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The Effect of Piperine Pro-Nano Liposphere on Direct Intestinal Phase II Metabolism: The Raloxifene Paradigm of Enhanced Oral Bioavailability

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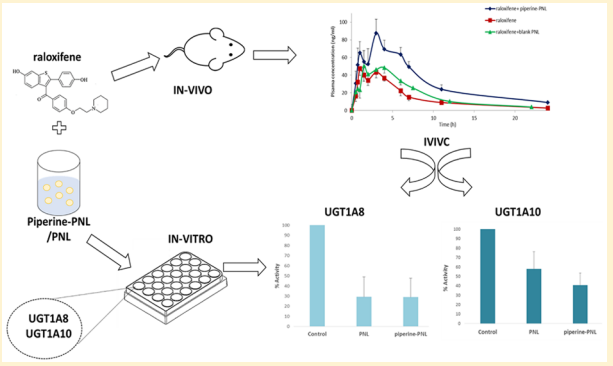
ABSTRACT: Phase II biotransformation reactions have been gaining more attention due to their acknowledged significance in drug bioavailability, drug development, and drug−drug interactions. However, the predominant role of phase I metabolism has always overshadowed phase II metabolism, resulting in insufficient data regarding its mechanisms. In this paper, we investigate the effect of an advanced lipid based formulation on the phase II metabolism process of glucuronidation, occuring in the enterocytes monolayer. The investigated formulation is a self-emulsifying drug delivery system, termed pro-nano lipospheres, which contains the natural absorption enhancer piperine. To evaluate the effect of this formulation on direct glucuronidation we chose the model molecule raloxifene. First, glucuronidation is the main clearance pathway of this compound without involvement of preceding mechanisms. Second, raloxifene's extensive glucuronidation site is primarily at the intestine. Raloxifene's oral bioavailability was determined in a series of pharmacokinetic experiments using the freely moving rat model. In order to test the effect of the formulation on the relevant UGT enzymes reported in the clinic, we used the *in vitro* method of UGT-Glo Assay. Coadministration of raloxifene and piperine pro-nano lipospheres to rats resulted in a 2-fold increase in the relative oral bioavailability of raloxifene. However, coadministration of raloxifene with blank pro-nano lipospheres had no effect on its oral bioavailability. In contrast to the difference found *in vivo* between the two vehicles, both formulations extended an inhibitory effect on UGT enzymes *in vitro*. Ultimately, these findings prove the ability of the formulation to diminish intestinal direct phase II metabolism which serves as an absorption obstacle for many of today's marketed drugs. Pro-nano lipospheres is a formulation that serves as a platform for the simultaneous delivery of the absorption enhancer and a required drug. The discrepancy found between the *in vivo* and *in vitro* models demonstrates that the *in vitro* method may not be sensitive enough to distinguish the difference between the formulations.



KEYWORDS: *direct phase II metabolism, glucuronidation, enterocytes, absorption, SNEDDS, piperine, raloxifene*

INTRODUCTION

Phase I metabolism and its effect on absorption have always been at the center stage of drug research. The more classical view is that cytochrome P450-catalyzed hydroxylation precedes phase II processes such as glucuronidation, which is true for many drugs.[1] However, there are parent compounds that are cleared by glucuronidation without the requirement of P450 enzymes. This type of metabolism is referred to as direct phase II metabolism.[2] The long harbored consensus regarding phase I and phase II metabolism led to limited information regarding the mechanisms involved in phase II metabolism as processes the parent drug undergoes directly.[3−5] Specifically, it was reported in 2002 that glucuronidation is an important clearance mechanism for approximately 1 in 10 of the top 200 prescribed drugs, making the investigation of this field a top priority.[1]

In phase II metabolism, molecules undergo conjugation reactions as part of their excretion route. Screening of phase II enzymes participating in the metabolism of clinically used drugs indicates that UDP-glucuronosyltransferases (UGTs) are the most dominant enzymes. They mediate glucuronidation process, in which glucuronic acid group, derived from the cofactor UDPGA, is transferred to the substrates, making it more hydrophilic.[2,6]

One of the practiced ways to inhibit the major drug metabolizing enzymes, such as UGTs, is the use of herbal absorption enhancers. These molecules are alkaloids, flavonoids and polyphenols such as curcumin, resveratrol, piperine, etc. However, there use is limited due to their solubility at the gastrointestinal tract milieu.[7,8]

We have developed a lipid-based formulation, which dissolves in its oily core lipophilic drugs with the natural absorption enhancer piperine. Piperine is an alkaloid, which constitutes a major active component in black pepper and is

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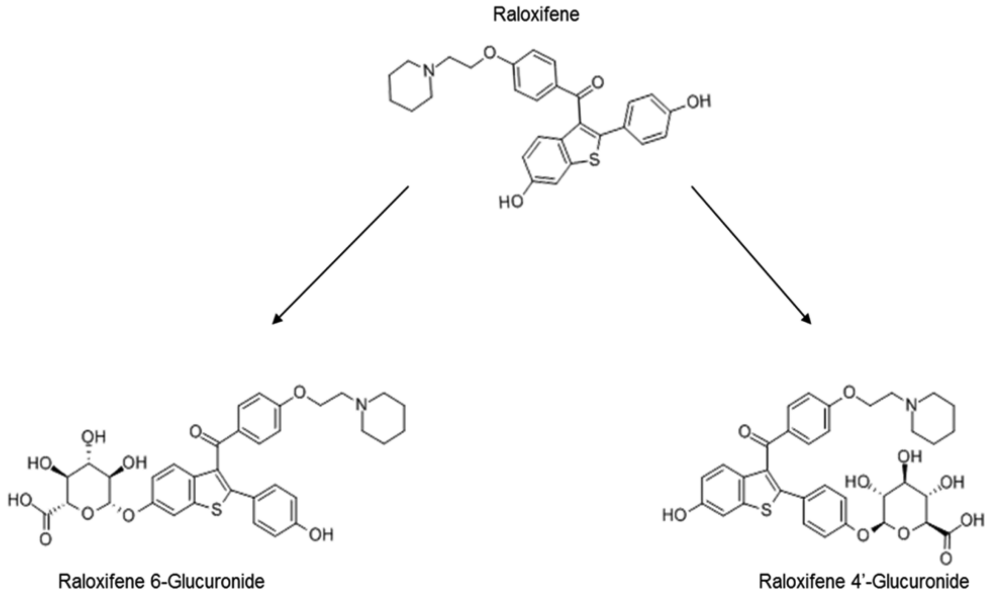

Figure 1. Structure of raloxifene and its two major metabolites.

often used an absorption enhancer. Previously, it has been reported that piperine has an effect on phase I and phase II metabolism, specifically CYP3A4, P-gp efflux and glucuronidation.[9−13] However, it is poorly soluble in water, leading to difficulty in using it in *in vivo* experiments.[8] The formulation presented here is termed piperine pro-nano lipospheres (piperine-PNL). This formulation, serves as a vehicle that enhances solubility and allows the delivery of the absorption enhancer and/or the drug to the enterocytes. As reported before, we investigated the effect of the piperine-PNL on the oral bioavailability of the major cannabinoids, Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), which are subjected to intestinal first pass metabolism.[14−16] Upon contact with water, the formulation self-emulsifies into nanoparticles. The particles entrap the drug and piperine in their core, improving their solubility in water.[17] In addition, excipients of the formulation, such as the surfactants and phospholipids, inhibit first pass metabolism mechanisms that affect the model molecules.[18−22]

Unlike THC or any other cannabinoid, a prominent metabolic route of CBD is direct glucuronidation of the parent compound, leading to the formation of an O-glucuronide.[23,24] We have shown that piperine-PNL increased CBD oral bioavailability by a further 2-fold in comparison to PNL while it enhanced THC's bioavailability by 1.47-fold compared to PNL administration.[17] We hypothesize that this difference might be a result of the effect piperine-PNL has on intestinal direct phase II metabolism, specifically, glucuronidation. In order to investigate this hypothesis, we selected the model molecule raloxifene. Raloxifene hydrochloride is a suitable compound since it is undergoes, exclusively, intestinal direct phase II metabolism.[5]

More than 90% of raloxifene intestinal metabolism and 50% of its hepatic metabolism are due to glucuronidation while phase I metabolism enzymes and P-gp effects on the compound are absent or negligible, as was found in *in vivo* studies.[25,26] Raloxifene is metabolized to two primary metabolites: raloxifene-4′-glucuronide (R-4-G) and raloxifene-6-glucuronide (R-6-G) (Figure 1). Clinical studies have shown that glucuronidation occurs primarily at the 4′ position of raloxifene.[5] The R-4-G formation is mediated mostly by UGT1A10 and UGT1A8. According to *in vitro* experiments, these enzymes are exclusively expressed at the human intestine.[5,27] We have examined the effect of the formulation on the oral bioavailability of raloxifene in a pharmacokinetic experiment using the freely moving rat model.[28] *In vitro* studies were carried out using UGT microsomes via the UGT-Glo Assay method,[29] in which we tested the effect PNL and piperine-PNL have on UGT 1A8 and 1A10.

EXPERIMENTAL SECTION

2.1. Materials. Ethyl lactate, Tween 20, Span 80, talinolol, piperine, raloxifene, methylcellulose 4000 CP, dimethyl sulfoxide (DMSO), and alamethicin were purchased from Sigma-Aldrich, Israel. Lecithin was purchased from Cargill, USA. Polyoxyl 40-hydroxy castor oil was purchased from BASF The Chemical Company, Germany. Tricaprin (CremerCOOR; MCT C10-95) was purchased from CREMER Oleo Division, France. Acetonitrile, ethanol, methanol, and formic acid were purchased from J.T. Baker, Phillipsburg, NJ, USA. UGT-Glo Assay kit was acquired from Promega, Madison, WI, USA. Corning supersomes were purchased from Corning, Corning, NY, USA. All chemicals were of analytical reagent grade, and solvents were HPLC grade.

2.2. PNL and Piperine-PNL Preparation. PNL and piperine-PNL were prepared by a preconcentrate method as previously described.[17] Briefly, an amphiphilic cosolvent (ethyl lactate) and soy phospholipid (lecithin) were placed in a clean scintillation tube at a ratio of 4:1, respectively. The mixture was heated to 40 °C until completely dissolved. Then, a triglyceride (tricaprin), polyoxyl 40-hydroxy castor oil, Tween 20, and Span 80 were added at the ratio of 1:1:1:1. The mixture was gently stirred and heated to 40 °C until a homogeneous solution was formed. In order to obtain piperine-PNL preconcentrate, piperine powder (2% w/w) was added to the PNL preconcentrate, stirred, and heated to 40 °C until completely dissolved.

2.3. *In Vitro* Studies. The effect of PNL and piperine-PNL on UGT activity was determined by using UGT-Glo Assay kit[29] (Promega, Madison, WI) and microsomes containing recombi-

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nant enzymes (Corning supersomes, Corning, NY). The assay was performed with two isoforms of UGT: 1A8 (n = 3) and 1A10 (n = 5). The assay is based on the use of a proluciferin substrate for various UGT enzymes, which reacts with Luciferin Detection Reagent (LDR) and D-cysteine to form a luciferin derivative. This derivative fluoresces and can be quantitated by a luminometer. To determine UGT activity, two reactions are set up in parallel for each sample. Both reactions contain UGT-containing microsomes as well as a proluciferin substrate, while only one of them contains the uridine-5′-diphosphoglucuronic acid (UDPGA) cofactor. During an incubation period with UGT enzyme and in the presence of UDPGA, a portion of the proluciferin substrate is glucuronidated. However, in the absence of UDPGA, the proluciferin is not glucuronidated. In the second step of the assay, addition of LDR and D-cysteine to both reactions results in conversion of the proluciferin substrate into the luciferin derivatives. While the luciferin produced from the unmodified proluciferin substrate will give light after this second step, the luciferin produced from the glucuronidated proluciferin substrate will not give light. Thus, the decrease in light output when comparing the reactions with and without UDPGA is proportional to the glucuronidation activity of the enzyme in the first step of the reaction.

2.3.1. UGT Reaction Mixture Composition. The reaction mixture contained UGT-Glo buffer, supplied in the UGT-Glo Assay kit. It consisted of 250 mM TES, pH = 7.5 and 40 mM magnesium chloride (8 μL per well), UGT-Multienzyme Substrate (50 μM), UGT or control microsomes (0.2 mg/mL), alamethicin (25 μg/mL), and water (up to a final volume of 20 μL per well).

2.3.2. UGT Reaction Assay. PNL and piperine-PNL were prepared as previously described and diluted with water to form nanoparticles. Piperine concentration in the prepared nanodispersion was 400 μM. 20 μL of UGT reaction mixture, containing one of the two UGT isoforms or control microsomes, was incubated with 10 μL of 16 mM UDPGA or 10 μL of water (for plus and minus UDPGA reactions, respectively) and 10 μL of PNL or piperine-PNL (final piperine concentration in reaction well was 100 μM) at 37 °C. After a 60 min incubation period, 40 μL of reconstituted LDR with 2 mM D-cysteine was added to each well and incubated for 20 min at room temperature. After this incubation period, the luminescence signal was read by a luminometer (Berthold Technologies, Bad Wildbad, Germany). The results were expressed as arbitrary units. Following suitable analysis, results were converted into percent of enzymatic activity, compared to control activity without PNL or piperine-PNL

2.4. *In Vivo* Studies. *2.4.1. Animals and Surgery.* All surgical and experimental procedures were approved by the Animal Experimental Ethics Committee of the Hebrew University, Hadassah Medical School, Jerusalem. Male Wistar rats (Harlan, Israel) weighing 275−300 g were kept under a 12 h light/dark cycle with free access to food (standard rat chow) and water prior to trial. Animals were anesthetized for the period of surgery by intraperitoneal injection of 1 mL/kg of ketamine−xylazine solution (9:1, respectively). An indwelling cannula was placed in the right jugular vein of each animal for systemic blood sampling, by a method described before.[28] The cannula was tunneled beneath the skin and exteriorized at the dorsal part of the neck. After completion of the surgical procedure, the animals were transferred to individual cages to recover overnight (12−18 h). During this recovery period, food, but not water, was deprived. Throughout the experiment, free access to food was available 4 h post oral administration. Animals were randomly assigned to the different experimental groups. Tramadol (5 mg/kg, 1 mg/mL) was given subcutaneously to rats before surgery and on the day of the experiment as part of the analgesia protocol.

2.4.2. Experimental Protocol. Raloxifene was orally coadministered with PNL and blank piperine-PNL. The small volume of gastric fluids found in rats is insufficient for proper self-emulsification required for PNLs. Thus, to enable proper formation of a nanodispersion system, for preclinical studies, dispersed formulations were prepared by 30 s vortex mixing of the preconcentrate in preheated water (1:4 v/v) prior to their administration to rats. This technique results in an o/w nanoemulsion which we term "dispersed PNL". All preparations were freshly prepared on the day of the trial. The PNL and piperine-PNL were administered at an equal volume of 0.6 mL dispersed form by oral gavage. The control group received water instead of PNL formulations (n = 6).

Raloxifene (20 mg/kg, 5 mg/mL) was orally coadministered with those formulations to all study groups in 2% methylcellulose (4000 CP) suspension containing 1% DMSO (n = 6 per each group). Combined oral volume of administration for both vehicles was less than 2 mL. For iv administration, raloxifene (1 mg/kg, 2 mg/mL) was dissolved in ethanol:polyethylene glycol (PEG) 300:water solution (1:4:5 v/v, n = 4). Systemic blood samples (0.35 mL) were obtained by intravenous cannula, placed in the jugular vein. In the case of oral administration, samples were taken at 5 min predose and 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 7, 11, and 24 h postdose. In the case of intravenous administration, samples were taken at 5 min predose and 3, 15, and 30 min and 1, 2, 4, 8, and 24 h postdose. To prevent dehydration, equal volumes of physiological solution were administered to the rats following each blood sampling. Sequential blood samples were collected into heparin-containing test tubes at predetermined time intervals. Plasma was separated by centrifugation (3220g, 7 min, 4 °C) and stored at −20 °C pending analysis.

2.5. Analysis of Raloxifene Concentration. Raloxifene was extracted by the following method: 150 μL of plasma was vortex-mixed for 1 min with 200 μL of NaOH (1 M) and 15 μL of talinolol (1 μg/mL in methanol) as internal standard. 3 mL of ethyl acetate was then added, followed by vortex-mixing for 2 min and separation by centrifugation (20124g, 10 min, 4 °C). The organic layer was transferred to fresh glass test tubes and evaporated to dryness (Labconco, Kansas City, MO). Finally, the samples were reconstituted in 80 μL of water−acetonitrile (75:25 v/v) and 0.1% formic acid mixture. Raloxifene amount was determined using a high performance liquid chromatography (HPLC) system (Waters 2695 separation module) with a mass spectrometer (Waters Micromass ZQ, Waters Corporation, Milford, MA). The HPLC−MS conditions were as follows: XTerra MS C8 column 3.5 μm 2.1 × 150 mm column (Waters, Milford, MA), an isocratic mobile phase, water−acetonitrile (75:25 v/v) with 0.1% formic acid, and flow rate of 0.2 mL/min at 35 °C. Retention time for raloxifene and talinolol was 4.36 and 2.91 min. The detection masses (m/z) were 474.38 and 364.5. Limit of quantification for raloxifene was 10 ng/mL.

2.6. Pharmacokinetic Analysis. Area under the plasma concentration−time curve (AUC) was calculated by using the trapezoidal rule with extrapolation to infinity by dividing the last measured concentration by the elimination rate constant (k_e). Elimination rate constant values were determined by a

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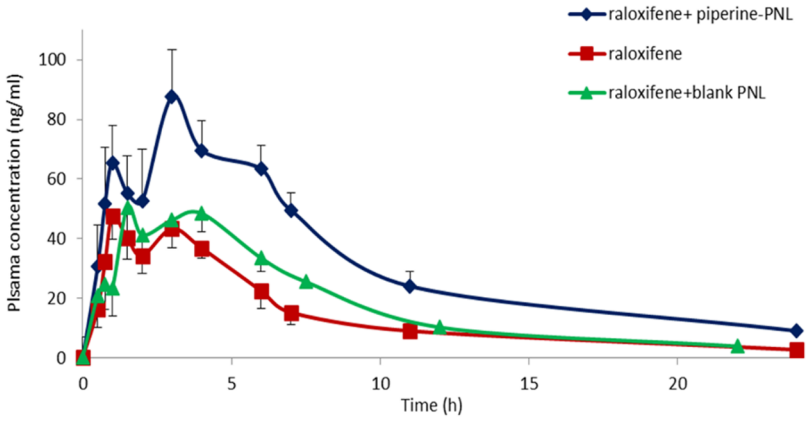

Figure 2. Plasma concentration vs. time plot (mean ± SEM) following PO coadministration of dispersed piperine-PNL (piperine 10 mg/kg), blank PNL, and water with raloxifene in 2% methylcellulose (20 mg/kg, n = 6).

linear regression analysis using the last points on the logarithmic plot of the plasma concentration versus time curve. Pharmacokinetic parameters such T_{max}, C_{max}, clearance (CL), volume of distribution (V), and bioavailability were calculated using noncompartmental analysis.

2.7. Statistical Analysis. All values are expressed as mean ± standard error of the mean (SEM) if not stated otherwise. To determine statistically significant differences among the experimental groups, one-way ANOVA, followed by Tukey's test, was used. P value less than 0.05 was termed significant.

RESULTS

The model compound raloxifene could not be incorporated into our PNL delivery system due to its incompatibility with the PNL components in terms of solubility. Thus, these studies were performed by the coadministration of raloxifene with PNL and piperine-PNL[18]

Coadministration of raloxifene with piperine-PNL to rats resulted in a 2-fold increase in the relative oral bioavailability of raloxifene. Similar results were obtained for the C_{max} values. Coadministration of raloxifene with blank PNL had no statistically significant effect on its oral bioavailability. These results (Figure 2 and Table 1) indicate that the oral bioavailability of intestinal UGT substrates can be enhanced by the piperine-PNL utilization. Additionally, these results demonstrate that the inhibition of intestinal UGTs by piperine-PNL is mediated by the piperine component.

Table 1. Pharmacokinetic Parameters Derived from Oral Coadministration of Raloxifene with PNL and Piperine-PNL to Rats (raloxifene 20 mg/kg, piperine 10 mg/kg, n = 6)

	raloxifene	raloxifene + PNL	raloxifene + piperine-PNL
AUC (h·ng/mL)	208 ± 21	310 ± 13	416 ± 53[a]
C_{max} (ng/mL)	58 ± 6	62 ± 5	111 ± 11[a]
T_{max}[b] (h)	1 (0.8–1.5)	1.5 (1.5–3.0)	3 (1–6)
V/F (L/kg)	1063 ± 159	586 ± 45	497 ± 49
CL/F (L/h/kg)	102 ± 11	76 ± 3	47 ± 5
k_{el} (h^{-1})	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.01
F%	0.6%	0.8%	1.1%[a]

[a]A significant difference (p < 0.05) from raloxifene corresponding values was found [b]Results are presented as median (range)

In order to determine the effect of PNL and piperine-PNL on the elimination phase of raloxifene, plasma concentration vs. time profiles were plotted in a semilogarithmic plot and elimination rate constants were calculated for all study groups (Figure 3, Table 1, Table 2). Comparison of the terminal slopes of plasma concentration in semilogarithmic plot indicates that the terminal elimination phase obtained following oral administration of raloxifene is not different from elimination phase in IV administration.

Since raloxifene bioavailability is limited predominantly by intestinal UGT activity, *in vitro* studies were performed to confirm the hypothesis that piperine-PNL or PNL components have inhibitory effect on UGT activity at the intestine. The glucuronidation activity of recombinant UGT1A8 and UGT1A10 was examined using a commercial assay kit (UGT-Glo Assay, Promega, Madison, WI), in the presence of PNL or piperine-PNL. The effect of piperine alone was not examined, due to practical difficulties of its solubility in the assay buffer. The metabolic activity of UGT1A8 is presented in Figure 4. The results are expressed as relative activity of the enzyme after addition of PNL or piperine-PNL, in comparison to the activity in the absence of these suspected inhibitors. The activity of UGT1A8 was 29.1% ± 19.9 (mean ± SD) of the control activity in the presence of PNL and 29% ± 18.7 with piperine-PNL (Figure 4). Both PNL and piperine-PNL significantly inhibited the activity of UGT1A8.

The glucuronidation activity of UGT1A10 was evaluated by the same method as UGT1A8. When enzymatic activity was measured relative to untreated controls, a significant inhibition by PNL and piperine-PNL was observed (Figure 5). UGT1A10 activity was 57.8% ± 18.5 and 40.6% ± 12.9 of the control activity, in the presence of PNL and piperine-PNL, respectively.

DISCUSSION

Single oral coadministration of raloxifene with blank piperine-PNL to rats resulted in a 2-fold increase in its relative oral bioavailability compared to raloxifene alone. Interestingly, oral coadministration of raloxifene with blank PNL to rats had no statistically significant effect on its bioavailability. It should be noted that as raloxifene is not commercially available in its base form, we used its hydrochloride (HCl) salt. *In vitro* studies conducted in order to facilitate the incorporation of raloxifene HCl into PNL and piperine-PNL failed. Raloxifene HCl

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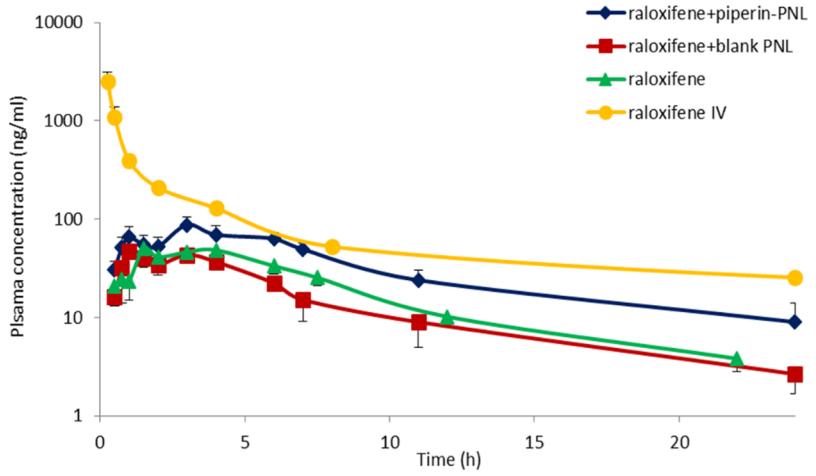

Figure 3. Semilogarithmic plot of plasma concentration vs. time (mean ± SEM) following IV (1 mg/kg, n = 4) and oral administration of raloxifene solution and coadministration of raloxifene (20 mg/kg, n = 6) with blank PNL and piperine-PNL to rats.

Table 2. Pharmacokinetic Parameters Derived from IV Administration (raloxifene 1 mg/kg, n = 4)

	AUC (h·ng/mL)	V (L/kg)	CL (L/h/kg)	k_{el} (h^{-1})
raloxifene IV	1830 ± 140	0.6 ± 0.04	8 ± 1	0.1 ± 0.01

showed no affinity neither toward the lipid core of the nanoparticle nor for its surfactant interface. Thus, in our studies raloxifene was coadministered with PNL formulations with two consecutive oral gavages.

So far, the prevailing notion was that self-nanoemulsifying drug delivery systems (SNEDDS) such as the PNL are effective at improving the oral bioavailability of a poorly soluble compound by their presentation and maintenance of the drug in a dissolved state. This way the drug is introduced in fine oil droplets at the molecular level, throughout the transit in the GI tract.[30−33]

However, there are compounds for which poor water solubility is not the main hurdle in their oral absorption process as opposed to presystemic metabolism. As raloxifene was not incorporated into PNL, it is evident that its enhanced absorption resulted from glucuronidation inhibition and not from effects on solubility. The method used to conduct bioavailability studies of raloxifene, i.e., its coadministration with PNL formulations, excludes the possibility of physical protection provided to the drug by the piperine-PNL. That is to say that piperine-PNL does not act as a shield, which prevents the introduction of the molecule to the metabolizing enzyme. Rather, the increased oral bioavailability is a result of inhibited presystemic metabolism. As blank PNL failed to affect the bioavailability of raloxifene, the increased AUC observed for piperine-PNL seems to be mediated by the piperine component. Different *in vitro* experimental models have shown that piperine has the potential to reduce phase I and phase II metabolism in the intestine and to inhibit P-gp efflux pumps. Piperine is a potent inhibitor of glucuronidation and as such can modify the metabolism of UGT substrates.[34] However, piperine is also a poorly soluble compound, rendering it insoluble in the aqueous environment of the intestine and ultimately hindering its potency.[8] While reported piperine solubility is 0.15 mg/mL (www.hmdb.ca),[35] we have

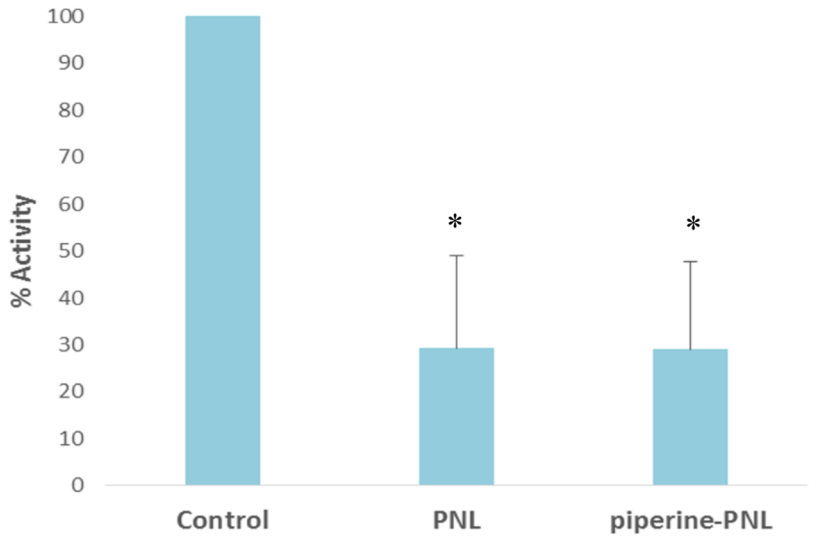

Figure 4. Glucuronidation activity of UGT 1A8, in the presence of 100 μM PNL or piperine-PNL, expressed as percent of control activity (mean ± SD) in a PNL and piperine-PNL free environment (n = 3). (*) A significant difference (p < 0.05) from control was found.

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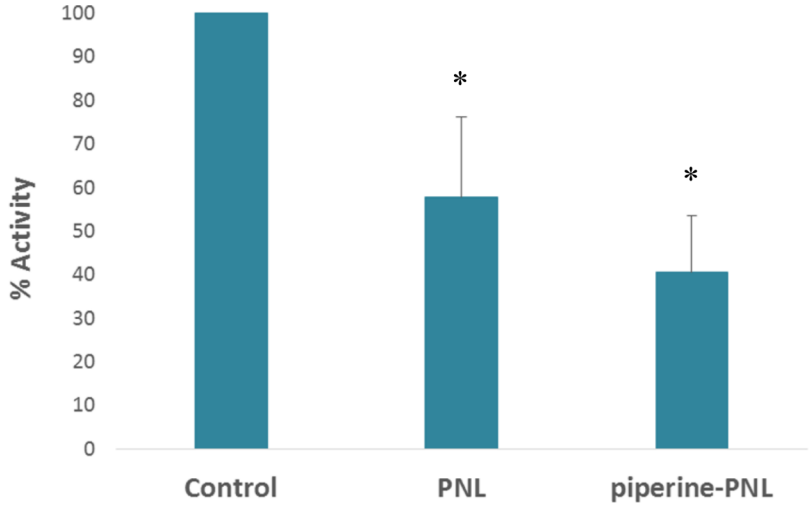

Figure 5. Glucuronidation activity of UGT1A10 recombinant enzymes, in the presence of 100 μM PNL or piperine-PNL, expressed as percent of control activity (mean ± SD) in PNL and piperine-PNL free environment (n = 5). (*) A significant difference (p < 0.05) from control was found.

increased piperine solubility to 20 mg/mL in the PNL formulation at room temperature. The PNL dissolves the piperine and serves as a platform for a successful delivery of the piperine as an absorption enhancer in needed concentrations.

When raloxifene was coadministrated with piperine solution (ethanol:polyethylene glycol 300:water), we received markedly erratic concentration profile, with high variability between rats, proving the necessity of the PNL. We hypothesize that, despite administration of dissolved piperine to rats, piperine precipitated in the aqueous GI media. This subsequently resulted in slow and unpredictable dissolution of the piperine precipitate and consequently extremely variable effect on raloxifen's bioavailability (data not shown).

Previously we have proven that the PNL components have inhibitory properties regarding phase I metabolism, specifically CYP3A4 as well as P-gp efflux.[18] Therefore, we hypothesized that the PNL excipients might have an effect on phase II metabolism as well. In the *in vivo* experiment presented here, PNL excipients were not sufficient to inhibit raloxifene glucuronidation.

Dong et al.[6] performed a screening of various pharmaceutical excipients in order to determine their effect on UGT1A1 using human and rat intestinal as well as liver microsomes. Focusing on the excipients that are part of the PNL, this group has proven that Span 80 increases UGT1A1 activity, and Tween 20 reduces its activity, while soybean lecithin does not have a noteworthy effect.[6] In light of these findings, there might be a possibility that combined PNL excipients do not reach a sufficient inhibitory effect for the UGTs participating in raloxifene metabolism.

Our studies also established that coadministration of raloxifene with either PNL or piperine-PNL does not affect the raloxifene elimination phases. This is evident by the parallel terminal slopes and similar k_{el} values obtained following IV raloxifene administration vs. oral administrations. It demonstrates that raloxifen's oral bioavailability was increased without altering its elimination phase, and the main difference in the pharmacokinetic profile is associated with an enhanced absorption phase. These results attest that the effect of piperine-PNL is attributed to decreased intestinal, rather than hepatic, glucuronidation.

In vitro, piperine-PNL caused a significant inhibition of UGT1A8 and 1A10, findings that fit the *in vivo* results. Piperine concentration for both enzymes was chosen based on previous reports regarding piperine inhibition potential in *in vitro* models.[36] The same diluting and preparation process was performed for blank PNL. The *in vitro* concentration of piperine was lower than the concentration in the animal model in order to avoid saturation of the enzymes. Although there was correlation with the piperine-PNL group in both models, there was a discrepancy between the effect of blank PNL *in vivo* and *in vitro*. While *in vivo*, blank PNL did not enhance raloxifene bioavailability, *in vitro* there is a statistically significant effect of the blank formulation on the UGT1A8 and 1A10 activity. The extent of inhibition of each enzyme was similar by blank PNL and piperine-PNL. *In vitro* experiments were performed with human intestinal microsomes since it is more clinically relevant to test the effect of PNL and piperine-PNL on this type of enzyme. As suggested by Oda et al., an animal isoform can be identified as a human orthologue, although it can still show different substrate specificity and tissue distribution.[4]

This type of inconsistency between *in vitro* and *in vivo* results regarding glucuronidation has been reported previously. Research explains that there are difficulties in predicting *in vivo* performance from *in vitro* data due to technical and physiological challenges, which affect UGT activity.[4,37−39] One of the difficulties at the center of these issues is the presence of alamethicin in the microsomal assay. In order to avoid latency of activity due to the intraluminal location of UGTs, microsomal assays are usually conducted with membrane-disrupting agents, including the pore-forming peptide alamethicin. Alamethicin is known to invade the membrane and form well-defined pores, allowing free diffusion of substrates, inhibitors, cofactors, and other products through the microsomal membrane, hence maximal enzyme activity can be observed. The pore formation does not affect the gross membrane structure and intrinsic enzyme catalytic activity.[40,41] However, this disruption leads to a nonphysiologic access of substrates and inhibitors to the active site of UGTs. It can play a crucial role in the miscorrelation in the effect of blank PNL on raloxifene metabolism *in vitro* and *in vivo*. It is conceivable that intact PNL, or some of the disassembled PNL components, can inhibit UGTs, in light of the inhibition reported regard CYPs

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and P-gp pumps and other types of UGTs.[22,42] On the other hand, the *in vivo* trial was performed in the absence of alamethicin, thus, the enterocyte and ER membranes constituted a barrier against PNL or PNL components from reaching UGTs and extending their effect.

CONCLUSIONS

In conclusion, intestinal direct phase II metabolism has a significant role in drug absorption and, thus, is of great importance in drug development. These are processes that affect the parent drug, as opposed to the held notion that phase II metabolism only follows oxidative metabolism. In this paper we present a solution for drugs undergoing extensive direct phase II metabolism in the form of the piperine-PNL formulation. This vehicle serves as a platform for the delivery of piperine in its solubilized state to the enterocyte monolayer. As a result, the piperine has the opportunity to execute its inhibitory effects on the UGT enzymes involved in raloxifene's metabolism. Ultimately, this formulation may be used for other model drugs that are subjected to direct phase II metabolism, thus improving their oral bioavailability. The raloxifene paradigm further established that the model drug is not necessarily dissolved in the PNL in order for the formulation to execute its effect. Although the *in vivo* model showed a difference between piperine-PNL and blank PNL, the *in vitro* model failed to distinguish the formulations. This suggests that, for this type of formulation in these methods, the *in vivo*−*in vitro* correlation cannot be used as a tool to predict *in vivo* performance of the PNL.

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Notes

The authors declare no competing financial interest.

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