High plasma levels and effective lymphatic uptake of docetaxel in an orally available nanotransporter formulation

Taher Nassar, Suha Attili-Qadri, Oshrat Harush-Frenkel, Shimon Farber, Shimon Lecht, Philip Lazarovici and Simon Benita *

The Institute for Drug Research, The School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, POB 12065, Jerusalem, 91120, Israel

Key words: oral, docetaxel, nanocapsules, bioavailability, lympha

*Correspondence

Email: benita@cc.huji.ac.il
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Abstract

Docetaxel, an efficient chemotherapeutic drug, exhibits low and variable oral bioavailability due to the active efflux by P-gp and more so to CYP3A4 gut metabolism. Using a spray-drying technique, docetaxel was incorporated in PLGA nanocapsules (NCs) which were embedded in entero-coated microparticles. An oral administration of the NC formulation elicited a higher absolute bioavailability than both a docetaxel solution (276%) and a free docetaxel NC formulation (400%) injected intravenously, a 5mg/kg dose. The BI and BII NC formulations elicited Cmax values that were 1735 and 2254% respectively; higher than the Cmax value of the oral docetaxel solution combined with blank microparticles, a 10mg/kg dose. No significant difference in AUC was observed between the batches. These unexpected results can be explained only if the pharmacokinetics of docetaxel had been modified. It was shown that NCs released from the microparticles penetrated the enterocytes, bypassing P-gp; apparently circumventing gut metabolism and accumulating within the lymphatic system from where both intact or biodegraded NCs and free docetaxel were progressively released into the circulation as plausibly supported by the fluorescent imaging results. Furthermore, the circulating docetaxel in plasma was unencapsulated and circulated either in free form or bound to albumin. Both free docetaxel NCs and microparticles exhibited in vitro efficacy on WRC 256 cells suggesting that the activity of docetaxel was not altered. This delivery concept has potential for clinical translation, perhaps allowing docetaxel chemotherapy to be switched from intravenous to oral delivery.

Key words: oral, docetaxel, nanocapsules, bioavailability, lymph
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Introduction

Docetaxel is an anticancer drug widely prescribed for the treatment of various cancers. The efficacy of a once-weekly small dose regimen is comparable to a large dose given once every three weeks yet with a lower hematologic toxicity profile (1,2). However, docetaxel administration is associated with unpredictable and hypersensitive reactions, due in part to polysorbate 80, the nonionic surfactant needed for solubilization (3). Efforts are being dedicated to find alternative polysorbate-free formulations for oral administration (4) offering patient convenience, eliminating the risk of infection (intravenous catheters), and reducing hospital admissions. Furthermore, the oral route is more appropriate for chronic treatment regimens (5). Unfortunately, docetaxel exhibits low and variable oral bioavailability due to active P-gp efflux and CYP3A4 metabolism in the gut wall (6,7). P-gp/CYP3A4 inhibitors succeeded in improving docetaxel oral bioavailability (8,9) but their clinical applicability is problematic because their administration often induces adverse effects (10). By co-administering docetaxel with ritonavir, which has CYP3A4 and P-gp inhibiting properties (11,12), docetaxel oral absorption has been markedly improved as recently confirmed in preliminary clinical studies (13,14). However, by combining two chemical entities, physicochemical incompatibilities or formulation difficulties may arise. We present a novel oral formulation containing PLGA docetaxel NCs embedded in entero-coated bioadhesive microparticles, which significantly improves docetaxel’s oral bioavailability without affecting the physiological activity of the P-gp and CYP3A4.
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Materials and Methods

1. Materials

We purchased docetaxel from Teva, Israel (Origin Sicor, Mexico batch number 4036001308C); Poly(DL-lactide-co-glycolide) at ratio 50:50, and 4,000 MW (PLGA4K) from Lactel (Pelham, AL, USA); Hydroxypropylmethylcellulose (Methocel E4M Premium or HPMC) from Dow Chemical Company (Midland, MI, USA); Glyceryl tributyratet from Sigma-Aldrich Chemical (St. Louis, MO). Oleoyl polyoxylglycerides (Labrafil M1944 CS) and Poly(methacrylic acid, Ethyl acrylate) 1:1 (Eudragit® L100-55) were donated by Gatetfossé (St. Priest, France) and Rohm (Darmstadt, GmbH, Germany) respectively. Other chemicals and solvents were of analytical reagent grade.

2. Methods

2.1 Preparation of nanocapsules

The primary NCs were prepared by dissolving in 100 mL of acetone: 1500 mg of glyceryl tributyratet, 300 mg of Labrafil M 1944 CS, 300 mg of PLGA4K and 180 mg docetaxel. With constant stirring, 70 mL of bi-distilled water were slowly added to the organic phase. When needed fluorescent NCs were prepared using identical experimental conditions as with docetaxel but substituting PLGA4K with PLGA4K-NIR-783, the synthesis of which is described in Supplemental Data.

2.2 Microencapsulation of the nanocapsules

To prepare double-coated NC formulations, a 200 mL 5 mM sodium phosphate buffer was prepared; pH was adjusted to 6.5 by 1N NaOH solution. 750 mg of Eudragit L were dissolved in this solution maintaining pH at 6.5. 100 mL of 1% HPMC solution were added. The combined solutions were added to the NC dispersed mixture. The final
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2.3 Physicochemical characterization of nanocapsules and microcapsules

2.3.1 Docetaxel nanoencapsulation evaluation

Triplicate PLGA NC suspensions (0.5 mL) were ultrafiltered using the cell device, Nanosep; cutoff 30K, centrifugation 4000 rpm for 30 min. To estimate the amount of docetaxel in both formulations, 1 mL from each was dissolved in 1.5 mL DMSO and 1 mL methanol and stirred vigorously for 1 h, centrifuged at 4000 rpm for 5 min. 250 l of the solution or 200 l of the combined ultrafiltrates were dissolved in 1 mL of acetonitrile (ACN) and the respective concentrations of docetaxel were determined by HPLC as described below.

2.3.2 Drug content in microparticles

12 mg of the dried formulations were completely dissolved in 1 mL of DMSO. Thereafter, 50 or 100 µl of the DMSO solution were dissolved in 950 or 900 µl of ACN and this solution was injected into HPLC accordingly: Mobile phase- 65% ACN and 35%
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water; flow rate- 0.8 mL/min; wavelength- 230 nm; column- Phenomenex Luna 5µC18 (2) 100 Å 250 x 4.60 mm, 5 micron.

A calibration curve was constructed from docetaxel concentrations ranging between 0 to 100 µg/mL yielding a linear correlation ($R^2 = 0.9976$) with a limit of docetaxel detection of 0.25 µg/mL.

2.3.3 Measurement of particle size and zeta potential

We measured NCs using an ALV Non-Invasive Back Scattering High Performance Particle Sizer (Langen, Germany). Zeta potential measurements were obtained using a Zetasizer Nano-ZS (Malvern, UK).

2.4 Morphological evaluation

2.4.1 Atomic Force Microscopy (AFM) studies

Microparticles were dried overnight in a cover glass. Analysis was performed using Scanning Probe Microscope Dimension 3100, Nanoscope V (Veeco, CA, USA). The topographic and phase images were obtained simultaneously.

2.4.2 Scanning Electronic Microscopy (SEM) studies

Morphological evaluation of NCs and spray-dried microspheres was carried out using a High-Resolution Scanning Electron Microscope - Sirion (HR-SEM) (FEI Company, The Netherlands). The specimens were fixed on an SEM-stub using double-sided adhesive carbon tape or alternatively, suspensions were poured into a cover glass to evaporate the water medium. After evaporation, standard coating by Au-Pd spattering (Pilaron E5100) under vacuum made the specimen electrically conductive. To evaluate the effect of
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2.5 Animal experimentations

The animal studies were approved by the Ethical Committee of Laboratory Animal Care at The Hebrew University of Jerusalem (MD-08-11329). Sprague Dawley male rats (300-325g) were used in all studies.

2.5.1 Pharmacokinetic study

3 groups of 4 rats were randomly divided and were either injected in the jugular vein a polysorbate 80 solution of docetaxel (Taxotere®) or a dispersion of docetaxel-PLGA NCs, or orally administered docetaxel NCs embedded in microparticles a single dose of 5 mg/kg. Blood samples (300µl) from the tail were collected in heparin containing tubes at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h and were immediately centrifuged at 10,000 rpm for 5 min, after which 150 µl of plasma samples were transferred to new tubes and stored at -80°C until analyzed by LCMS/MS (Shimadzu LC-20 HPLC system coupled with a Sciex Qtrap 3200 TurboIonSpray detector in positive ionization mode).

The assay was based on protein precipitation of docetaxel and internal standard (IS = paclitaxel) using ACN (100 µL plasma sample mixed with 300 µL ACN containing IS). Separation was carried out under reverse-phase conditions using Phenomenex Synergi column (MAX-RP, 50×2 mm, 2.5 µm, 100A) in gradient mode (mobile phase A- methanol/water/formic acid 20/80/0.2 and mobile phase B-methanol/water/formic acid 80/20/0.2). Flow rate was maintained at 0.3 mL/min, and the column was maintained at
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40°C. Detection and quantitation were carried out by multiple-reaction monitoring with transitions from m/z 830.5 to 549.4 and 876.5 to 308.0 for docetaxel and IS respectively. Tested samples were quantified against a calibration curve in the range of 5-500 ng/mL. The correlation coefficient values were better than 0.990 indicating high linearity, accuracy and specificity.

2.5.2 Docetaxel absorption studies

Five groups of 4 rats were used to evaluate the oral absorption and docetaxel blood levels. They were fasted overnight with free access to water. Taxotere® (Batch D9A118, exp. 03/2012, kindly donated by Sanofi-Aventis, Israel), was administered either intravenously or orally at a dose of 10 mg/kg docetaxel (3.3 mg/rat). 83.33µl Taxotere® were diluted in sterile saline to 0.5 mL and injected into the jugular vein or dispersed in 2.5 mL DDW and delivered orally. A third group of rats was orally administered 76 mg of blank-NC formulation and 83.33 µl Taxotere® suspended in 2.5 mL DDW to determine the blank formulation’s effect on P-gp activity. Finally, 76 and 80.2 mg of docetaxel-NCs BI and BII formulation respectively were suspended in 2.5 mL DDW and delivered orally per animal. Thirteen blood samples (400 µl) were collected and treated as described above for up to 96 hours.

2.5.3. Determination of encapsulated and free docetaxel

Three fasted rats were orally administered docetaxel NCs formulation (BI) at a dose of 5 mg/kg. At time 0 (before gavage) and 3 h post administration, blood samples were collected, centrifuged and plasma fractions (500µl) were immediately injected into the gel filtration column Sepharose CL-4B (AKTApriime®, apparatus equipped with fraction collector, UV, conductivity and pH monitors; Amersham, Uppsula, Sweden) eluted at a
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A flow rate of 1mL/min of saline. The NCs and plasma protein fractions were separately collected and tested for docetaxel by LC-MS/MS following appropriate separation process validations.

### 2.5.4 Nanocapsule fluorescent labeling

Six groups of 3 rats, assigned to time intervals of 0.5, 1, 1.5, 4, 8 and 24 h were orally administered the same dose of 10 mg/kg of docetaxel incorporated in the fluorescent PLGA-NIR783 NCs embedded in the microparticles (80 mg suspended in 2.5 mL of DDW). They were shaved around the stomach area to increase NIR signal intensity and anesthetized with an injected ketamine (10%) and xylazine (2%) solution (1:9 respectively) at a dose of 1 mL/kg. They were then scanned using MousePOD small-animal adaptor (LI-COR Biosciences, Lincoln, NE, USA) mounted on top of the Odyssey instrument (15). During in vivo imaging the animal adaptor was maintained at 37°C. The animals received a constant supply of fresh air. Fluorescent imaging allowed monitoring of the biofate of fluorescent NCs in whole-animal and specific exposed organs. Before surgery, the animals were administered one dose of pentazocine (Talwin) as an analgesic. Following surgery they were immediately euthanized and tissue specimens; blood, plasma, stomach, intestine, liver, heart, kidney, mesenteric lymph node and spleen were exposed and monitored for imaging signal by the Odyssey system. Appropriate time intervals between each animal were allowed. Fluorescence value quantitation in mesenteric lymph node and plasma was carried out following preliminary calibration and fixation of the scanning parameters in terms of intensity, sensitivity and focus. The quantitation of the fluorescent images was performed using the Odyssey version 3.0 image analysis program.
2.5.5 Walker carcinoma cell line in vitro experiments

The effect of free and docetaxel-loaded NCs and respective microcapsules on Walker carcinoma cell line was examined. Walker 256 cells were obtained from the American Type Culture Collection (ATCC cat no. CCL-38) on July 2010, characterized by morphology and growth curve analysis by ATCC. The cells were seeded at $10^5$ cells/mL for 4 h in M199 complete growth medium (M199 with 5% Horse Serum and Pen-Strep solution). Docetaxel loaded in free NCs or NCs embedded in microparticles or docetaxel in solution (DMSO) were incubated for 72 h at different concentrations from 0.1 to 50 $\mu$g/mL. The concentration of blank NCs was equivalent to the concentration of NCs loaded with docetaxel. The cell viability was tested by MTT.

2.5.6 Statistical analysis

Differences were analyzed using unpaired t test with Welch correction or one-way analysis of variance (ANOVA). Analysis was determined: Tukey–Kramer multiple-comparisons test calculated by InStat software (version 3.01). The level of significance was corrected using a post-test analysis.

Results

Evaluation of microparticles embedding docetaxel NCs

The average diameter and zeta potential values of the NCs formed were 304, 293, 352 nm and -54.4 ±2.3, -60.1± 4.3, -37.7±3 mV for blank NCs and docetaxel-loaded BI and BII NCs respectively. The drug contents in the NC-loaded microparticles were 4.34±0.15 and 4.14±0.12% w/w for BI and BII respectively. The morphological properties of the NC formulations were determined by SEM and AFM techniques. In Fig. 1A most of the
Lymphatic uptake of docetaxel in an orally available nanotransporter formulation microparticles from BII can be observed deflated with sizes ranging between 2-5 µm. Furthermore, despite incubation of 1 h in pH 1.2, the microparticles essentially kept their original structure even though numerous NCs were exposed (Fig. 1B). Following incubation in pH 7.4, the microparticles lost their outer coating and no defined structures could be noted (Fig. 1C). The release of NCs, as a result of dissolution of the microparticle polymeric shells, can be seen partly embedded in matrix polymers that precipitated due to the evaporation of the aqueous medium needed for SEM evaluation. The AFM results confirmed the SEM observations since phase-image analysis showed the presence of numerous spherical NCs following incubation of 1 h at pH 7.4 (Fig. 1D). The microparticles were designed to degrade at pH above 6 since they need to release docetaxel-loaded NCs. The stability of docetaxel in the microparticles was therefore evaluated using a validated HPLC method at pH 7.4 and no degradation of docetaxel was observed over 8 hours.

**Pharmacokinetic evaluation**

Docetaxel plasma levels from 4 rats, for each formulation in both pharmacokinetic studies are presented in Fig. 2. At 24, 48 and 96 h docetaxel plasma concentrations were below the detection limit (less than 5 ng/mL), hence data are not shown. The AUC and clearance (CL) values of the docetaxel solution and injected NCs at a dose of 5mg/kg were not significantly different. The terminal ($T_{1/2}$) value of docetaxel in solution was significantly smaller than that in NCs. The oral administration of the same NCs in entero-coated microparticles elicited a higher absolute bioavailability than both the docetaxel solution (276%) and the docetaxel NCs (400%) injected intravenously, whereas the $T_{1/2}$ and CL values were significantly smaller than those of the docetaxel solution and NCs.
Lymphatic uptake of docetaxel in an orally available nanotransporter formulation (Fig. 2A and Table 1A). At a dose of 10mg/kg, Taxotere® oral solution elicited an absolute bioavailability of docetaxel of 5.5% (Table 1B) confirming results reported in the literature. Furthermore, administration of docetaxel solution with an equivalent quantity of blank microparticles elicited a $C_{max}$ value of 132.5 ±120.4ng/mL, well below those elicited by the trojan NCs formulations (2299.6 ± 615.5 and 2986.9 ± 1859.4 ng/mL).

Docetaxel NC organ distribution

To elucidate the intestinal absorption route of the NCs, fluorescent-labeled NCs were prepared and monitored by the Odyssey system. Fig. 3 shows the cumulative fluorescence in the liver and spleen over time was not substantial but reached the circulation (Fig. 4) while the fluorescence in selected organs and more particularly the calculated mean-integrated intensities of the fluorescence in the mesenteric lymph node and plasma increased with time, reached a maximum and declined significantly over 24 h (Fig. 5A & B). Fig. 4 illustrates that the circulating fluorescent NCs remained partly intact for 8 h as fluorescence was detected in the blood and plasma but not in the hematocrit suggesting that NC coatings did not biodegrade rapidly into fragments that could be adsorbed or entrapped in the erythrocytes although the quenching or elimination of the fluorescence cannot be totally excluded. Similarities in the calculated mean-integrated intensities of the fluorescence profiles suggest a relationship between lymph and plasma compartments (Fig. 5A & B).

Docetaxel NC formulation effect on Walker carcinoma cell

Fig. 6A shows that the WCR 256 viability following exposure to docetaxel NC embedded in microparticles was more affected compared to blank microparticles but less than the
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docetaxel solution, whereas Fig. 6B exhibits an increase in cytotoxicity with docetaxel-
loaded NCs compared to the docetaxel solution.

Discussion
Optimized conditions were identified for NC preparation. Nano oil droplets, surrounded
by a thin polymeric membrane (2-3 nm thick) were observed (TEM data not shown). A
good reproducibility was achieved between the batches. The difference in morphological
structures of the microspheric matrices as a function of pH is due to the aqueous-
solubility properties of the polymers. While HPMC is soluble in aqueous solution
irrespective of pH, Eudragit L dissolves only above pH 5.5. The release of the NCs
should be attributed to the massive erosion of the microparticle matrices due to complete
dissolution of HPMC and Eudragit L under pH 7.4 conditions. Furthermore, SEM
evaluation of BI microparticles yielded similar morphological results and no difference
could be detected between BI and BII.
The injected NCs, 5mg/kg dose, did not modify the in vivo PK profile of docetaxel and
suggested that the NCs either do not accumulate in specific organs or immediately release
most of the docetaxel in the plasma, exhibiting a similar profile as the docetaxel solution
(Fig. 2A). Non-significant decrease of 30% in absolute bioavailability and increase of
58% in the CL value of injected docetaxel NCs compared to docetaxel solution were
observed (Table 1A). These findings are similar to those published by Sparreboom et al,
2005 (16) who suggested that the increase in the CL value of the paclitaxel nanoparticles
should be attributed to the presence of cremophor in the Taxol formulation which formed
micelles and prevented paclitaxel from leaving circulation and entering the tissues.
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Similarly here, the presence of polysorbate 80 in the docetaxel aqueous formulation formed micelles and prevented docetaxel from leaving circulation, whereas with the NCs following the release of most of docetaxel in the plasma, the drug could circulate freely since it was not incorporated within the surfactant micelles. Notably, 5 mg/kg is the maximum dose of docetaxel in NCs for intravenous injection. At 10 mg/kg, NCs elicited marked side effects probably due to the large amount of NCs in the injected dose since the same docetaxel dose in solution was well tolerated. The docetaxel-loaded NCs embedded in the microparticles (10mg/kg) increased the docetaxel’s oral absorption tremendously compared to the polysorbate-docetaxel solution alone or combined with the blank microparticles, confirming the findings observed with the 5 mg/kg docetaxel dose (Table 1B). Although AUC values for the 10mg/kg dose are presented (Table 1B), they could not be compared to the corresponding NC injected dose. Oral Taxotere® solution elicited low absolute bioavailability confirming that the colocalization of P-gp with CYP3A in the villous tips of the enterocytes provides a highly effective barrier against systemic absorption of P-gp substrate drugs including docetaxel (17,18). Thus, docetaxel is only administered intravenously in the clinic and is metabolized by CYP3A to several metabolites that are all considered to be therapeutically less effective (19, 20). Although a 6-fold difference in C_{max} and more than a 2-fold effect on AUC values were noted in the presence of blank formulation compared to those elicited by the docetaxel-polysorbate solution alone, these values were not statistically different. Nevertheless, the increase in C_{max} values noted in the presence of blank formulation should be attributed to the presence of numerous blank lipophilic oil cores that enhance docetaxel solubility in the aqueous environment rather than to the inhibition
Lymphatic uptake of docetaxel in an orally available nanotransporter formulation of the P-gp which leads ultimately to significantly smaller values than those observed when ritonavir or cyclosporine were used to inhibit the pump (8, 11, 21). There are other pharmaceutical approaches being used to increase docetaxel oral absorption. $C_{\text{max}}$ and AUC values achieved by orally administering docetaxel microemulsions to SD rats (10 mg/kg) were much smaller than those achieved in the present study (22). Furthermore, nanoparticles (NPs) prepared with a polymer which inhibits P-gp efflux to increase oral docetaxel absorption, achieved a 26.4-times longer half-life than intravenous administration of Taxotere® at the same dose. One orally-administered dose of the NP formulation sustained docetaxel levels for almost three weeks compared to 22 h of intravenous administration of Taxotere®. Oral bioavailability was enhanced from 3.59 to 78% (23). In our case, we found no trace of docetaxel in the blood after 24 h. Thus, the biofate of the docetaxel NPs remains unclear since the authors didn’t explain how their NPs elicited docetaxel levels for so long (23).

The BI and BII formulations elicited $C_{\text{max}}$ values that were 1735 and 2254% respectively higher than the $C_{\text{max}}$ value of the oral docetaxel solution combined with blank microparticles (Table 1B). If we assume, excluding side effects, that the injected 10 mg/kg corresponding docetaxel NCs exhibit a similar pharmacokinetic profile as the 5 mg/kg dose, then the extrapolated absolute bioavailability values of BI and BII were 665 and 870% respectively compared to the docetaxel injected solution. These deductions should be carefully considered since they’re based on assumptions which need to be verified. Statistical analysis of the data indicates that no significant difference was apparent between the NC formulation batches. Although the %CV in AUC of Batch II is high (around 50%), this variability is probably due to the intrinsic batch properties and
Lymphatic uptake of docetaxel in an orally available nanotransporter formulation should not yet represent any concern. It should be emphasized that the %CV in C_max was 30%. We did not observe the same variability with Batch I. These deviations can be considered reasonable taking into consideration that laboratory formulation process parameters are better controlled and markedly improved during scaling up and validation phases. Nevertheless, these observations suggest that the positive results achieved are formulation related and merit further improvement and optimization. Additional experimentation is warranted prior to making any firm conclusions.

The unexpected enhanced absolute bioavailability achieved with NC formulations at 5 and possibly 10 mg/kg of docetaxel can be explained if the PK had been modified. Figs. 3 and 4 demonstrate that NCs penetrated the enterocytes and entered circulation, bypassing the normal pathway (lymphatic system), subsequently protecting the docetaxel from CYP enterocyte metabolism and systemic degradation.

It apparently could be deduced from the fluorescent-labeled NC monitoring that NCs accumulated within the lymphatic system from where both intact or biodegraded NCs and free docetaxel were progressively released into the central compartment (Fig. 3 and 4). Figs. 5C and D support these findings: Fig. 5C shows NCs separated from plasma protein fractions even if a small peak is observed in the free docetaxel plasma fraction probably associated with the presence of chylomicrons; Fig. 5D indicates that practically all plasma-circulating docetaxel is not nanoencapsulated (1109 versus 5 ng/mL). Unsurprisingly, the docetaxel cannot be retained in the NCs when circulating in the plasma under infinite dilution since drugs incorporated within NCs partition rapidly in favor of the releasing medium when sink conditions prevail (24). This is further supported by the extensive binding of docetaxel to plasma albumin (25).
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The higher T₁/₂ value noted with the NCs as compared to the docetaxel solution (5mg/kg) can be explained by the residual NC docetaxel content of 5 ng/mL as it prolonged the residence time in circulation (Fig. 5D). 10 mg/kg dose data should be taken with precaution because it can be noted that the behaviors between the 5 and 10 mg/kg doses differ (Fig. 2B), probably because of the nonlinearity of docetaxel pharmacokinetic properties. The rapid decline from 1000 to 10 ng/mL, within 4 h (Fig. 2B), should be attributed to the complete depletion of the reservoir effect in the lymphatic system. Furthermore, the docetaxel circulating in plasma was unencapsulated and circulated freely or bound to albumin (Fig. 5C & D). Thus, the terminal phase represents the rapid degradation/elimination of docetaxel in the plasma and differs from Taxotere® since it is unprotected by the polysorbate micelles as was Taxotere® (25).

Clearance results also support these conclusions. Intravenously-injected Taxotere® elicited CL values of 5.11±0.75 L/h.kg close to those of van Waterschoot et al, (7) while the BI and BII NC formulations exhibited values of 0.59±0.23 and 0.67±0.25 L/h.kg respectively, showing that docetaxel elimination was markedly reduced because of the formation of an additional compartment in the lymph system that progressively released encapsulated or free docetaxel into plasma protecting the drug from CYP4A3 degradation effect. Docetaxel and NC release from the lymph compartment was not markedly retarded since most of the docetaxel was eliminated within less than 12 h. This kinetic deduction is also supported by the fact that NPs of 150-300 nm could not be eliminated or filtered intact by the kidneys and consequently their elimination process should be different from the docetaxel-elimination process (26). Thus, for docetaxel to be eliminated within 12 h, implies that the drug was released from the NCs in the plasma as
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shown here. The presented data are indirectly confirmed by van Waterschoot et al, (7) who used a transgenic mice model lacking CYP3A and P-gp genes. When orally challenged with docetaxel, a disproportionate (>70-fold) increase in systemic exposure was observed compared with the drug increases in single CYP3A−/− (12-fold) or Mdr1a/1b−/− (3-fold) mice, whereas in the present study the trojan NCs increased the docetaxel systemic exposure by more than 276-400%. The marked docetaxel enhancement exposure by the NC formulation suggests that NCs both circumvented P-gp and protected the drug from CYP3A intestinal and systemic metabolism. Despite exposure to high docetaxel blood levels following oral administration the rats showed no side effects; neither losing weight nor developing any visual lesions. We hypothesize that the NCs were internalized intact in the enterocytes through an endocytosis process (27), enhancing docetaxel absorption and preventing damage to mucosal tissue that could be elicited by high free-docetaxel molecule concentration.

With these findings, the question of docetaxel's cytotoxicity on Walker 256 carcinoma cell line was addressed. Fig. 6A illustrates that the control microparticles elicited toxic effects on the cells because of HPMC and Eudragit L that are not approved for injection. However, the nanocapsulated docetaxel microparticles exhibited more pronounced cytotoxic effects than blank microparticles, at least up to 5 µg/mL, showing additional intrinsic cytotoxic activity due to the presence and release of docetaxel. These results indicate that docetaxel activity does not change following nanoencapsulation and embedding in the microparticles. It should be emphasized that microparticles are not expected to come in contact with cancerous cells since only the docetaxel NCs should penetrate the enterocytes, reach the systemic circulation through the lymphatic system.
Lymphatic uptake of docetaxel in an orally available nanotransporter formulation and encounter them. Thus, to mimic normal physiological conditions expected following treatment, the effect of docetaxel-loaded NCs on Walker 256 was evaluated (Fig. 6B). Cytotoxicity increased notably with docetaxel-loaded NCs compared to the docetaxel solution, whereas the respective blank NCs elicited a moderate lethal effect at 5 µg/mL equivalent concentration (Fig. 6B); suggesting that the cytotoxic effect induced by blank NCs is probably due to their penetration into the cells by an endocytosis-mediated process. These deductions are further supported by the enhanced effect of the docetaxel-loaded NCs, with an IC_{50} improved value of 70% (4µg/mL instead of 6.8µg/mL) confirming the marked toxicity of the docetaxel NCs.

In conclusion, the significant improvement in docetaxel oral absorption should be attributed to the novel nanoplatform. This novel nanocarrier apparently behaves as a nanotransporter without markedly affecting the physiological activity of P-gp since the NCs penetrated the enterocytes and masked docetaxel which reached circulation. The lymphatic route is involved in NC absorption therefore circumventing the first-pass effect and progressively releasing the encapsulated or free docetaxel into the plasma protecting the drug from CYP4A3 degradation and retarding docetaxel elimination. It was further shown that by incorporating docetaxel into the innovative formulation its intrinsic activity remained unaltered since both NCs and microparticles exhibited in vitro efficacy on WRC 256 cells. The efficacy of the orally-administered NCs formulations in different animal models is being addressed and is currently under investigation.
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Legends

**Figure 1**: SEM visualization of docetaxel nanocapsule formulation (BII) at different magnifications and pH following 1 h incubation, (A) dry formulation; (B) pH=1.2; (C) pH=7.4 and (D) AFM, topography and phase-image visualization at pH=7.4.

**Figure 2**: Mean plasma docetaxel concentration–time profiles (A) following intravenous jugular administration of Taxotere® and docetaxel nanocapsules or oral administration of docetaxel nanocapsule formulation (BI) at the dose of 5 mg/kg; (B) following intravenous jugular administration of Taxotere® or oral administration of docetaxel solution with and without blank nanocapsule formulation, or BI and BII nanocapsule formulation at a dose of 10 mg/kg docetaxel in fasted rats; (N= 4).

**Figure 3**: Fluorescent images of various isolated rat organs at given time intervals following oral administration of NIR-PLGA nanocapsule formulation (80 mg/kg). Scanning was performed using a NIR Odyssey (LI-COR) system.

**Figure 4**: Fluorescent images of plasma; hematocrit and whole blood at given time intervals following oral administration of NIR-PLGA nanocapsule formulation (80 mg/kg). Scanning was performed using a NIR Odyssey (LI-COR) system.

**Figure 5**: Fluorescence-integrated intensity, as measured by an Odyssey system, of rat’s mesenteric lymph node (A) and plasma (B) over time following oral administration of NIR-PLGA nanocapsule formulation (80 mg/kg). Free plasma (C) and circulating docetaxel plasma (D) collected three hours after oral administration of docetaxel nanocapsule formulation (5 mg/kg) in 3 rats following size exclusion chromatography using sepharose CL-4B column.

**Figure 6**: The effect of docetaxel in DMSO solution on the viability of WCR 256 cells and blank microparticle formulations (A) following first incubation over 3 h in the cell growth medium and then added to the cell culture and blank and docetaxel nanocapsules (B) following 72 h incubation.
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Lymphatic uptake of docetaxel in an orally available nanotransporter formulation


Acknowledgements:
This research is supported in part by the Israel Science Foundation (grant 576/08)
Lymphatic uptake of docetaxel in an orally available nanotransporter formulation

Table 1: (A) Average pharmacokinetic parameter values (mean±SD) following iv injection (jugular) of docetaxel in marketed diluted solution (Taxotere®) and docetaxel-loaded nanocapsules (NCs) or oral administration of docetaxel-loaded NCs embedded in microparticles at the same dose of 5 mg/kg, N=4.
(B) Average pharmacokinetic parameter values (mean±SD) following iv injection of 10 mg/kg of docetaxel in marketed diluted solution (Taxotere®) or following oral administration in either aqueous diluted solution or combined with blank NCs embedded in microparticles at a dose of 250 mg/kg or docetaxel-loaded NCs embedded in microparticles (BI and BII), N=4.

<table>
<thead>
<tr>
<th>(A) IV pharmacokinetic study</th>
<th>C_{max}, ng/mL</th>
<th>AUC, h. ng/mL</th>
<th>T_{1/2}, h</th>
<th>CL, L/h/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Docetaxel solution (iv)</td>
<td>-</td>
<td>2080±81.2</td>
<td>2.9±0.4</td>
<td>2.4±0.1</td>
</tr>
<tr>
<td>2. Docetaxel NCs (iv)</td>
<td>-</td>
<td>1441.9±485.6</td>
<td>4.7±0.1</td>
<td>3.8±1.3</td>
</tr>
<tr>
<td>3. Docetaxel-PLGA 4K double-coated NCs, BI (oral)</td>
<td>882.6±111.9</td>
<td>5754.5±1338.8</td>
<td>1.9±0.7</td>
<td>0.9±0.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(B) Oral absorption study</th>
<th>C_{max}, ng/mL</th>
<th>AUC, h. ng/mL</th>
<th>T_{1/2}, h</th>
<th>CL, L/h/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Docetaxel solution (iv)</td>
<td>-</td>
<td>1988.3±307.9</td>
<td>5.4±1.2</td>
<td>5.1±0.7</td>
</tr>
<tr>
<td>2. Docetaxel solution (oral)</td>
<td>19.5±15.4</td>
<td>109.5±86.3</td>
<td>2.9±2.2</td>
<td>76.5±63.2</td>
</tr>
<tr>
<td>3. Docetaxel solution + blank microparticles (oral)</td>
<td>132.5±120.4</td>
<td>232.8±102.4</td>
<td>4.4±1.9</td>
<td>37.1±16.5</td>
</tr>
<tr>
<td>4. Docetaxel-PLGA 4K double-coated NCs, BI (oral)</td>
<td>2299.6±615.5</td>
<td>13236.5±1702.6</td>
<td>0.7±0.04</td>
<td>0.59±0.23</td>
</tr>
<tr>
<td>5. Docetaxel-PLGA 4K double-coated NCs, BII (oral)</td>
<td>2986.9±1859.4</td>
<td>17280.3±8438.1</td>
<td>0.6±0.02</td>
<td>0.67±0.25</td>
</tr>
</tbody>
</table>

Table 1A: AUC and CL of 1 vs 2 p>0.05 ns
AUC of 1 vs 3 p<0.01 significantly (*)
AUC of 2 vs 3 p<0.001 (**)CL of 1 vs 3 p>0.05 ns
CL of 2 vs 3 p<0.01 significantly (*)

Table 1B: C_{max} of 2 not significantly different from 3
C_{max} and CL of 4 & 5 significantly higher and smaller respectively than C_{max} and CL of 2 & 3 (p<0.001).C_{max} and CL of 4 not significantly different from 5
Figure 1
docetaxel NCs 5 mg/kg iv
Taxotere 5 mg/kg iv
PLGA
Kf orm - BI
5 mg/kg PO

Figure 2A

Figure 2B
Figure 3
Figure 4
Figure 5
Figure 6
High plasma levels and effective lymphatic uptake of docetaxel in an orally available nanotransporter formulation

Taher Nassar, Suha Attili Qadri, Oshrat Harush- Frenkel, et al.

Cancer Res  Published OnlineFirst March 1, 2011.

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