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RESEARCH PAPER

Enhancement of Docetaxel Absorption Using Ritonavir in an Oral Milk-Based Formulation

K. Soulele[1] • T. Karampelas[2] • C. Tamvakopoulos[2] • P. Macheras[1,3]

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ABSTRACT

Objective The current study aimed to develop a novel milk-based formulation of docetaxel, a sparingly soluble antineoplastic agent, administered so far exclusively by the intravenous route and evaluate its oral bioavailability.

Methods Pre-formulation studies included the determination of docetaxel solubility in water-alcohol mixtures as well as short-term content uniformity experiments of the final formulation. The pharmacokinetic (PK) performance of the developed milk-based formulations was further evaluated in vivo in mice using ritonavir, a potent P-glycoprotein inhibitor, as an absorption enhancer of docetaxel and the marketed intravenous docetaxel formulation, Taxotere®, as a control.

Results In vivo PK results in mice showed that all the administered oral docetaxel formulations had limited absorption in the absence of ritonavir. On the contrary, ritonavir co-administration given as pre-treatment significantly enhanced oral bioavailability of both the marketed and milk-based docetaxel formulations; an even more marked increase in drug exposure was observed when ritonavir was incorporated within the docetaxel milk-based formulation. The fixed-dose combination also showed a more prolonged absorption of the drug compared to separate administrations.

Conclusions The current study provides insights for the discovery of a novel milk-based formulation that could potentially serve as an alternative, non-toxic and patient-friendly carrier for an acceptable docetaxel oral chemotherapy.

KEY WORDS docetaxel · milk-based formulation · oral bioavailability · ritonavir

INTRODUCTION

Docetaxel is a hydrophobic antineoplastic compound that demonstrates significant antitumor activity and is widely used in the treatment of various malignancies (1). Docetaxel disrupts microtubule functioning in cells, resulting in the inhibition of cell division. It has been approved as an injectable formulation in the form of *Taxotere*® by the FDA and EMA for use in treating 5 types of cancer: metastatic and adjuvant breast cancer, metastatic androgen independent prostate cancer, advanced non-small cell lung cancer, advanced gastric adenocarcinoma and locally advanced squamous cell carcinoma of the head and neck. One of the major limitations of docetaxel therapy arises from its extremely low aqueous solubility (2).

Docetaxel is currently distributed in a blister carton package consisting of one single-dose Taxotere vial containing docetaxel dissolved in polysorbate 80 and one single-dose solvent for Taxotere vial containing 13% (*w*/w) ethanol, wherein the above two are mixed together to prepare a premix with a solubility of 10 mg/mL and then added into an infusion bag containing physiological saline solution. However, hypersensitivity reactions may occur due to the use of polysorbate 80 and the presence of ethanol may cause additional side reactions (3, 4).

Due to the problems of the intravenous administration, there is an increasing interest in the development of an oral docetaxel formulation (5–9). However, oral administration of docetaxel is hampered by its affinity for drug transporters,

✉ P. Macheras
macheras@pharm.uoa.gr

[1] Laboratory of Biopharmaceutics – Pharmacokinetics, Faculty of Pharmacy, National and Kapodistrian University of Athens, Athens, Greece

[2] Division of Pharmacology-Pharmacotechnology, Center for Clinical, Experimental Surgery and Translational Research, Biomedical Research Foundation of the Academy of Athens (BRFAA), Athens, Greece

[3] PharmaInformatics Unit, ATHENA Research Center, Artemidos 6 & Epidavrou , 15125, Marousi, Athens, Greece

especially ABCB1 (P-glycoprotein, P-gp), extensive first-pass metabolism by cytochrome P450 3A (CYP3A) and poor solubility (10). Studies with mice in which oral docetaxel was administered in combination with ritonavir indicated that ritonavir dramatically enhances the bioavailability of docetaxel (11). It was found that both P-gp and CYP3A4 caused the low oral bioavailability of docetaxel, and ritonavir was capable of inhibiting both systems significantly. This effect was also confirmed in patients with solid tumors (12), where it was found that the apparent bioavailability of docetaxel combined with ritonavir was 131 ± 90% compared with the bioavailability of docetaxel alone, whereas co-administration of oral docetaxel in patients with solid malignancies with another P-gp and CYP3A4 inhibitor, cyclosporine (15 mg/kg) led to an absolute bioavailability of 90% for docetaxel (13). In all oral animal and human studies of docetaxel, the intravenous formulation was administered per os since there is no approved oral formulation of docetaxel. However, the intravenous formulation, has excipients that are not appropriate for oral administration, an unpleasant taste and a limited shelf half-life (10).

In this work, we utilized milk as a potential formulation vehicle (14) in order to develop a novel formulation containing not only docetaxel, which has very low solubility, but also the P-gp inhibitor ritonavir in a dissolved form in the mice gastrointestinal tract. Milk is particularly useful for highly non-polar drugs because is a medium with high solubilization (15, 16) and various freeze-dried drug-milk formulations have been studied in the past (17–20).

MATERIALS AND METHODS

Materials

Docetaxel anhydrous (HPLC grade 99.7%, Sicor de Mexico S.A.), Ritonavir USP (HPLC grade 99.6%, Emcure Pharmaceuticals Limited) and Taxotere 20 mg/ml concentrate for solution for infusion (Sanofi S.A.) were kindly provided by Vianex S.A. (Greece). Ultra-high temperature processing (UHT) milk: full fat milk (3.5% fat, 3.3% proteins, 4.7% carbohydrates, Ennstal, Austria) was used for the preparation of milk-based formulations. Ethanol absolute was obtained from Panreac Quimicasau (Barcelona), t-butyl alcohol, ammonium acetate (LC/MS grade ≥ 99.0%) and formic acid (LC/MS grade 98%) were purchased from Fluka Analytical (Germany), acetonitrile RS LC/MS grade from CARLO ERBA reagents S.A. (France). All other chemicals were of analytical grade and included propylene glycol (≥99.5% grade, Sigma-Aldrich), cremophor EL (Acros Organics) and sodium citrate monobasic anhydrous (≥99.5% grade, Sigma Life Sciences, Germany). Ultra-purified water was obtained through a Barnstead NANOpure Diamond water purification system (Dubuque, Iowa, USA). Regenerated cellulose target syringe filters of 0.45 μm pore size and 17 mm outside diameter (Titan® Scientific Resources Inc., Eatown, NJ, USA) were also used in the solubility experiments.

In Vitro Studies

For the development of this novel docetaxel milk-based formulation a series of pre-formulation studies was performed in order to determine the excipients and processes required for the preparation of a final formulation that could provide docetaxel in a solubilized form to be utilized for in vivo studies, maintaining also the required homogeneity and acceptable characteristics for oral administration. Initially, the solubility of docetaxel in different water-alcohol solutions was determined in order to find the vehicle that could best incorporate docetaxel in a solubilized form into the final formulation. In a next step, various docetaxel milk-based formulations were prepared which differed in their composition and also the applied manufacturing processes. As a final step, the optimal docetaxel formulation was further tested for its short-term homogeneity through the performance of content uniformity experiments.

Solubility Measurements

For the determination of intrinsic solubility of docetaxel in the different water-alcohol mixtures, the shake flask methodology was applied. Ethanol and t-butyl alcohol were chosen as alcohol constituents, based on certain properties required for further formulation development, like the solubilization capacity, the low toxicity and the suitability as a freeze-drying medium. In this context, ethanol/water and t-butanol/water mixtures in ratios of 10%, 20%, 40%, 60%, 80% alcohol were tested as media for the determination of equilibrium solubility of docetaxel. According to the applied methodology, docetaxel was added in surplus to a certain volume of medium and sealed flasks were shaken within a water shaking bath at 50 rpm and temperature 25°C for 48 h. Following this time period and after confirming saturation by observing the presence of undissolved material within the flasks, filtration of the samples was performed and filtrates were further diluted with mobile phase in order to be quantified. Solubility experiments in each medium were performed in either triplicates or sextuplicates. Statistical analysis of the obtained results was performed with the statistical package SigmaPlot 10.0 (Systat Software Inc., US).

Preparation of Docetaxel-Milk Based Formulations

In order to investigate the potential of docetaxel oral bioavailability enhancement through the administration of a milk-based formulation, the results of the solubility experiments were used to prepare a number of different docetaxel milk-

based formulations. From the developed formulations, three were finally tested for their performance in vivo. The first developed formulation (Formulation A) included a 60–40% ethanol/water docetaxel solution mixed with 80% *v*/v full fat (3.5%) milk, in order to evaluate the ability of milk to maintain docetaxel in a solubilized form in vivo, without further processing. Additional testing of several formulation strategies resulted in the development of the second formulation, which differed in both composition and manufacturing process compared to the first formulation. In this respect, the second formulation (Formulation B) included the initial incorporation of docetaxel in a 85–15% t-butyl alcohol - water solution 93% v/v full fat (3.5%) milk, a mixture that was further freeze-dried through an optimized lyophilization cycle (−80 °C, 0.160 mbar, for 72 h) until complete removal of frozen solvents and formation of a proper cake. The freeze-dried formulation was then reconstituted with a certain amount of water to reach the desired concentration just prior to drug administration. The third developed formulation (Formulation C) was the same as Formulation B, with the addition of the absorption enhancing agent, ritonavir (marketed product Norvir®, AbbVie Ltd.) within the milk-based formulation. Studies have shown that ritonavir, a CYP3A/P-glycoprotein inhibitor, can strongly enhance the apparent oral bioavailability of docetaxel. For this reason, ritonavir was added in the solution of docetaxel consisting of 85–15% t-butyl alcohol - water and the formulation was further processed as described above. It has to be noted that ritonavir exerts similar solubility properties to docetaxel, therefore, the agent was incorporated into the initial vehicle without performing any additional solubility experiments in this respect. Similarly, the freeze-dried formulation was also reconstituted with water prior to drug administration. The reconstitution of the freeze dried powdered pharmaceutical compositions in water prior to oral administration was made by short agitation. Macroscopic observation of the ethanol-based formulation (A) and the t-butyl formulations (B, C) before and after freeze-drying did not appear to present any differences, something that could suggest an effect of a specific solvent or freeze-drying on the milk proteins.

Content Uniformity

Following product development, Formulation C containing both docetaxel and ritonavir, as described above was chosen to be further tested for its short-term homogeneity after reconstitution. In this context, the freeze-dried docetaxel/ritonavir milk-based formulation was delivered in flasks where the content was reconstituted with the appropriate volume of water. Following powder reconstitution, samples of the product were taken from the surface, middle and bottom of the milk solution at 2 min, 2 and 4 h. The experiment was performed in triplicates at room temperature. Following extraction, samples were analyzed by an LC-MS/MS. Homogenous dispersion of the developed formulation was assessed by showing content uniformity of docetaxel as a function of time during the defined time period.

In Vivo Studies

The oral in vivo performance of the prepared docetaxel milk-based formulations was studied on male C57BL/6 mice of 10–12 weeks old and weighing 25–30 g, under controlled temperature and humidity conditions, which were fasted overnight for 12–14 h with free access to water. Animals were maintained at the BRFAA animal facility. All animal procedures were approved by the Bioethical Committee of BRFAA based on the Presidential Decree 56/2013 in harmonization with the European Directive 2010/63/EU for the protection of animals used for scientific purposes. Apart from the characterization of the pharmacokinetic (PK) profile of the developed milk-based formulations, comparative bioavailability with the marketed intravenous docetaxel formulation (Taxotere® 20 mg/ml concentrate for solution for infusion) given by the oral route, with or without ritonavir pre-treatment, was also determined.

Mice ($n = 6$ per administration) were dosed by oral gavage with approximately 250 μL (depending on the exact concentration of the dosing solution) of one of the above-mentioned formulations on separate PK studies. Formulations were either reconstituted or diluted with the appropriate physiological vehicle right before administration to obtain the desired drug concentration. To reduce the number of animals and generate individual animal PK profiles, a serial blood sampling technique via tail vein bleeding in mice was developed, in which only 10 μL of blood was collected per time point. Sampling times included 15 min, 30 min and 1, 2, 4, 6 and 8 h post-dose. Samples were collected in tubes containing sodium citrate 0.1 M as anticoagulant and were stored in -80°C until sample analysis.

Pharmacokinetic parameters of docetaxel were estimated using the classic non-compartmental methods (WinNonlin® v.5.0.1 / Pharsight Corp, Menlo Park, CA). These parameters referred to the first recorded maximum blood concentration value (Cmax), the time at which Cmax occurs (Tmax) and the area under the concentration-time curve from time zero to the last quantifiable sample (AUCt). AUCt was calculated using the linear trapezoidal rule. Descriptive statistics (arithmetic mean and standard deviation) were calculated for these PK parameters and relative bioavailability between the different docetaxel formulations was estimated.

Analytical Methodology

The identification and quantification of docetaxel from the in vitro and in vivo studies were performed using two different

Liquid Chromatography / Mass Spectrometry (LC-MS/MS) methods, an isocratic method in case of in vitro study samples and a gradient elusion method for the in vivo samples. HPLC was performed using a Dionex Ultimate 3000 system (Dionex Corporation, Germering, Germany) equipped with three pumps (two for nano and one for micro LC), a temperature controlled column compartment and an autosampler. A Waters Atlantis HPLC Column, dC18 3 μm, 2.1 × 50 mm at a flowrate of 0.3 mL/min for the separation of analytes of interest was used. The mobile phase consisted of A: 10% ACN, 90% water, 2 mM ammonium acetate, and 0.1% FA and B: 90% ACN, 10% water, 2 mM ammonium acetate, and 0.1% FA. Mass spectrometry was performed on an API 4000 QTRAP LC-MS/MS system fitted with a TurboIonSpray source and a hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Concord, Ontario, Canada). The standard parameters of the mass spectrometer ion source were adjusted as follows: temperature of heated capillary: 550°C, ion spray voltage (ISV): 5500 V, dry nitrogen curtain gas: 20 units, collision gas: 12 units, ion source gas 1: 40 units and ion source gas 2: 45 units. Both developed analytic methodologies (for in vitro and for in vivo studies) showed adequate sensitivity, precision, accuracy, specificity, and linearity.

In the case of in vitro sample analysis, an isocratic method consisting of 55% Mobile Phase A – 45% Mobile Phase B was used, providing a linear calibration curve with concentration range of 25–2500 ng/mL.

Quantification of samples from the in vivo experiments required the development of a gradient elution methodology in order to be able to simultaneously detect ritonavir and quantify docetaxel, using paclitaxel as an internal standard (IS), in the same analytical run. The gradient methodology used was as follows: i. 0–2 min: 90%A-10% B, ii. 2–5 min: 50%A-50% B, iii. 5–9.2 min: 90%A-10% B. Samples were extracted using protein precipitation with cold acetonitrile. This methodology allowed the adequate separation and characterization of the three molecules and provided a linear calibration curve with the concentration range of 10–1000 ng/mL for the quantification of docetaxel in the obtained blood samples.

RESULTS

In Vitro Studies

Solubility Measurements

As expected, the solubility of docetaxel in both ethanol/water and t-butyl alcohol/water mixtures studied increased by increasing the proportion of alcohol content in the mixture (Table 1). Fig. 1(A and B) also shows graphically the saturation solubility of docetaxel in the different ethanol-water (10%, 20%, 40% and 60% ethanol) and t-butyl alcohol water (20%, 40%, 60% and 80%) mixtures, respectively, at temperature of 25°C. The observed curves derived from nonlinear regression analysis of the exponential model on the experimental data.

As can be seen in Fig.1, the solubility of docetaxel in both water-alcohol media increases exponentially with increasing alcohol content. More specifically, docetaxel shows low solubility (less than 1 mg/mL) in all ethanol content ratios except for the 60% content, where solubility sharply increases (more than 10 mg/mL). From the nonlinear regression of the exponential model to the data, it can be derived that for 0% ethanol content, docetaxel solubility in water is approximately 1.8 μg/mL at 25°C, which is in line with literature reported values (9), (http://www.drugbank.ca/drugs/DB01248). Accordingly, in t-butyl alcohol/water mixtures docetaxel also shows an exponential increase with increasing alcohol content. In this case, however, this increase is gradual and smoother, reaching up to 20 mg/mL at 80% t-butyl alcohol content. The amount of docetaxel that could be dissolved in this latter mixture was considered adequate for the development of the final formulation, since from the nonlinear fitting of exponential model to the observed solubility data, a relatively good estimation of the amount of drug dissolved at 85% t-butyl alcohol content could be provided.

Content Uniformity

Based on the content uniformity results of docetaxel/ritonavir milk-based formulation (Formulation C), docetaxel appears to be homogeneously dispersed in milk during the study duration (Fig. 2). The determined concentration at all time-points and from all three sampled levels (surface, middle and bottom) of the milk formulation was very close to the theoretical one (1285.7 μg/mL). No sedimentation was observed, since the drug concentration remained constant for all milk samples taken from surface, middle and bottom at all time-points up to 4 h.

In Vivo Studies

The seven docetaxel formulations that were assessed in the in vivo PK studies are listed in Table 2.

The PK profiles of docetaxel following oral administration of the different drug administrations in mice are shown in Fig. 3. It has to be noted that even though Formulation A, consisting of a simple 60–40% ethanol/water docetaxel solution mixed with milk without further processing (e.g., freeze-drying), was administered in quite higher dose (29.5 mg/kg) compared to the other formulations, the observed blood concentrations were below the lower limit of detection of the

Table I Saturation solubility values (mean ± SD) of docetaxel in different ethanol and t-butyl alcohol mixtures with water at temperature 25°C

Alcohol content (%)	Docetaxel Solubility (μg/mL) Mean (± SD)
Ethanol	
10 (*n* = 6)	9.2 (0.48)
20 (n = 6)	44.7 (4.71)
40 (n = 6)	769.3 (75.6)
60 (*n* = 3)	16,000.0 (1050.1)
T-butyl alcohol	
20 (n = 3)	0.059 (0.007)
40 (n = 3)	1.86 (0.20)
60 (n = 3)	8.26 (0.17)
80 (*n* = 4)	20.20 (0.64)

bioanalytical method, and were not included in the presented results.

The docetaxel PK parameters are summarized in Table 3. Oral bioavailability of docetaxel remained low when the drug was administered without the ritonavir co-administration, irrespectively of the formulation strategy followed. The AUCt of docetaxel in milk Formulation B, however, increased 1.6-fold compared with that of oral Taxotere®, taking into consideration the different administered doses (199.6 ng*h/mL vs. 89.0 ng*h/mL). AUCt of Formulation B was further enhanced in the presence of ritonavir vehicle (containing ethanol/Cremophor EL/Propylene glycol/water as excipients) demonstrating a 1.7-fold and 2.6-fold increase as compared to Formulation B alone and oral Taxotere®, respectively. Cmax was also enhanced in the presence of ritonavir vehicle by about 1.5-fold as compared to both Formulation B

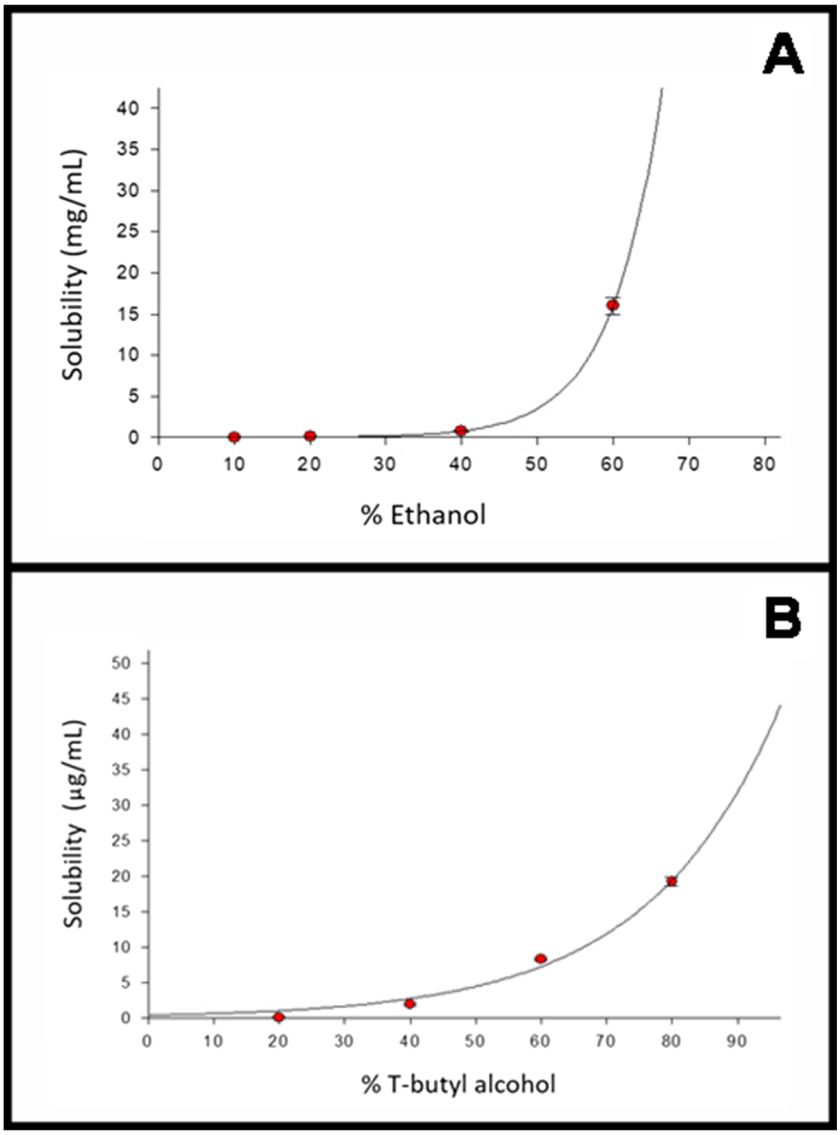

Fig. 1 Saturation solubility of docetaxel in different A. water/ethanol solutions (10–60% ethanol content) and B. water/t-butyl alcohol solutions (20–80% t-butyl alcohol content) at 25°C

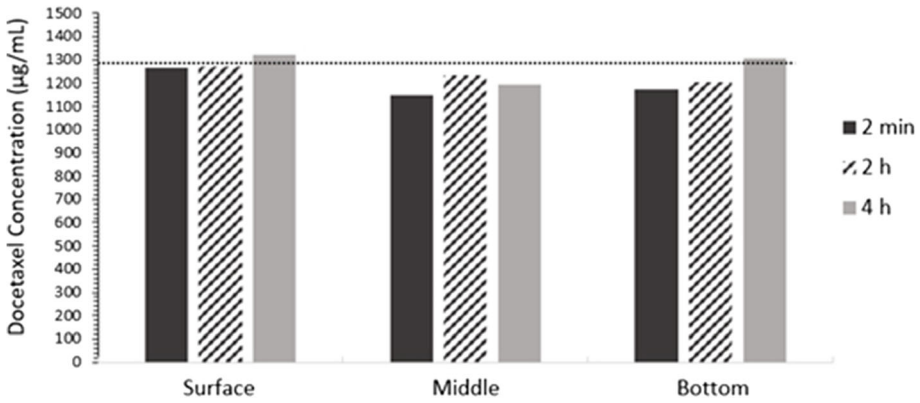

Fig. 2 Concentration of docetaxel measured in the reconstituted milk-based Formulation C. Samples were taken from surface, middle and bottom of the milk volume at 2 min, 2 and 4 h. The dashed horizontal line indicates the theoretical content 1285.7 μg/mL for docetaxel. For details about the preparation of formulation see Section Preparation of Docetaxel-Milk Based Formulations

and oral Taxotere® (62.1 ng/mL vs. 38.3 ng/mL vs. 28.1 ng/mL, respectively).

When ritonavir, an established absorption enhancer of docetaxel, was co-administered either as a pretreatment or incorporated in the milk formulation (Formulation C), a dramatic increase of the bioavailability of docetaxel was observed in all cases. Thus, in the case of oral Taxotere®, pre-treatment with ritonavir formulation resulted in a 45-fold increase in AUCt (3982.4 ng*h/mL vs 89.0 ng*h/mL). A significant but relatively lower increase in bioavailability was also observed in the case of milk Formulation B, where introduction of ritonavir pre-treatment led to a 21-fold increase of AUCt (4110.0 ng*h/mL vs 199.6 ng*h/mL). Similar increases were also noted for Cmax, in both cases.

Table 2 Oral Docetaxel formulations investigated in the PK studies in mice

n	Docetaxel Formulation	Dose (mg/kg)	Ritonavir [a] (dose mg/kg)
1	Taxotere®[b]	6.2	No
2	Taxotere®	6.2	Pre-treatment (12.5)
3	Formulation A[c]	29.5	No
4	Freeze-dried milk Formulation B	9	No
5	Freeze-dried milk Formulation B	9	Pre-treatment (12.5)
6	Freeze-dried milk Formulation B	9	Pre-treatment with ritonavir vehicle
7	Freeze-dried milk Formulation C (docetaxel + ritonavir)	9	Co-administration (12.5)

[a] *Ritonavir pretreatment was given 30 min before docetaxel administration formulated in a vehicle similar to the marketed product Norvir® containing (43/9.8/25/22.2) %v/v ethanol/Cremophor EL/Propylene glycol/water for injections as excipients*

[b] *Taxotere® formulation: (25/9.75/65.25) %v/v polysorbate 80 citric acid/ethanol/water for injection*

[c] *Formulation A: Milk mixed with docetaxel solution consisting of 60–40% ethanol-water*

The highest bioavailability of docetaxel, however, was observed when ritonavir was actually incorporated with docetaxel in the milk-based formulation (Formulation C), which led to a 36-fold increase in AUCt (7156.8 ng*h/mL vs 199.6 ng*h/mL) and 29-fold in Cmax (1102.2 ng/mL vs 38.3 ng/mL), respectively, when compared to Formulation B, where only docetaxel was administered. When Formulation C was compared with oral Taxotere®, a substantial increase in docetaxel absorption was observed with 55-fold higher AUCt values (7156.8 ng*h/mL vs 89.0 ng*h/mL), whereas in the case of Taxotere® with ritonavir pre-treatment, bioavailability of Formulation C remained higher by 1.2-fold (AUCt: 7156.8 ng*h/mL vs 3982.4 ng*h/mL), following dose correction. In addition to that, docetaxel levels from Formulation C had a slower decline compared to other formulations, suggesting a more prolonged absorption of the drug which would lead to even higher bioavailability if a more extended sampling was performed. Based on the above findings it is clear that from a quantitative perspective, ritonavir appears to offer a greater bioavailability enhancement effect compared to milk.

Importantly, including ritonavir in the administered schemes did not lead to any delay in Tmax values with most treatments showing their peak concentrations at around 1 h post-dose. This suggests that even though ritonavir has a significant effect in docetaxel bioavailability, it does not seem to notably affect its absorption rate. On the other hand, a Tmax of 2 h was observed in the case of two milk-based formulations (Formulation B + ritonavir and Formulation C), which further suggests a more prolonged absorption of docetaxel from the milk formulations. This finding is in accordance with previous findings showing that in the case of microemulsions, the drug needs to first be released out from the lipid phase, thereby resulting in a delayed Tmax and a more prolonged drug absorption (21). In addition, the levels of Ritonavir were estimated based on measurements of area count of analyte/internal standard in the three studies in which ritonavir was administered (Supplementary Fig. 1). A similar pattern was observed in all treatments, suggesting that its incorporation in Formulation C, did not affect its pharmacokinetics.

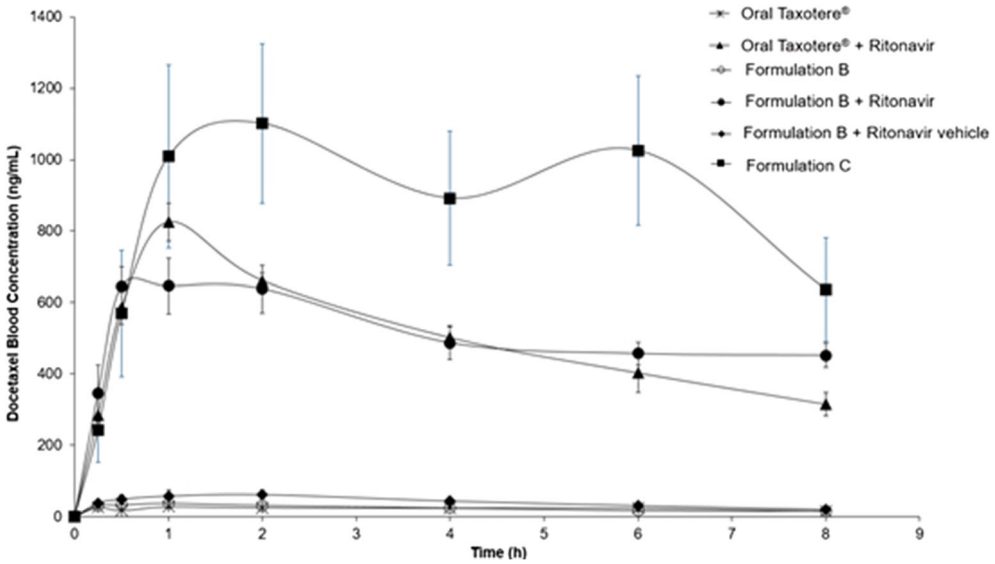

Fig. 3 Blood concentration-time profiles of docetaxel after oral administration of 6 different docetaxel administration schemes. Each data point represents the mean ± SD of six mice. *Key: Formulation C (freeze-dried milk-based formulation of docetaxel 9 mg/kg + ritonavir 12.5 mg/kg), Formulation B (freeze-dried milk-based formulation of docetaxel 9 mg/kg), Ritonavir (12.5 mg/kg) was formulated in a vehicle similar to the marketed product Norvir® containing (43/9.8/25/22.2) %v/v ethanol/Cremophor EL/Propylene glycol/water and was given as pretreatment 30 min prior to docetaxel administration, Taxotere® (6.2 mg/kg) contains (25/9.75/65.25) % v/v polysorbate 80, citric acid/ethanol/water for injection as excipients*

DISCUSSION

Milk is an abundant and inexpensive carrier that presents the desired characteristics for its use as an oral drug delivery platform (14). It is a natural oil-in-water emulsion where both water-soluble and lipophilic substances can be incorporated and retained in solubilized form for an improved oral bioavailability. Importantly, milk appears to have attractive formulation properties, especially with respect to novel oral formulations. Specifically, the complex interfacial layer of milk known as the milk fat globule membrane (MFGM) stabilizes the fat globule in milk, constituting an effective natural surface-active medium (22). In addition, fat globules of milk present stability under gastric conditions, and several MFGM proteins seem to be resistant to gastric digestion (23). Notably, fat globules in milk present extended bioavailability in the stomach (23). The above-mentioned properties of milk could potentially enhance the pharmacokinetics of a therapeutic entrapped in a milk formulation. In the current work, milk was used as the basic component for the development of a novel milk-based formulation of docetaxel.

Table 3 Pharmacokinetic parameters after oral administration of six different docetaxel administrations to mice

Formulation	Dose (mg/kg)	Docetaxel PK Parameters (Mean ± SD)		
		Cmax (ng/mL)	AUCt (ng*h/mL)	Tmax (h)
Oral Taxotere®	6.2	28.1 (11.2)	89.0 (72.3)	1.0
Oral Taxotere® + Ritonavir	6.2 + 12.5	825.2 (52.7)	3982.4 (303.1)	1.0
Formulation B	9	38.3 (8.5)	199.6 (34.2)	1.0
Formulation B + Ritonavir	9 + 12.5	646.6 (79.3)	4110.0 (325.6)	1.0
Formulation B + Ritonavir vehicle	9	62.1 (12.7)	333.0 (66.4)	2.0
Formulation C	9 + 12.5	1102.2 (224.5)	7156.8 (499.2)	2.0

Key: Formulation C (freeze-dried milk-based formulation of docetaxel 9 mg/kg + ritonavir 12.5 mg/kg), Formulation B (freeze-dried milk-based formulation of docetaxel 9 mg/kg), Ritonavir (12.5 mg/kg) similar to Norvir® formulation as 30 min pretreatment; AUCt, the area under the concentration-time curve from time zero to the last quantifiable sample; Cmax, the first recorded maximum blood concentration value; Tmax, the time at which Cmax occurs

Docetaxel is a poorly water-soluble antineoplastic agent, administered exclusively by the intravenous route, with multiple acute and long-term adverse reactions (24, 25). Various efforts have been made so far to introduce an oral formulation of docetaxel based on emulsion formulation technology to reduce these administration side effects (8, 9, 26). However, oral administration of docetaxel is still clinically not feasible, restricted by its poor oral bioavailability, which is in part due to its practically insoluble properties in aqueous media, but also its high affinity to the multidrug efflux pump P-glycoprotein (P-gp) and hepatic first-pass metabolism (11).

Several literature reports have indicated that the solubility and rate of dissolution of lipophilic drugs is much higher in milk than in aqueous media (15, 27). A number of in vitro and in vivo studies with different drug–milk formulations have also demonstrated their superiority both with respect to solubility and dissolution performance as well as an enhancement of bioavailability compared to conventional oral preparations of lipophilic drugs (17–20). It was, therefore, considered that milk, as a natural emulsion could potentially increase docetaxel solubility and serve as a favorable carrier for enhancing its oral bioavailability.

Efflux phenomena by the intestinal P-gp transporter and first pass metabolism are also crucial for developing an oral formulation of docetaxel. In this respect, ritonavir, a potent CYP3A/P-glycoprotein inhibitor, was used as an absorption enhancer, either administered as a pre-treatment or by incorporating the agent within the milk-based formulation (28). Ritonavir has been shown to strongly enhance the apparent oral bioavailability of docetaxel in both animal and human pharmacokinetic studies (11, 12).

In the present study, three docetaxel milk-based formulations were developed and tested for their PK performance, with or without ritonavir co-administration. For the development of these formulations a series of in vitro pre-formulation studies was performed in order to investigate the appropriate solvent composition that could adequately solubilize the required amount of docetaxel, but also retain the desired properties. The solubility experiments performed with various ethanol and t-butyl alcohol mixtures with water, showed an exponential increase of docetaxel solubility with increasing alcohol content in both cases. Based on these experiments, the use of 85–15% t-butyl alcohol/water solution was considered as the most appropriate solvent for docetaxel initial solubilization, that could also attain the desired properties for the subsequent freeze-drying process of the milk-based formulation. T-butyl alcohol is a low toxicity, high vapor pressure and low melting solvent, which has shown to be an excellent freeze-drying medium (29). Further experiments investigating the homogeneity and short-term content uniformity of the most complex milk-based formulation (Formulation C), also indicated that the developed formulation could adequately retain docetaxel in solubilized form following reconstitution without any precipitation issues for the assessed period of time. Importantly, these experiments aimed to assess the short-term homogeneity of the final docetaxel milk-based freeze-dried formulation following reconstitution with water and identify potential precipitation issues. In those experiments no precipitation of the drug substance was observed during the studied time points (2 min, 2 and 4 h) with the drug concentration remaining constant for all milk samples taken from surface, middle and bottom at all time-points up to 4 h. These results could suggest that the developed milk-based formulation may adequately retain docetaxel in a solubilized form following reconstitution without significant precipitation issues occurring for the assessed period of time.

In the in vivo PK studies in mice, seven different docetaxel treatments were evaluated. Apart from the developed formulations, oral Taxotere® with or without ritonavir was also administered in order to serve as control. As could be seen form the obtained results, the bioavailability of docetaxel following oral administration of either the marketed i.v. formulation (Taxotere®) or the milk-based formulations without ritonavir pre-treatment was low. When the milk-based formulation B was administered with ritonavir vehicle pre-treatment, there was a slight increase in docetaxel bioavailability, attributed possibly to the surfactants included as excipients in the ritonavir formulation, however, absorption of docetaxel was still limited. On the contrary, co-administration of ritonavir as a pre-treatment significantly increased docetaxel systemic exposure of both Taxotere® and milk-based Formulation B by 45- and 21-fold, respectively, compared with each docetaxel formulation alone.

The increase in oral bioavailability of docetaxel was even more marked when ritonavir was incorporated within the docetaxel milk-based formulation (Formulation C) with an increase in docetaxel exposure by 36-fold compared to Formulation B and 55-fold compared to plain oral Taxotere®. A comparison between Taxotere® plus ritonavir and Formulation C also favored the milk-based formulation with a 1.2-fold higher exposure for the latter preparation over the 8-h study period. It should be further noted, however, that docetaxel blood levels following administration of Formulation C declined much slower than with Taxotere® plus ritonavir, showing a more prolonged absorption of the drug from the milk-based formulation. This finding suggests that the comparative bioavailability of the milk-based Formulation C would be even higher if a more extended sampling had been applied. It should be noted that no side effects were observed in the mice that received Formulation C (or with any other formulation), suggesting that addition of ritonavir in the formulation did not cause any toxicity. Docetaxel in Taxotere® is readily exposed in the gastrointestinal track since it is solubilized in Tween 80 and ethanol (9). However, in case of the milk-based formulation which is a natural microemulsion, the drug needs to be released out from the

lipid phase, thereby resulting in a delayed Tmax and a more prolonged drug absorption.

If we had to address a potential head-to-head comparison of ritonavir and milk on their effect in enhancing the bioavailability of docetaxel, our results show that ritonavir would prevail. Nevertheless, the benefits of our milk formulation are multidimensional as besides the obvious quantitative outcome of the enhanced bioavailability, it also provides a viable solution for formulating not only docetaxel, but also ritonavir, a molecule with challenging solubility in order to be administered orally. Moreover, the combination of the two factors (milk and ritonavir) provided the greatest bioavailability, something that further highlights the importance of their mutual contribution in the final formulation in order to achieve the optimal outcome.

The enhanced oral bioavailability of docetaxel observed in Formulation C could be explained by the combination of the following attributes. Firstly, it can be derived from the obtained in vitro and in vivo results that the developed milk-based formulation was able to significantly increase solubility of docetaxel. Most importantly this improved solubility could be further maintained during the gastrointestinal dilution and permeation process. In addition to that, the synergistic effect of milk lipid phase as well as the presence of natural surfactants in milk, further acted as a favorable vehicle for docetaxel absorption. Nevertheless, the more marked effect in docetaxel bioavailability was exerted by the introduction of the P-gp and CYP3A4 inhibitor, ritonavir, which significantly increased the oral bioavailability of docetaxel, especially when incorporated within the milk-based formulation. Therefore, Formulation C could effectively increase docetaxel oral bioavailability by the combined effects of improved solubility, inhibition of intestinal efflux transporters as well as permeability enhancing effects of milk.

Finally, it should be noted that in a relevant study in mice in which docetaxel, following the dosing of apatinib, was administered IP (five times every 4 days) and plasma samples were collected 0.5 h following the last administration, plasma concentration of docetaxel was approximately 1.5 μg/mL (30), levels that are comparable with levels generated from Formulation C in the present work. In addition, in the Feng et al., 2018 manuscript, docetaxel was efficacious in A549 xenografted mice and the IC_{50} value of docetaxel in A549 cancer cells was approximately 50 nM, suggesting that the dosing strategies proposed in the current study could potentially lead to efficacious anticancer treatment.

Further research for the optimization and better characterization of the developed milk-based formulation are certainly required. These studies should include solubility and stability studies of the water-alcohol solutions and the final milk-based formulation, optimization of the freeze-drying and reconstitution processes, as well as performance of PK studies in larger animal models or humans. In any case, this study provides the first evidence that the developed milk-based formulation could potentially serve as an alternative, non-toxic and patient-friendly carrier for an acceptable docetaxel oral chemotherapy.

CONCLUSIONS

A novel milk-based formulation was developed, which was able to enhance the oral bioavailability of docetaxel through the combined effects of improved solubility, P-gp inhibition and potential enhancement of intestinal permeability. Co-administration of the P-gp inhibitor, ritonavir, was, therefore, deemed necessary. Incorporation of ritonavir in the milk-based formulation has shown even higher bioavailability when compared with ritonavir administered as a pre-treatment. Docetaxel blood levels were also shown to remain high over a more prolonged period of time in this case, suggesting an even more favorable overall synergistic effect of the final milk-based formulation.

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