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Evaluation of Tributyrin Lipid Emulsion with affinity to Low Density Lipoprotein: Pharmacokinetics in Adult Male Wistar Rats and Cellular Activity on Caco-2 and HepG2 Cell Lines

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In vitro and *in vivo* evaluation of tributyrin lipid emulsion

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3. List of nonstandard abbreviations used in the paper:

C_{max}, maximal plasma concentration; FITC, Fluorescein isothiocyanate; K_a, first-order absorption rate constant; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; $T_{1/2}$, elimination half-life; TAE: Tris-Acetate-EDTA; TE, Tris-EDTA; T_{max}, time to reach maximal plasma concentration.

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Abstract

The tributyrin lipid emulsion was proved able to bind to low-density lipoprotein (LDL) *in vitro*. The aim of this study was to investigate the pharmacokinetics of the emulsion *in vivo* and the cellular activity *in vitro*. The pharmacokinetics of tributyrin and its metabolite, butyrate was evaluated in male Wistar rats after administration with pure tributyrin or tributyrin emulsion. After oral administration, C_{max}, T_{max} and $T_{1/2}$ of butyrate were 87.6 μM, 25.3 min and 63.0 min for the pure tributyrin compared to 1344.5 μM, 8.5 min and 19.8 min for the 10% (v/v) tributyrin emulsion. C_{max} and MRT of tributyrin were 2.74 μM and 87.9 min, 4.2 μM and 132.0 min for pure tributyrin and 10% emulsion, respectively. The bioavailabilities of the pure tributyrin vs tributyrin emulsion were 15.3% vs 65.7% and 34.9% vs 64.5% calculated from butyrate and tributyrin, respectively. After the rats were treated with 17α-ethynylestradiol (a LDL receptor upregulator), the distribution volumes calculated from both butyrate and tributyrin were significantly increased after oral administration or infusion of the 10% tributyrin emulsion. The increased distribution volume after co-administration with a LDL receptor upregulator suggested the increased uptake of tributyrin/butyrate by tissues with increased expression of LDL receptors. The selective uptake of the emulsion by the cellular LDL receptors was further confirmed by testing the cellular viability in the presence of competing LDL. The viable cells can reach 92% of control at IC_{50} in Caco-2 and 77% in HepG2 incubated with emulsion in the presence of LDL.

Introduction

Low-density lipoprotein (LDL) is the major transport protein for cholesterol in human plasma. LDL is a spherical particle with a diameter of 20-25 nm. Each LDL particle contains cholesteryl esters in its core which is surrounded by a hydrophilic coat composed of phospholipids, cholesterol and one molecule of hydrophobic protein called apolipoprotein B-100 (apoB-100), the ligand for binding of the lipoprotein to specific receptors on the cell plasma membrane. After binding to the receptors, LDL is internalized via receptor-mediated endocytosis and degraded in lysosomes (Brown and Goldstein, 1986) and the cholesterol is liberated for use in the synthesis of steroid hormones and new plasma membranes. In the rapidly proliferating malignant cells, the increased need of cholesterol for new membrane synthesis may result in over-expression of the LDL receptor (Ho et al., 1978; Gueddari et al., 1993; Leppälä et al., 1995; Maletinska et al., 2000; Sviridov et al., 2003), which allows greater uptake of the lipoprotein by the neoplastic cells. Several investigators showed the uptake of LDL-drug complexes by malignant cells, leading to the possibility of using LDL as drug carrier in cancer chemotherapy (Maranhão et al., 1994; Shawer et al., 2002; Chung and Wasan, 2004). This would increase the anti-neoplastic effects while reducing the toxic effects upon normal cells. However, the native LDL is difficult to obtain and handle, so that its introduction into clinical practice would be rather problematic. In contrast, lipid emulsion, which structurally resembles the lipid portion of the native LDL can acquire apolipoprotein B or apolipoprotein E in plasma (Wilson et al., 1991; Maranhão et al., 1994 and 2002; Shawer et al., 2002; Su and Ho, 2004). The modified lipid emulsion particle enables it to be taken-up by the cell via the LDL receptor-mediated pathway.

Tributyrin, a prodrug of butyrate with a half-life of about 6 min has more favorable pharmacokinetic properties and is better tolerated when compared with butyrate (Planchon et al., 1993). Liquid tributyrin filled gelatin capsules were administered orally resulting in millimolar concentrations of butyrate both in plasma and inside the cell (Conley et al., 1998). In *in vitro* study, tributyrin by itself was found able to induce growth inhibitory and differentiating effects on carcinoma cells and showed even more potent activity than butyrate (Heerdt et al., 1999; Clarke et al., 2001; Schroder and Maurer, 2002; Yan and Xu, 2003). It has also been shown to be an effective anti-tumor agent in combination with other agents *in vitro* (Witt et al., 2000; Gaschott et al., 2001).

In our previous paper (Su and Ho, 2004), we proved the lipid emulsion of tributyrin can bind with LDL in plasma. The purpose of the present work was to determine the pharmacokinetics of the emulsion in rats. And we also performed the uptake study of lipid emulsion of tributyrin in Caco-2 and HepG2 cells, which have been reported to express upregulated LDL receptors (Sviridov et al., 2003).

Materials and Methods

Drugs and Chemicals. Tributyrin (99%) was a product of Kasei Kogyo Co. Ltd. (Tokyo, Japan). Lipoid E80 (containing 80% phosphatidylcholine) was obtained from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (99%) and cholesteryl oleate (>98%) were all obtained commercially from Sigma Co. (St. Louis, MO). Minimum essential medium (MEM) was purchased from Invitrogen Corporation (Grand Island, NY). MEM Non-essential amino acid, anti-human β-lipoprotein and anti-goat IgG FITC conjugate were obtained from Sigma Co. (St. Louis, MO). All other chemicals were of reagent grade and Milli-Q water was used throughout the experiment.

Animals. Adult male Wistar rats, weighing 200 to 300 g were used. The rats were obtained from Laboratory Animal Center (National University of Singapore, Singapore), at least 1 week before the experiments, and they were housed in a temperature-controlled room (22 ± 2°C) with a light cycle of 12 h. The lights were on from 8:00 am to 8:00 pm, during which time all the experiments were conducted. The animals had free access to standard laboratory chow and tap water, unless otherwise stated.

Preparation of Tributyrin Emulsion. The emulsion was prepared according to the procedures developed by our previous study (Su and Ho, 2004). Briefly, after Lipoid E80, cholesterol and cholesteryl oleate (65.8:13.7:20.5, w/w) were dissolved in chloroform, the solvent was evaporated and the residue was placed in desiccator overnight. The dry residue was fully suspended in water and the obtained dispersion was added to tributyrin. The tonicity of the emulsion prepared was adjusted with glycerol. Then the mixtures were sonicated using a probe sonicator (Sonics & Materials Inc., Newtown, CT), with the probe placed vertically in the center of the mixture. The sample

container was cooled in an ice bath during sonication to minimize the heat generated. Sonication was carried out for 20 min with 1 second pulse at a constant 35 W. The pH value of the emulsion was adjusted to pH 7.0 with 1 M NaOH. The emulsion prepared would have particle size of 230 to 250 nm and zeta potential of -40 to -50 mV.

Pharmacokinetic Experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee (National University of Singapore, Singapore). At least 1 day before the experiments, the rats underwent surgery for the implantation of vascular cannulas. The right jugular vein was exposed and cannulated with a heparinized polyethylene-50 tube for blood sampling. The tip of the cannula was advanced to the right atrium of the heart. When intravenous administration was required, the left femoral vein was also exposed and cannulated with a heparinized polyethylene-10 tube, which was advanced to the inferior *vena cava*. With oral route of administration, the femoral vein was left uncannulated. The cannulas were then passed under the skin and fixed near the base of the neck.

The pharmacokinetics of tributyrin and its metabolite, butyrate was evaluated in male Wistar rats after administration with pure tributyrin or tributyrin emulsion at the dose of 2 g/kg. Each dose of tributyrin was calculated on the basis of the fasted body weight of individual rat. Oral doses were given by 1.5-inch, 18-gauge, curved gavage needle and infusion was delivered by Terufusion syringe pump TE-331 (Terumo, Tokyo, Japan) for two hours. To confirm the effective binding of the lipid emulsion particles to the specific LDL receptors *in vivo*, pharmacokinetics of tributyrin was also evaluated in rats previously treated subcutaneously for 5 days with 17α-ethynylestradiol, a compound that is known to upregulate the activity of the LDL receptors in tissues (Chao et al., 1979).

Blood samples (200 µl) were drawn into a heparinized syringe via the jugular vein-cannula. Samples were obtained at 0 (2–10 min before drug administration), 5, 10, 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 240 and 360 min after oral dosing. For infusion, samples were obtained at 30, 60, 90 and 120 min during infusion and 5, 10, 15, 30, 45, 60, 90, 105 and 120 min after infusion. The collected blood volume was replaced with 200 µl of warmed saline after each sample. The blood samples were stored in vials with phenylmethyl sulfonyl fluoride (PMSF) in ice bath and the plasma was separated within 1 hour. The separated plasma was stored at -80°C and analyzed within 7 days using GC/MS method reported previously (Su et al., 2004).

Pharmacokinetic Analysis. The plasma concentration-time profiles after oral administration were characterized by the following poly-exponential equation:

$$C(t) = \sum_{i=1}^{n} A_i \cdot e^{-\lambda_i \cdot t} \qquad (1)$$

where $C(t)$ is the plasma concentration of drug at time t, A_i and λ_i are the coefficients and the exponents of the equation, respectively. The plasma concentration-time profiles after infusion were characterized by another two poly-exponential equations:

$$C(t) = \sum_{i=1}^{n} \frac{A_i}{\lambda_i \cdot T}(1 - e^{-\lambda_i \cdot t}) \qquad t < T \qquad (2A)$$

$$C(t) = \sum_{i=1}^{n} \frac{A_i}{\lambda_i \cdot T}(1 - e^{-\lambda_i \cdot T}) \cdot e^{-\lambda_i \cdot (t-T)} \qquad t \geq T \qquad (2B)$$

where T is the duration of the infusion. The plasma data were modeled using WinNonlin version 4.1 pharmacokinetic software (Pharsight Corporation, Mountain View, CA), to calculate pharmacokinetic parameters of pure tributyrin and tributyrin emulsion from the plasma concentrations of both butyrate and tributyrin. When fitting to compartment

model, the goodness of fit and the most appropriate model were determined by assessing the randomness of the scatter of actual data points around the fitted function and by using Akaike's information criterion (Akaike, 1976) and coefficient of variation percentage. The butyrate and tributyrin data from oral administration of pure tributyrin or tributyrin emulsion were best described with a one-compartmental model and model independent, respectively. Both the butyrate and tributyrin data from infusion of tributyrin emulsion were analyzed by one-compartmental model. The area under the curve (AUC) and total clearance (CL) were estimated through the non-compartmental method. AUC was calculated using the trapezoidal method. The mean residence time (MRT), bioavailability (F; using the average AUC of the intravenous data as the reference value), and volume of distribution (V) were calculated using standard formulas (Rowland and Tozer, 1995). Statistical significance testing was done using analysis of variance (ANOVA) (SPSS for Windows, release 12.0, SPSS Inc., Chicago, IL). A difference between mean values was considered significant if the *p*-value obtained was ≤ 0.05.

DNA Fragmentation Assay. To detect the activity of tributyrin after prepared as emulsion *in vitro*, the apoptotic DNA fragments was performed as described previously with minor modification (Herrmann et al., 1994). Following incubation with 4 μM pure tributyrin or tributyrin emulsion at 37°C for 96 h, the adherent and nonadherent cells were harvested after centrifugation. The harvested cells were washed with phosphate buffered saline (PBS) and the cell pellets are treated with 3 ml lysis buffer (1% NP-40 in 20mM EDTA, 50mM Tris-HCl, pH 7.4) at 37°C overnight. The supernatant was brought to 1% SDS and treated for 2 h with 0.5 mg/ml RNase at 56°C followed digestion with 0.25 mg/ml proteinase K for at least 2h at 37°C. After addition of 0.5 vol 10M

Ammonium acetate the DNA was precipitated with 2.5 vol isopropanol at -20°C overnight. DNA was collected and thoroughly rinsed with 15 ml 70% ethanol for 2 h followed by centrifugation for 20 min at 14,000 rpm. The pellet was dried in a SpeedVac to remove the residual ethanol. DNA was dissolved in 25 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 37°C overnight and run for gel electrophoresis on 1% agarose gel in TAE buffer (40 mM Tris-HCl, 20 mM acetatic acid, 1 mM EDTA pH 7.8) with 0.5 µg/ml ethidium bromide at 100 v for 1 hour.

LDL Receptors in Caco-2 and HepG2 Cell Lines. To prepare for the studies of tributyrin emulsion growth inhibition on neoplastic cells, the presence of LDL receptors was first investigated in Caco-2 and HepG2 cell lines. LDL receptors in these two cell smears were detected by the immunofluorescence technique with the following steps: the cells were cultured up to semi-confluency in culture dishes with cover glasses. After fixation with 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature, the smears were incubated in 3% (w/v) bovine serum albumin (BSA) pH 7.5 for 1 h at room temperature to block unspecific binding of the antibodies. Then goat IgG anti-human LDL receptor (1:50) in 1% (w/v) BSA pH 7.5 was incubated with smears at 4˚C overnight, followed by incubation with anti-goat IgG FITC conjugate (1:50) in 1% (w/v) BSA pH 7.5 for 60 min at room temperature. After each step the smears were washed with PBS and, finally, the smears was mounted in 2.5% 1,4-diazabicyclo [2.2.2] octane (DABCO) mounting medium (50% glycerol in PBS). The LDL receptor stained with FITC was observed under fluorescence microscope with blue filter (Ex: 330-380 nm; Em: 420 nm). Negative control smears were performed in the same cell lines by omitting the primary antibody.

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Growth Inhibition in Caco-2 and HepG2 Cell Lines. For determining cell proliferation, the viable cell numbers were counted using MTT assay. 10^4 cells were grown in 96-well microtiter plates. After an incubation time of 24 h at 37°C in an atmosphere containing 5% CO_2, the cells were incubated in the media with tributyrin concentrations ranging from 0.1 to 4 mM using tributyrin solution (tributyrin was first dissolved in DMSO and then diluted with culture medium. The final concentration of DMSO in the cell culture was less than 0.2% v/v) or tributyrin emulsion for 48 h. After that, 100 µl 0.5 mg/ml MTT in PBS was added to each well. The microtiter plate was incubated for another 2 h before measuring absorbance at 590 nm of the samples using microtiter plate reader (Spectroflour, Tecan, Austria). The cell viability of each well (expressed as survival index) was determined using equation (1).

$$Survival\ Index\,(\%) = \frac{A_{test} - A_{blank}}{A_{contol} - A_{blank}} \times 100\% \qquad (1)$$

The 50% inhibitory concentration (IC_{50}) was determined as the drug concentration required to inhibit 50% of the cell growth.

Competitive Uptake of Lipid Emulsion with LDL. This experiment was performed with Caco-2 and HepG2 cells as described above, but with the addition of increasing amounts of native human LDL to each well of microtiter plate. The concentration of tributyrin emulsion was kept constant at 0.75 mM for Caco-2 and 1.0 mM for HepG2 in each well. The concentrations of native LDL ranged from 10 to 100 µg/ml. After 48 h incubation, the cell viability of each well was determined using MTT assay as described above.

Results

Pharmacokinetics of Butyrate and Tributyrin after Oral Administration. The plasma concentration-time profiles of butyrate after oral administration of pure tributyrin or 10% emulsion of tributyrin to the healthy rats or rats treated with 17α-ethynylestradiol are illustrated in Figure 1A. Key pharmacokinetic parameters were calculated from these data and are presented in Table 1. The C_{max} and K_a of butyrate concentration from oral administration of 10% tributyrin emulsion were significantly higher than those from administration of pure tributyrin to healthy group. And the AUC_{0-360} for oral administration of pure tributyrin was only 20.1% of that for administration of emulsion to healthy rats. The results suggest that the emulsion prepared has the ability to enhance the oral absorption of tributyrin. The C_{max} and AUC of butyrate concentration after oral administration of emulsion to the treated rats were significantly lower than that of the healthy rats. The CL and V of tributyrin after administration of emulsion to the treated rats were 4.8 and 3.0 folds higher than that of the healthy rats. As 17α-ethynylestradiol was reported to upregulate the activity of the LDL receptors in tissues (Chao et al., 1979) and the tributyrin emulsion has the ability of binding to LDL (Su and Ho, 2004), the higher values of CL and V in the treated rats were possible due to higher uptake of tributyrin in the form of emulsion and the consequent metabolism of the compound in these tissues.

The faster T_{max} and higher AUC of tributyrin attained by the 10% emulsion when compared to the pure tributyrin (Figure 1B, Table 1) indicate that the emulsion can enhance the oral absorption of tributyrin. However, after administration of the 10% emulsion, the concentration of tributyrin decreased to 11.9% of C_{max} in 30 min in healthy

rats, while the concentration after administration of the pure tributyrin dropped slowly after C_{max} (Figures 1B). In comparison to the pure drug, the 10% emulsion achieved much higher butyrate concentration before 105 min in the healthy rats (Figures 1A). The results further illustrated the enhanced absorption of tributyrin in emulsion, leading to formation of more butyrate after hydrolysis during the absorption process and in the blood circulation (O'Connor and Bailey, 1988). After the rats were treated with 17α-ethynylestradiol, a LDL receptor upregulator, the tributyrin clearance after administration of the emulsion was increased by 3.3 times, the volume of distribution increased by 7.1 times; whereas the $AUC_{0\text{-}360}$ was reduced by 4.6 times. These results suggested that the induction of LDL receptors in the tissues enhanced the uptake of tributyrin in form of lipid emulsion.

Pharmacokinetics of Butyrate and Tributyrin after Infusion. During the continuous infusion of emulsion to healthy rats for 2 hours, the butyrate concentration from the 10% emulsion remained steady and fluctuated within 7.0% (RSD), which indicated that the elimination rate was roughly equal to the infusion rate. In contrast, the butyrate concentrations from the 5% emulsion decreased 70.9% and from the 15% emulsion increased 32.2%, respectively (Figure 2A). The results suggest that continuous infusion of 10% tributyrin emulsion can maintain a steady plasma butyrate concentration in the healthy rats. The rapid decline in butyrate concentration despite continuous infusion of 5% emulsion could be due to rapid uptake of the drug by the peripheral tissues and metabolic organs at a rate faster than the infusion rate. With an infusion of 15% emulsion, when these sites presumably to be the LDL receptors were saturated, continuous infusion caused a substantial increase in the butyrate concentration. The

decrease in the butyrate concentration during infusion of 10% tributyrin emulsion to the rats pre-treated with the LDL receptor inducer indicated faster elimination of tributyrin from the blood through higher uptake of emulsion by tissues with upregulated expression of lipoprotein receptors. The significant differences ($p < 0.05$) in the clearance and distribution volume between the healthy and the pre-treated rats infused with 10% emulsion suggested that 17α-ethynylestradiol could enhance the uptake of the tributyrin emulsion at both the peripheral and clearance sites (Table 2). Despite the AUC values for butyrate concentration were found to be proportional to the increasing doses of the 5, 10 and 15% emulsions ($r^2 > 0.99$), the extrapolated line of the plot of the AUC against dose was significantly far away from the zero intercept ($p < 0.05$) (Figure not shown). The volume of distribution and clearance after infusion of the 10% and 15% tributyrin emulsion were also lower than that after the 5% emulsion, indicating nonlinear kinetics was involved in the disposition and elimination of tributyrin during infusion (Table 2). It may be noted that the time to reach steady state for drugs with nonlinear kinetics is affected by the dosing rate in addition to the metabolic rate and volume of distribution of the drug (Mehvar, 2001). That could be the reason for the 5, 10 and 15% emulsion to have different steady state profiles.

During infusion of the 10%, 15% emulsion to the healthy rats and 10% emulsion to the treated rats, the tributyrin concentrations fluctuated minimally within 11.2, 10.4 and 8.0%, (Figure 2B). In the respective healthy group receiving the emulsion infusion, a peak in the tributyrin plasma concentration always appeared after the infusion was terminated. This phenomenon could be due to the release of the drug from the LDL receptor binding sites back to the blood circulation after termination of the infusion. This

phenomenon was absent in the rats treated with the LDL receptor upregulator, probably due to much higher tissue binding capacity to lipid emulsion in tissues (Figure 2B).

DNA Fragmentation Assay. Internucleosomal DNA degradation is a very specific event in apoptosis, leading to DNA fragmentation. To confirm the apoptotic activity of tributyrin was not affected by formulating it into a lipid emulsion, DNA fragmentation assay was conducted with both pure tributyrin and tributyrin emulsion on Caco-2 and HepG2 cell lines. The DNA from Caco-2 and HepG2 cells treated with either pure tributyrin or tributyrin emulsion displayed the characteristic internucleosomal ladder of DNA fragments (Figure 3). In contrast, the control Caco-2 and HepG2 cells showed no DNA fragments. The results suggest that tributyrin in the form of emulsion still possesses the apoptotic activity on Caco-2 and HepG2 cells.

LDL Receptors in Caco-2 and HepG2 Cell Lines. An intense immunoreaction was observed in both Caco-2 and HepG2 cells stained with the antibody specific to the LDL receptors indicating the presence of the receptors in these two cell lines (Figure 4). Omitting the primary antibody resulted in a negative reaction in these cells confirming the specificity of the reaction (data not shown). This finding supported the previous report that both Caco-2 and HepG2 expressed the mRNA of LDL receptor (Sviridov et al., 2003).

Growth Inhibition in Caco-2 and HepG2 Cell Lines. Growth inhibition studies were performed with Caco-2 and HepG2 cells. Pure tributyrin and tributyrin emulsion were tested at increasing concentrations of 0.1 to 4 mM. Figure 5A shows the inhibitory effect of increasing concentrations of pure tributyrin and tributyrin emulsion on Caco-2 cells after a 48 h exposure. The IC_{50} for pure tributyrin and tributyrin emulsion were 0.90

mM and 0.75 mM, respectively. Figure 5B shows the inhibitory effect of increasing concentrations of pure tributyrin and tributyrin emulsion on HepG2 cells. In this cell line, the IC_{50} were 1.6 mM and 1.0 mM for pure tributyrin and tributyrin emulsion, respectively. Both Caco-2 and HepG2 cells appeared to be slightly more sensitive to the treatment by tributyrin emulsion than pure tributyrin.

Competitive Uptake of Lipid Emulsion with LDL. The tributyrin emulsion prepared here has previously been proved able to bind to LDL *in vitro* (Su and Ho, 2004). To determine the uptake mechanism of lipid emulsion by Caco-2 and HepG2 cells, the medium was added with increasing amounts of native LDL, while keeping the pure tributyrin or tributyrin emulsion concentration constant (the pure tributyrin or tributyrin emulsion concentration was kept at 0.75 mM and 1.0 mM in the Caco-2 and HepG2 cell culture medium, respectively). After incubation for 48 h, the viable cells were measured with MTT assay. In both cell lines, addition of increasing amounts of native LDL progressively increased the percentage of viable cells treated with tributyrin emulsion (Figure 6). At the maximal concentration of LDL (100 μg/ml), the viable cells reached over 92% and 77% for Caco-2 and HepG2 cells, respectively, while the addition of native LDL did not show any influence on the viability of the cells treated with pure tributyrin. The results suggest that competitive uptake existed between the emulsion and LDL uptake in Caco-2 and HepG2 cells. It may be noted that in the absence of LDL, pure tributyrin and tributyrin emulsion had identical effect on the viability of Caco-2 cells (Figure 6). Thus, the mechanism involved in the uptake of pure tributyrin by Caco-2 cells might be as efficient as that through the LDL receptors. Another possibility is that tributyrin has very mild cytotoxic effect on colonic cancer cells. It first induced

differentiation in colonic cancer cells before leading to their death from apoptosis (Schroder and Maurer, 2002). The present growth inhibition system might not be sensitive enough to discern the selective uptake of the tributyrin emulsion by the Caco-2 cells from the pure tributyrin.

Discussion

The major goal in cancer chemotherapy is to selectively kill the tumor cells without damaging normal tissues and organs. Therefore, the achievement of vehicles capable of delivering drugs to targeted cells has long been pursued. In the last decades, several authors suggested the possibility of using of LDL to shuttle antitumor drugs to neoplastic cells, based on the increased expression of LDL receptor in certain types of cancer. Although it was successful in experimental trials, the difficulties in isolating LDL from plasma and handling the preparation are serious drawbacks for its use in routine treatment.

LDL-resembling emulsions however, can mimic the metabolism of plasma lipoprotein *in vivo* (Redgrave and Maranhão, 1985; Redgrave et al., 1988; Maranhão et al., 1994 and 2002; Shawer et al., 2002). Maranhão et al. (1993) demonstrated in rats such emulsion has a plasma kinetic behavior resembling that of native LDL and that the emulsion is probably taken up by the LDL receptor that sequestrate LDL into the cell. This type of emulsion normally is devoid of apo B, but it acquires LDL or apo E from the circulating lipoproteins after injection into the blood. Because LDL receptor can also recognize apo E, the emulsion is thus taken up by the tissues via the LDL receptor-mediated endocytic pathway. In patients with familial hypercholesterolemia, LDL-resembling emulsion plasma clearance was pronouncedly reduced compared to normal subjects (Roland et al., 1991). This is also expected for native LDL, because LDL receptor is defective in this disease (Brown and Goldstein, 1986). The lipid emulsion used here has been well characterized in our previous study (Su and Ho, 2004). It is constituted of stable homogeneous spherical particles with a cholesteryl ester core surrounded by a phospholipids monolayer. We have previously shown that the lipid

emulsion of tributyrin has the ability of binding to LDL *in vitro*, it is reasonable to assume that the emulsion may concentrate in tissues or organs with higher LDL receptor expression.

In the present investigations we determine whether differences in pharmacokinetics exist between the lipid emulsion of tributyrin and pure tributyrin together with the influence of 17α-ethynylestradiol on the pharmacokinetics of the emulsion. Between the emulsion and pure tributyrin, statistically significant differences in pharmacokinetic parameters calculated from both tributyrin and butyrate plasma concentrations were observed. Emulsion prepared in our study significantly enhanced the oral bioavailability of tributyrin. Both the oral administration and infusion of emulsion resulted in substantial higher concentrations of tributyrin and butyrate in plasma, which were maintained for at least 6 hours and 4 hours, respectively. The pharmacokinetic parameters (clearance, biodistribution volume and AUC) between healthy and rats treated with 17α-ethynylestradiol also showed significant differences. The changing of the balance process between emulsion and LDL caused the concentration-time profiles in the healthy rats infused with emulsion generated a peak after termination of infusion. In our previous report, the emulsion binding with LDL was proved a reversible process (Su and Ho, 2004). During infusion, part of emulsion infused bound with the over-expressed-LDL-receptor organs, such as liver. While dissociation predominated in balance after infusion, when the emulsion concentration in blood decreased, causing the release of emulsion back to blood from these organs. As the LDL receptors in treated rats have higher LDL receptor activity, the phenomenon of dissociation of drug from the binding sites leading to the appearance of a peak plasma concentration was not observed in these animals.

The elimination of butyrate and tributyrin was found nonlinear in this study. Egorin et al. (1999) also reported the saturable clearance and nonlinear pharmacokinetics of butyrate when using much higher doses of butyrate in both mice and rats, although the nonlinear nature of butyrate pharmacokinetics was less striking in rats. In their study, the very high concentration of butyrate (about 10 mM) in mice possibly saturated the activity site of enzyme for metabolizing butyrate, which caused the saturable clearance. Although the highest plasma butyrate concentration observed in this study was only about 1.8 mM, the tributyrin emulsion with affinity to LDL receptors might enhance the uptake and cause saturation at both the tissue binding site and clearance site, causing nonlinear pharmacokinetic profiles. .

In vitro, tributyrin was reported to inhibit the growth of some tumor cells by inducing apoptosis and DNA synthesis arrest (Maier et al., 2000; Schroder and Maurer, 2002; Yan and Xu, 2003; Kuefer R et al., 2004). Although several pathways were reported, the molecular mechanisms by which tributyrin and/or its metabolite, butyrate lead to a differentiated phenotype of tumor cells are still poorly understood (Heerdt et al., 1999; Guang et al., 2000; Clarke et al., 2001). The studies proposed modulation of nuclear functions such as gene expression, acetylation of histones with altered chromatin conformation and DNA cleavage of internucleosomal regions that are typical for apoptotic cells (Boffa et al., 1981; Heerdt et al., 1999). In this study, the DNA fragmentation assay was applied to the cells treated with tributyrin or tributyrin emulsion. The observations suggested that similar to the pure tributyrin, the tributyrin emulsion can also induce apoptosis in Caco-2 and HepG2 cell lines.

The physiologic role of the LDL receptor is to transport cholesterol-carrying lipoprotein particles into cells. The primary ligand for the receptor is LDL, which contains a single copy of apolipoprotein B-100 (apo B-100); approximately 65-70% of plasma cholesterol in humans circulates in the form of LDL. The LDL receptor also binds tightly to β-migrating forms of very low-density lipoprotein, which contains multiple copies of apolipoprotein E (apo E). Receptor-ligand complexes enter the cell by endocytosis at clathrin-coated pits, where receptor molecules cluster on the cell surface. The N-terminal domain of the LDL receptor is the ligand-binding domain and all other domains serve for internalization of the ligand bound to the receptor (Motley et al., 2003; Chung and Wasan, 2004; Ehrlich et al., 2004). Bound lipoprotein particles are subsequently released in the low-pH milieu of the endosome, and the receptors then return to the cell surface in a process called receptor recycling (Davis et al., 1987; Rudenko et al., 2002).

The current study provided the profiles of both butyrate and tributyrin in rats after oral administration and intravenous infusion of tributyrin emulsion. For the first time, the tributyrin concentration in plasma could be followed for up to 6 hours after oral administration. Infusion of tributyrin emulsion provided another possible route of administration for studying the pharmacokinetics of tributyrin. The uptake mechanism of tributyrin emulsion was proved to be mediated, at least partially, by the LDL receptor pathway in Caco-2 and possibly also in HepG2 cell lines.

The limitation of this study was that only the pharmacokinetics and *in vitro* cell culture was studied. Investigation of the tissue distribution of labeled tributyrin would

further clarify whether the tributyrin emulsion was actually delivered to the tissue and up-taken through the LDL receptors.

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Legends for Figures

Fig. 1. Plasma concentrations of butyrate (**A**) and tributyrin (**B**) in rats after oral administration of pure tributyrin in healthy rats (+) or 10% tributyrin emulsion in both healthy rats (×) and rats treated with 17α-ethynylestradiol (■). Data represent the means of plasma concentration from six rats.

Fig. 2. Plasma concentrations of butyrate (**A**) and tributyrin (**B**) in rats after continuous infusion of 5% (+), 10% (×) or 15% (Δ) tributyrin emulsion to healthy rats or 10% emulsion to rats treated with 17α-ethynylestradiol (■). Data represent the means of plasma concentration from six rats. (Insert provides the enlarged profiles)

Fig. 3. Induction of DNA fragmentation by incubation of the cells in medium with pure tributyrin or tributyrin emulsion. **M**: 1 Kb DNA maker; **1**: Caco-2 incubated in medium (control); **2**: Caco-2 incubated in medium with 4 μM tributyrin emulsion; **3**: Caco-2 incubated in medium with 4 μM pure tributyrin; **4**: HepG2 incubated in medium (control); **5**: HepG2 incubated in medium with 4 μM tributyrin emulsion; **6**: HepG2 incubated in medium with 4 μM pure tributyrin

Fig. 4. Immunofluorescence images of Caco-2 (**A**) and HepG2 (**B**) cell lines detected by FITC conjugate

Fig. 5. Inhibitory effect of pure tributyrin or tributyrin emulsion in Caco-2 (**A**) and HepG2 (**B**). The cells were treated with increasing concentrations of pure tributyrin or tributyrin emulsion for 48 h and the antiproliferative effect was measured by MTT assay.

Fig. 6. Inhibitory effect of pure tributyrin or tributyrin emulsion in the presence of LDL in Caco-2 (**A**) and HepG2 (**B**). The cells were treated with pure tributyrin or tributyrin emulsion (0.75 mM in Caco-2 and 1.0 mM in HepG2) in the presence of various concentrations of LDL for 48 h. The antiproliferative effect was measured by MTT assay.

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TABLE 1 Pharmacokinetic parameters describing butyrate and tributyrin concentrations achieved in rat plasma after oral administration of pure tributyrin or 10% tributyrin emulsion at the dose of 2 g/kg (n = 6/group)

Parameters	*Pure Tributyrin*	*10% Emulsion*	*10% Emulsion*[a]
Butyrate concentration		One-compartmental model	
C_{max} (μM)	87.6 ± 6.0[†]	1344.5 ± 107.7[†‡]	347.2 ± 53.4[‡]
T_{max} (min)	25.3 ± 3.9	8.5 ± 1.1	6.2 ± 1.1
K_a (1/min)	0.10 ± 0.03[†]	0.28 ± 0.07[†]	0.27 ± 0.06
K_e (1/min)	0.011 ± 0.002	0.035 ± 0.005	0.086 ± 0.018
V/F (L/kg)	59.4 ± 17.4	3.8 ± 0.5[‡]	11.5 ± 3.0[‡]
CL/F (L/min/kg)	0.52 ± 0.12	0.12 ± 0.04[‡]	0.57 ± 0.15[‡]
$AUC_{0\text{-}360}$ (μM·min)	11214 ± 2231[†]	55907 ± 5808[†‡]	11560 ± 2122[‡]
$AUC_{0\text{-}\infty}$ (μM·min)	13225 ± 2588[†]	56766 ± 6012[†‡]	11950 ± 2284[‡]
F (%)	15.3 ± 3.0	65.7 ± 7.0	13.8 ± 2.6
Tributyrin concentration		Non-compartmental model	
C_{max} (μM)	2.74 ± 0.66[†]	4.20 ± 1.16[†‡]	0.36 ± 0.08[‡]
MRT (min)	87.9 ± 20.9	132.0 ± 30.9	161.9 ± 30.5
V/F (L/kg)	16586 ± 3344	4682 ± 1500[‡]	33213 ± 8081[‡]
CL/F (L/min/kg)	39.8 ± 10.2	21.6 ± 5.9[‡]	71.3 ± 20.0[‡]
$AUC_{0\text{-}360}$ (μM·min)	134.5 ± 30.6[†]	226.1 ± 50.1[†‡]	49.3 ± 5.5[‡]
$AUC_{0\text{-}\infty}$ (μM·min)	172.0 ± 39.5[†]	317.3 ± 60.6[†‡]	95.9 ± 10.9[‡]
F (%)	34.9 ± 8.0	64.5 ± 12.3	19.5 ± 2.2

[†] *$p < 0.05$ between oral and infusion administration;*

[‡] *$p < 0.05$ between healthy and treated rats.*

[a]*: Administrated to rats treated with 17α-ethynylestradiol.*

The data are presented as mean ± SD.

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TABLE 2 Pharmacokinetic parameters describing butyrate and tributyrin concentrations achieved in rat plasma after infusion of 5%, 10% and 15% emulsion of tributyrin at the dose of 1, 2 and 3 g/kg, respectively (n = 6/group)

Parameters	*5% Emulsion*	*10% Emulsion*	*10% Emulsion*[a]	*15% Emulsion*
Butyrate concentration		One-compartmental model		
K_e (1/min)	0.254 ± 0.055	0.208 ± 0.012[‡]	0.080 ± 0.023[‡]	0.162 ± 0.014
V (L/kg)	0.65 ± 0.16	0.39 ± 0.03[‡]	1.49 ± 0.52[‡]	0.32 ± 0.04
CL (L/min/kg)	0.173 ± 0.031	0.080 ± 0.007[‡]	0.118 ± 0.042[‡]	0.055 ± 0.008
$AUC_{0\text{-}240}$ (μM·min)	11414 ± 3117	84184 ± 12420[‡]	57885 ± 15267[‡]	183203 ± 23750
$AUC_{0\text{-}\infty}$ (μM·min)	19814 ± 3288	86358 ± 12454	58184 ± 15386	186442 ± 23764
Tributyrin concentration		One-compartmental model		
K_e (1/min)	0.13 ± 0.02	0.23 ± 0.03[‡]	0.10 ± 0.02[‡]	0.16 ± 0.03
V (L/kg)	154 ± 38	74 ± 16[‡]	525 ± 97[‡]	121 ± 18
CL (L/min/kg)	17.0 ± 4.3	13.9 ± 1.8[‡]	42.9 ± 5.8[‡]	16.4 ± 3.8
$AUC_{0\text{-}240}$ (μM·min)	123.5 ± 20.7	452.7 ± 90.5	139.2 ± 41.0	593.0 ± 100.6
$AUC_{0\text{-}\infty}$ (μM·min)	201.7 ± 33.3	492.2 ± 96.7	159.4 ± 41.4	626.4 ± 106.9

[‡] $p < 0.05$ between healthy and treated rats.

[a]*: Administrated to rats treated with 17α-ethynylestradiol.*

The data are presented as mean ± SD.

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Figure 1

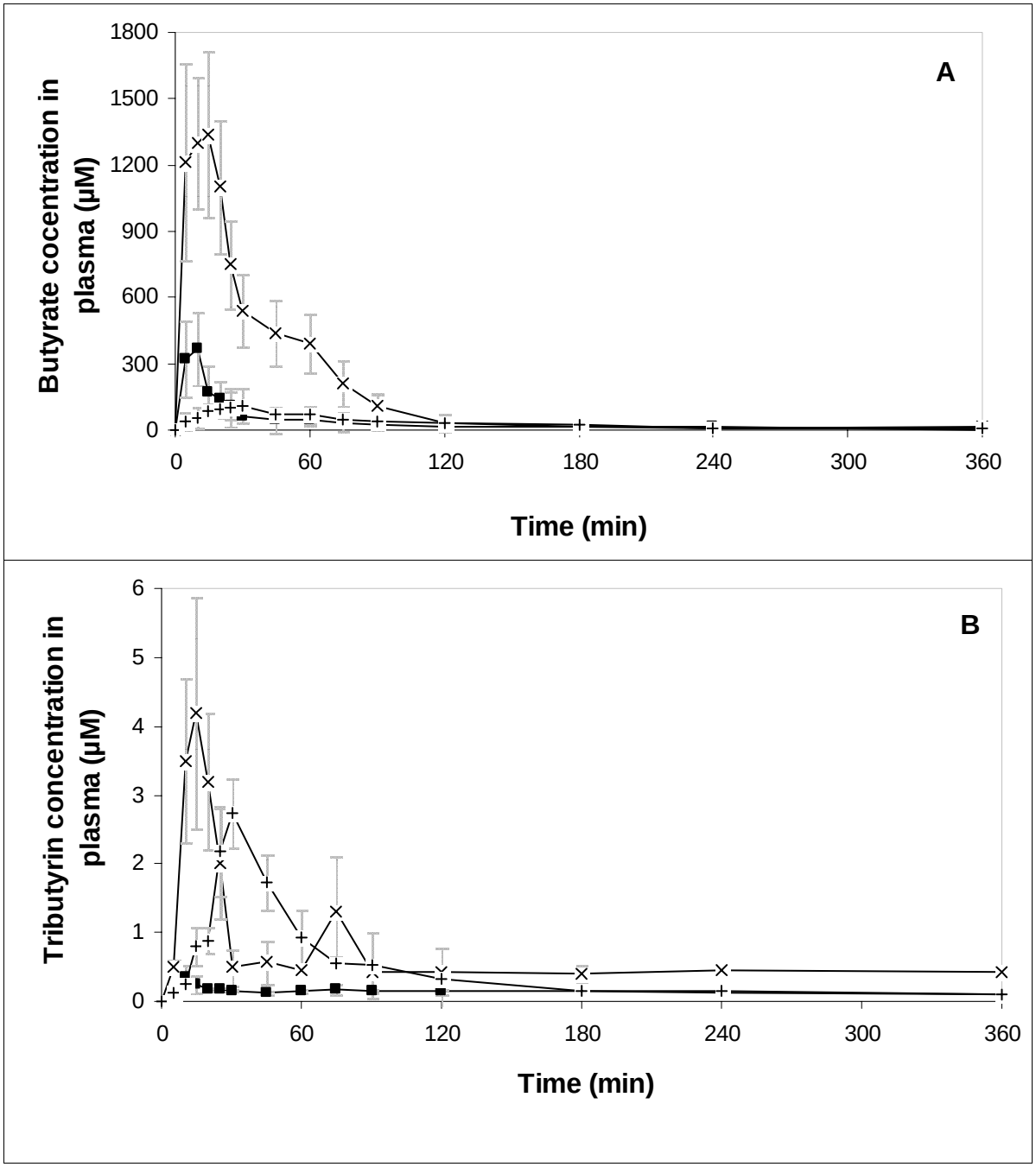

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Figure 2

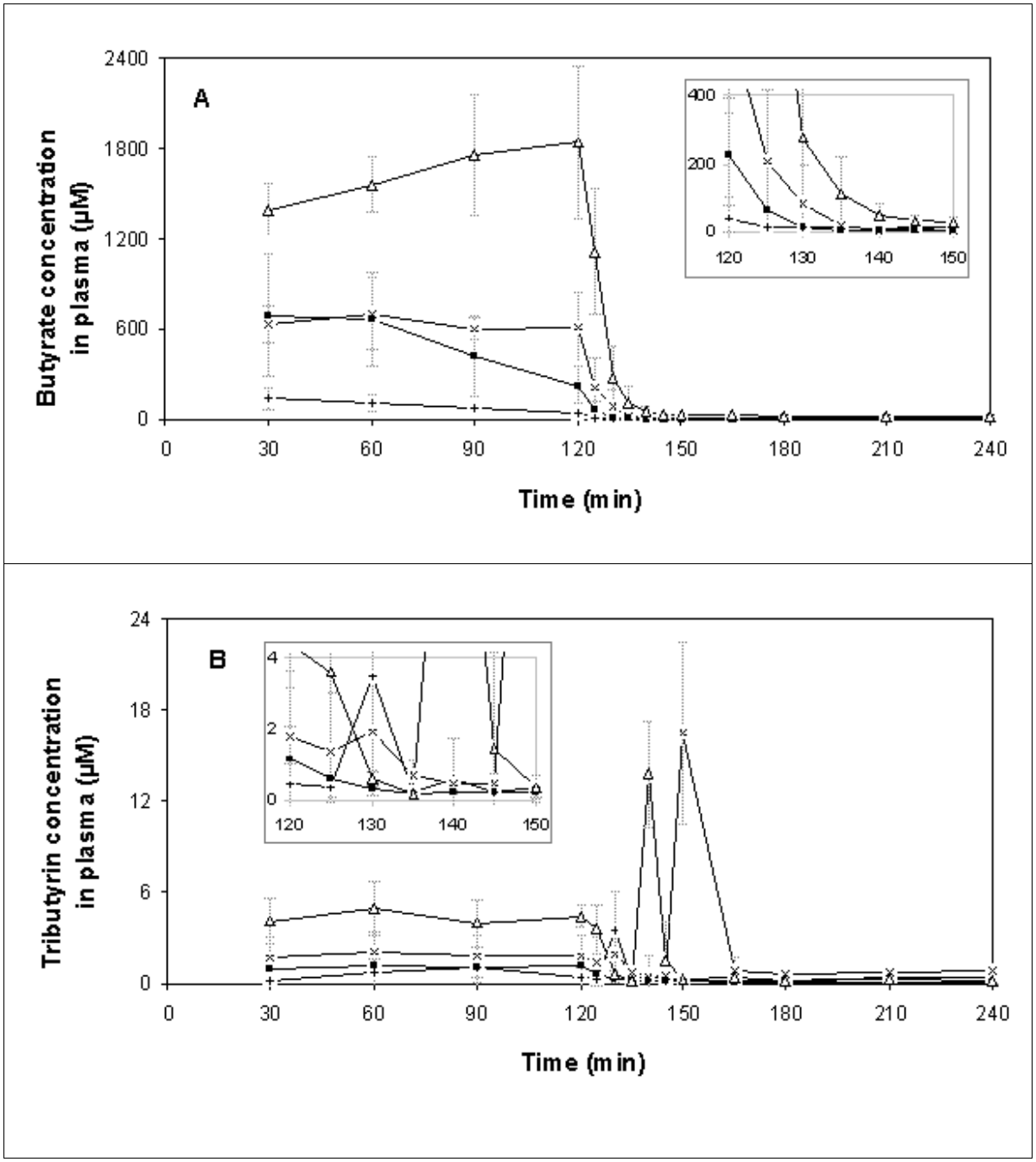

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Figure 3

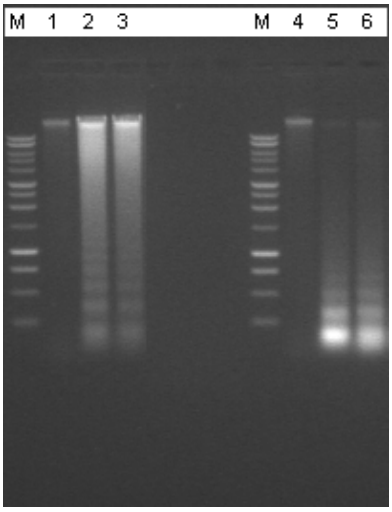

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Figure 4

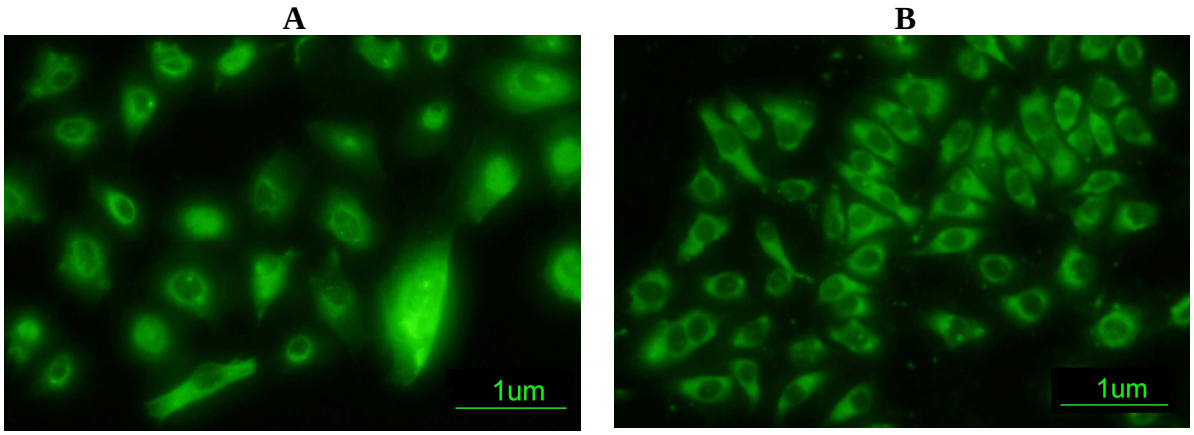

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Figure 5

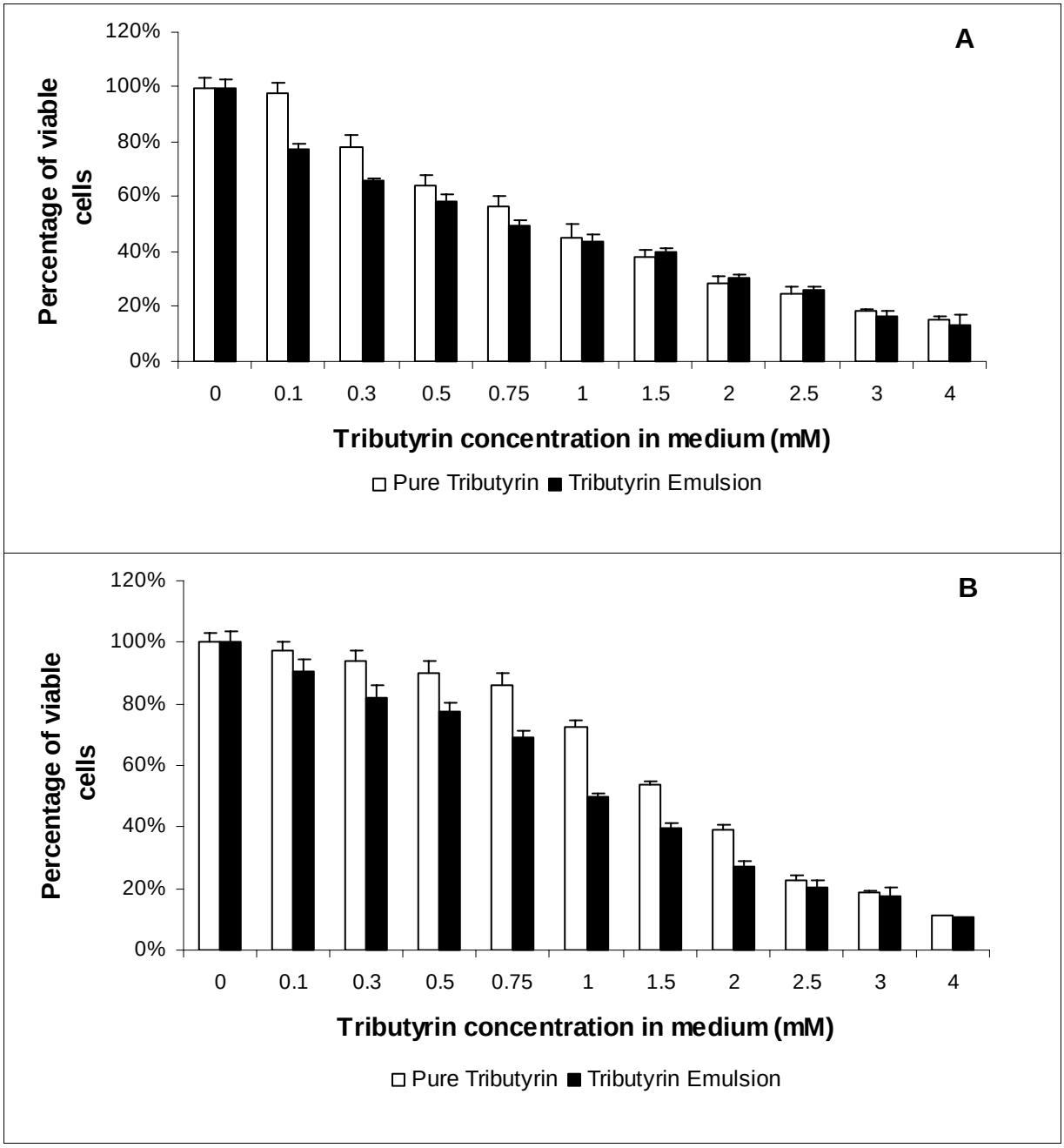

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Figure 6