chemical additives on the membrane integrity of monolayers (Artursson et al., 1996). The cells grown on transwell filters were treated with T4 and fatty acids as described for transport studies for 2 h. At the end of the 2 h, the monolayers were then washed twice with phosphate buffered saline (PBS) and incubated with 30 μg/mL of PI in PBS for exactly 3 min. The cells were then fixed for 10 min in a 4% paraformaldehyde solution in PBS. The monolayer supporting the filter membranes was then cut out from the cell culture inserts and mounted on glass slides in a 1:1 glycerol:PBS solution. The monolayers were examined under a Nikon Eclipse TE2000 E inverted microscope (Nikon Instruments, Inc., Melville, NY) at excitation wavelength = 536 nm and emission wavelength = 617 nm.

2.9. Data analysis

The apparent permeability coefficients of T4 and LY were calculated according to the following equation:

$$Papp = \frac{dc}{dt} \cdot \frac{V_r}{60AC_0}$$

where $Papp$ is the apparent permeability coefficient (cm/min), dc/dt is the cumulative flux (μg/mL/min) determined by the slope of the regression line of cumulative receiver concentration versus time, V_r is the volume of the receiver compartment (0.8 mL). A is the surface area of the monolayer (0.3 cm^2) and C_0 is the initial concentration of T4 or LY added to the donor side (μg/mL).

All results are expressed as mean ± standard deviation of three replicates. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni multiple comparison tests using SPSS version 17 (SPSS Inc., Chicago, IL).

3. Results

3.1. Transport studies

Apparent permeability coefficients (*Papp*) were calculated to estimate the enhancing effects of C10, C12 and C18 on the transport of T4 (Table 1). The *Papp* value of T4, without any enhancer, was $0.54 \pm 0.06 \times 10^{-6}$ cm/min while LY was transported with a *Papp* of $0.84 \pm 0.53 \times 10^{-6}$ cm/min (Table 2). In the presence of fatty acids, significant accumulation of T4 and LY occurred in the basolateral compartment leading to significantly higher *Papp* values as compared to the control, with p-values <0.05. A concentration dependant increase in the *Papp* values of both T4 and LY was seen among each fatty acid studied, shown in Fig. 1. Increase in the concentration of C10 from 1:0.5 to 1:3 resulted in a 2–17-fold increase in the *Papp* value of T4. Similarly, there was a 2–20-fold and a 2–35-fold increase with C12 and C18, respectively. All three fatty acids studied showed similar effects on *Papp* of both T4 and LY, which indicates that transport of T4 is increased by the paracellular route. Amongst the three fatty acids studied, the increase in T4 transport was in the order of C12 ≈ C18 > C10.

3.2. TEER measurements

TEER measurements were made both prior to and at the end of transport studies (2 h of exposure to enhancers). Tables 1 and 2 show the TEER measurements for T4 and LY, respectively, expressed as percentage of initial values. The increase in *Papp* values was accompanied with a drop in TEERs indicating the opening of tight junctions. TEER measurements taken 2 h after removal of enhancers and re-supplementing with complete media represent recovery of monolayers from the effects of fatty acids. As seen in Table 1, the TEER values for most fatty acids were restored to >95% of the initial value, except for 1:3 concentration of C12 and C18. Irreversible decrease in TEER values suggests permanent damage to the cells. Similar results were seen for LY (Table 2), with incomplete or no recovery of TEER values with C12 and C18 at concentration of 1:3.

3.3. LDH measurement

The toxic effects of fatty acids on the cells were evaluated by measuring the amount of LDH released into the apical compartment, the results of which are shown in Table 1. The values are expressed as a percentage of LDH released by cells treated with a cytotoxic reagent, the value of which was set as 100%. C10 was the least toxic with no significant difference between

Table 1
Permeation enhancing effect of fatty acids on the transport of T4 and related toxicity values. All values expressed as mean ± s.d. of three replicates.

Treatment	*Papp* × 10^{-6} (cm/min)	TEER 2 h (Ω cm^2)	TEER 2 h Posttreatment (Ω cm^2)	LDH (%)	Viability (%)
HBSS	n/a	97.97 ± 3.15	100.51 ± 2.91	9.59 ± 0.12	n/a
T4	0.54 ± 0.06	99.15 ± 1.19	111.36 ± 13.40	9.20 ± 2.85	100.59 ± 5.48
C10					
1:0.5	1.06 ± 0.13	93.33 ± 5.09	105.62 ± 5.44	6.34 ± 1.87	104.95 ± 2.20
1:1	1.24 ± 0.12*	85.28 ± 1.18*	99.54 ± 1.05	11.02 ± 3.73	102.87 ± 7.97
1:2	3.23 ± 0.65*	82.36 ± 2.83*	105.78 ± 2.04	7.49 ± 1.91	97.72 ± 6.28
1:3	9.38 ± 0.80*	76.88 ± 3.53*	97.31 ± 1.82	8.69 ± 3.75	100.32 ± 1.83
C12					
1:0.5	1.46 ± 0.16*	82.19 ± 0.95*	103.80 ± 3.15	9.01 ± 3.69	97.78 ± 0.75
1:1	3.06 ± 0.73*	80.25 ± 0.57*	105.37 ± 7.78	9.20 ± 1.96	100.70 ± 3.39
1:2	9.49 ± 0.83*	75.02 ± 1.51*	105.94 ± 15.69	18.74 ± 0.80*	86.02 ± 3.37*
1:3	11.23 ± 0.84*	55.79 ± 0.41*	75.04 ± 4.38*	25.92 ± 2.40*	82.03 ± 4.22*
C18					
1:0.5	1.34 ± 0.13*	82.49 ± 2.73*	102.11 ± 2.85	6.83 ± 2.79	100.31 ± 3.62
1:1	4.69 ± 0.66*	78.75 ± 1.01*	97.92 ± 1.05	7.76 ± 2.53	99.02 ± 1.14
1:2	8.26 ± 0.88*	67.07 ± 0.53*	103.12 ± 1.77	8.01 ± 1.20	99.52 ± 1.13
1:3	19.24 ± 1.30*	41.06 ± 0.30*	40.48 ± 0.82*	75.86 ± 6.02*	41.88 ± 4.86*

n/a – not applicable.
* $p < 0.05$; significant difference from control.

Table 2
Apparent permeation coefficients and TEER values for LY transport in the presence of fatty acids.

Treatment	*Papp* × 10^{-6} (cm/min)	TEER 2 h (Ω cm^2)	TEER 2 h posttreatment (Ω cm^2)
HBSS	n/a	97.97 ± 3.15	100.51 ± 2.91
LY	0.84 ± 0.53	97.16 ± 0.49	103.03 ± 3.24
C10			
1:0.5	1.07 ± 0.53	98.25 ± 0.33	103.00 ± 0.43
1:1	1.45 ± 0.26*	86.25 ± 1.04*	106.94 ± 7.55
1:2	4.15 ± 0.68*	79.92 ± 1.98*	107.63 ± 4.53
1:3	10.92 ± 0.76*	78.58 ± 1.98*	90.36 ± 7.44
C12			
1:0.5	1.42 ± 0.44*	81.08 ± 2.19*	102.86 ± 1.32
1:1	1.78 ± 0.44*	82.41 ± 1.28*	103.63 ± 3.47
1:2	5.91 ± 0.67*	71.99 ± 4.57*	75.10 ± 4.27*
1:3	8.49 ± 0.92*	57.87 ± 2.19*	76.30 ± 3.93*
C18			
1:0.5	1.38 ± 0.58*	84.02 ± 1.23*	100.04 ± 2.01
1:1	4.40 ± 0.54*	80.01 ± 1.99*	102.34 ± 2.46
1:2	9.89 ± 1.51*	65.19 ± 1.98*	117.57 ± 4.11
1:3	25.29 ± 2.04*	32.20 ± 5.34*	33.40 ± 5.76*

n/a – not applicable.
* $p < 0.05$; significant difference from control.

LDH amounts released by cells exposed to C10 when compared to untreated cells. C12 did not show toxicity at 1:0.5 and 1:1 molar ratio but the LDH release increased significantly at higher concentrations. The highest toxicity, however, was seen at 1:3 concentration of C18 though lower concentrations did not show any toxicity.

3.4. Cell viability

The percentage of viable cells was measured after exposure to fatty acids as another means of evaluating toxicity. Activity of a live-cell protease was studied as a marker of cell viability. The results are shown in Table 1 where cell viability is expressed as percentage of live-cell protease activity measured in untreated cells (100% viable). The results are in agreement with TEER and LDH values. C10 showed 100% viable cells. However, the viability decreased to 86% and 82% at 1:2 and 1:3 concentration of C12, respectively. Only about 41% of cells remained viable after exposure to C18 at a concentration of 1:3 indicating severe toxicity.

3.5. PI staining

To visually evaluate the damage to cell monolayers upon exposure to fatty acids, the cells were stained with PI and monitored under a fluorescence microscope. PI stains the nucleus of dead cells and therefore can be used to study toxicity. The results are consistent with the LDH measurements and the viability values. Fig. 2 shows the PI stained monolayers treated with various fatty acids at concentration of 1:3. C18 shows the most pronounced effect with a high number of dead cells visible.

4. Discussion

Current estimates suggest that 10–12 million Americans suffer from hypothyroidism affecting their everyday quality of life (Klein and Danzi, 2003). *Synthroid*®, an oral tablet formulation of T4, is the second most widely prescribed drug in the United States after Lipitor™ with more than 25 million prescriptions filled every year (DrugTopics, 2007). The oral bioavailability of T4 is highly variable with a serious bioequivalence problem between the various

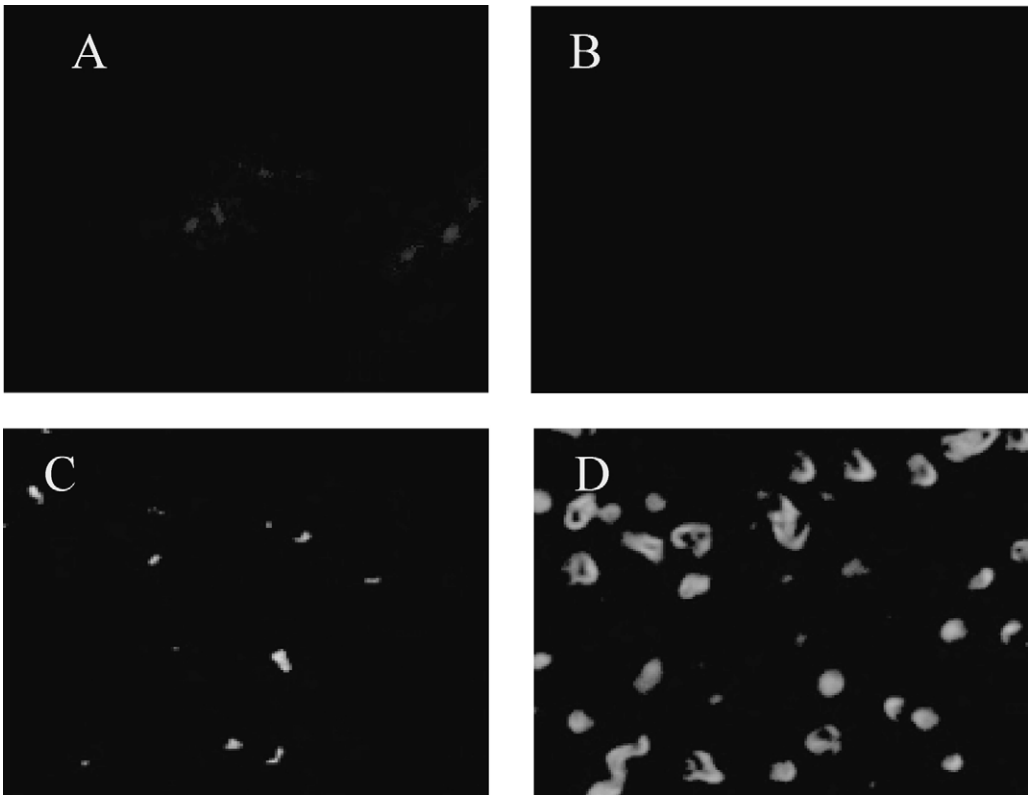

Fig. 2. PI staining of monolayers after transport experiments with 1:3 concentration of (A) No enhancer, (B) C10, (C) C12 and (D) C18. Uptake of stain indicates dead cells. Note the cell damaging effects are most pronounced for C18.

available products. In our study, we examined the intestinal permeability enhancing effects of medium chain fatty acids – C10 and C12 and a long chain unsaturated fatty acid – C18 on T4 across MDCK cell monolayers. The currently available formulations of T4 do not contain any permeation enhancing excipients. MDCK cells are used as a common model for studying drug transport mechanisms in small intestine (Hidalgo et al., 1992; Irvine et al., 1999; Kim et al., 1993).

Fatty acids occur physiologically in the gastrointestinal tract because of which they have been studied frequently as absorption enhancers for hydrophilic drugs. Thyroid hormones including T4 are generally regarded as lipophilic. However, T4 can exist as a highly polar zwitter-ionic molecule at its absorption site in small intestine, which can seriously restrict its absorption via passive diffusion (Bohets et al., 2001; Hidalgo, 2001). This explains the low *Papp* value of T4 observed without an enhancer.

The influence of the three fatty acids was studied in molar ratios T4:fatty acid of 1:0.5, 1:1, 1:2 and 1:3. The transport of Lucifer yellow, a hydrophilic marker that is predominately transported via paracellular route was also studied to characterize the pathways of absorption enhancement. Most absorption enhancers mediate their effects by changes in cell membrane integrity resulting in cytotoxicity (Swenson and Curatolo, 1992). A complete recovery of cells after exposure to absorption enhancers is highly desirable to prevent permanent cell damage due to repeated administration. Hence, the cytotoxicity of the fatty acids was also evaluated by recovery of TEER measurements, LDH release, % cell viability and PI staining.

In the presence of fatty acids, significant increase in the transport of T4 was seen as indicated by the higher *Papp* values ($p < 0.05$). All three fatty acids showed a concentration dependent increase in *Papp* values. Addition of C10 at a ratio of 1:0.5 (T4:C10) resulted in an approximately 2-fold increase in *Papp* value of T4. The *Papp* values further increased with the increase in concentration of C10 leading to a 17-fold increase when added at a ratio of 1:3. These results are in agreement with the steep dose–effect relationship of C10 seen by Anderberg et al. and Lindmark et al. (Anderberg et al., 1993; Lindmark et al., 1995). The decrease in TEER values upon exposure of cells to C10 indicates modulation of tight junctions and paracellular integrity. Similar increase in LY transport and decrease in TEER values in the presence of C10 further suggest that C10 primarily affects the paracellular pathway. Upon removal of C10 at the end of transport studies, the TEER values recovered back up to initial values indicating a temporary, fully reversible effect of C10 on the tight junctions. These results are further supported by almost 100% cell viability and negligible amounts of LDH released as compared to the control. All concentrations of C10 studied showed no cytotoxicity.

Lauric acid (C12) showed a similar concentration–effect relationship as C10. However, C12 had a more pronounced effect on transepithelial transport of both T4 and LY. C12 has been previously shown to result in a higher increase in absorption of various hydrophilic drugs in comparison to C8, C10 and C14 (Higaki et al., 2001; Ogiso et al., 1991). The increase in *Papp* values was accompanied by decrease in TEER values, again indicating alteration of tight junctions. The cells when exposed to higher concentrations of C12, however, did not recover fully. At molar ratios of 1:2 and 1:3 (T4:C12), TEER values recovered to 75% of the baseline value. This was accompanied with higher LDH release and a decrease in % cell viability. Therefore, the benefits of increased *Papp* values at higher concentrations of C12 are nullified by increased toxicity to the cells.

Oleic acid (C18) is a long chain unsaturated fatty acid which has been studied as an absorption enhancer alone or in combination with other enhancers. In the present study, C18 showed comparable increase in transport of T4 and LY to C12. Fusogenic fatty

acids such as oleic acid have the ability to alter the lipid structural dynamics of biological membranes and induce permeability changes (Murakami et al., 1988; Werner et al., 1996). Increase in C18 concentration lead to a 35-fold increase in *Papp* but toxicity studies revealed irreversible damage to the cells confirmed by massive staining of cells by PI indicating large number of dead cells.

A promising absorption enhancer is expected to augment transepithelial transport of drugs with a lack of toxicity and complete recovery of cells. The increase in transport of T4 as reported above can be explained by common mechanisms of absorption enhancement by medium chain fatty acids including structural deformations of tight junctions in the form of dilatations, mobilization of intracellular Ca^{2+} and/or contraction of actin microfilaments leading to enhanced transport via paracellular route (Nishimura et al., 1985; Tomita et al., 1995). On the other hand, increased membrane fluidity reported by long chain fatty acids including oleic acid can enhance drug absorption also via transcellular route in addition to paracellular pathway (Murakami et al., 1988; Werner et al., 1996).

The present study gives valuable information on the enhancement effect of straight chain fatty acids on the epithelial transport of T4. However, further studies are needed to design a viable oral formulation of T4 with enhanced and more predictable bioavailability. The shorter residence time of the drug at the site of absorption and the presence of mucus on the intestinal wall *in vivo*, may act as a barrier to drug diffusion. The presence of intestinal fluids may also lead to dilution of the enhancer further reducing its local permeation enhancement effects (Larhed et al., 1997; Takatsuka et al., 2006). The degree of cytotoxicity observed with C12 and C18 in the present study might also be reduced *in vivo* owing to the shorter residence time of the enhancer and high cell turnover of intestinal epithelia. Nevertheless, daily administration of high doses of fatty acids accompanying the drug might lead to an accumulative toxic effect, which needs to be carefully evaluated while designing an effective formulation. Future studies shall, therefore, involve evaluating the *in vivo* intestinal absorption of T4 in rats following peroral administration of the drug in the presence of currently studied enhancers.

5. Conclusion

Levothyroxine is a pro-hormone administered orally as a sodium salt for the treatment of various types of hypothyroidism. It has a highly variable bioavailability and severe bioequivalence concerns. Improving the intestinal permeability of T4 may lead to higher and less variable absorption, which in turn can potentially lead to improved bioavailability. In this study, we evaluated the effect of three fatty acids, C10, C12 and C18 on epithelial transport enhancement of T4 in MDCK cell line. Permeability enhancement of T4 by fatty acids was in the order of $C12 \approx C18 > C10$. Similar transport enhancement was observed for LY, a hydrophilic marker, indicating the modulation of tight junctions and enhancement via paracellular pathway. At lower concentrations, none of the fatty acids showed toxicity. However, C12 and C18 were highly toxic at higher concentrations causing irreversible damage to the cells. Hence, addition of fatty acids at low concentrations can significantly improve the transepithelial transport of T4 without any cytotoxicity. These results may prove to be useful in developing a new oral formulation for T4 with improved bioavailability characteristics.

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