

# Improvement of Biodistribution with PEGylated Liposomes Containing Docetaxel with Degradable Starch Microspheres for Hepatic Arterial Infusion in the Treatment of Liver Metastases: A Study in CC-531 Liver Tumor-bearing WAG RIJ Rats

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**Abstract.** *Aim: To improve the drug concentration in liver metastases, docetaxel was encapsulated in polyethyleneglycol-liposomes and administered regionally with degradable starch microspheres (DSM). Materials and Methods: A rodent model of solitary metastasis (CC-531 adenocarcinoma) was studied. The animals were randomized into six groups and treated with 15 ng/kg docetaxel: I: intravenous (i.v.). II: PEG-liposomes i.v.; III: intraarterial (i.a.) via the hepatica artery; IV: i.a.) + DSM; V: PEG-liposomes i.a.; and VI: PEG-liposomes i.a. + DSM. The docetaxel concentration in the serum, liver and liver tumor at defined times (5, 15, 30, 60, 120, 240 min and 24 h) was measured using HPLC. Results: The area under the concentration (AUC) versus time curves showed an 11-fold higher concentration in the tumor tissue when comparing the docetaxel-PEG-liposomes i.a. + DSM group to the i.v. group ( $p < 0.01$ ). Conclusion: Compared to intravenous therapy, i.a. therapy with docetaxel-PEG-liposomes + DSM results in higher tumor tissue concentrations.*

Gastric cancer with hepatic metastases continues to be a difficult disease to manage, and there is no effective treatment available at this time. The prognosis of these patients continues to be very poor (1).

Unfortunately, the liver is the organ that is most frequently involved in the hematogenous dissemination of gastric cancer (2). Moreover, 50% of patients with breast cancer develop distant metastatic disease (3-5) and 20-30% of patients with

metastatic breast cancer develop liver metastases and have a poor prognosis, with a median survival of 1-2 years and a 5-year survival rate of approximately 20% (6, 7).

The most frequently applied chemotherapeutic agent for advanced breast cancer, ovarian cancer and gastric cancer is docetaxel (8-10).

Docetaxel inhibits cell proliferation by inducing a sustained mitotic block at the metaphase/anaphase anticancer agents in this class promote the polymerization of stable microtubules, inhibit their disassembly and profoundly affect a number of key cellular functions that depend on the turnover of tubulin. *In vitro* docetaxel is an inhibitor of microtubule depolymerization (11-15).

For advanced carcinomas with liver metastases, regional chemotherapy with intraarterial (i.a.) administration of the cytostatic agent into the target region is a promising approach. Additionally, the administration of degradable starch microspheres (DSM) slows down the blood flow in the unaffected residual liver in favor of the liver tumor and the reduced blood flow rate is accompanied by a concomitant increase of the contact time of the cytostatic agent with the tumor (16, 17).

A further increase of the cytostatic agent concentration can be achieved by applying liposomes as a drug carrier (18, 19). Liposome-encapsulated cytostatic agents have been shown to be therapeutically more effective in tumors, since they are able to overcome both systemic toxicity and drug resistance (20-22). Furthermore, a number of authors (23-25), including ourselves (26), have shown that liposome-encapsulated cytostatic agents change the pharmacokinetic behavior and accumulation of the active substance in the tumor and influence the dose-limiting toxicity.

Liposomes are lipid vesicles formed from natural and synthetic phospholipids of different size, load and composition (27). They are defined as vesicular structures consisting mainly of amphiphilic, biologically degradable

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phospholipids and can thus encapsulate both water-soluble and lipid-soluble effective agents. A greater or lower affinity for the reticuloendothelial system (RES) can be observed depending on the size, composition, fluidity and load of liposomes. Small unilamellar vesicles (SUV), reversed-phase-evaporated vesicles (REV) and multilamellar vesicles (MLV) liposomes are used for cytostatic agent encapsulation. However, Papahadjopoulos and Allen have demonstrated that modifying the SUV liposome membrane by adding polyethylene glycol (PEG) markedly reduces the interaction of the vesicles with the stationary macrophages in the liver and spleen after *i.v.* application (28). This increased the circulation half-time of the so-called 'stealth liposomes'. When superparamagnetic iron oxide particles were enclosed in PEG-modified liposomes as contrast medium, and accumulation in the tumor was examined by magnetic resonance imaging (MRI), the best tumor accumulation was achieved with SUV-PEG liposomes (26). This liposome preparation was used for the following experiments.

The docetaxel concentration in various tissues using SUV-PEG docetaxel liposomes was compared to that using non-encapsulated docetaxel in systemic and regional administrations with and without DSM.

## Materials and Methods

**Experimental animals.** The experimental animals were 270 WAG-RIJ (Wistar Albino Glaxo/Rij) rats (breeder: Charles Ribber, Extertal, Germany). The animals were 80-125 days old and weighed 180-250 g. The animals were housed individually in rooms maintained at  $21 \pm 1^\circ\text{C}$  with a 12-hour dark/light cycle. They were fed a standard rat chow with free access to water. Care was provided in accordance with the national guidelines for the care and use of laboratory animals. The study was approved by the local Ethics Committee.

**Tumor cell preparation.** The tumor cell line CC-531 is a moderately differentiated adenocarcinoma originating from the colon of rats exposed to methylazoxymethanol. The cells were obtained from the German Cancer Research Center (DKFZ), Heidelberg, Germany. The tumor cells were cultivated at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$  in an incubator in 20 ml complete medium, RPMI-1640 (Gibco, Life Technologies, Eggenstein, Germany), 10% fetal calf serum (Seromed, Biochrom, Berlin, Germany), and 1% penicillin/streptomycin (Seromed). An *in vitro* study was performed to examine the effectiveness of docetaxel at inhibiting the growth of CC531 cancer cells using the MTT assay.

**MTT assay.** Logarithmically growing CC-531 cells were trypsinised and 100  $\mu\text{l}$  cell suspensions containing 3,000 cells were seeded in 96-well plates. After 1 day, the cells were exposed to different concentrations of docetaxel in 100  $\mu\text{l}$  medium. Docetaxel (Taxotere; Sanofi-Aventis, Frankfurt/Main, Germany) was added to the CC-531 cells at 0, 3, 30 or 300  $\mu\text{g/ml}$ . The effect on cell growth was measured at 48 and 72 h after addition of the drug using the 3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide (MTT) assay. With this test, viable cells were detected by incubation with MTT,

with formazan crystals forming in the tumor cells that had survived. Ten microlitres of MTT were added to the cells and incubated for 3 h at  $37^\circ\text{C}$ ; the medium containing the drug and MTT was then discarded. The cells were lysed and the formazan crystals dissolved by the addition of acidified 2-propanol (0.04 N HCl). Absorption was measured at 540 nm with a reference filter of 690 nm in an automated microtiter plates spectrophotometer. The absorption was expressed as a percentage of that of the untreated control cells.

**Tumor induction.** After 3 days' culture, the CC-531 cells were washed twice with PBS and were detached with 1 ml trypsin. The trypsin was deactivated by adding 5 ml complete medium. After centrifugation, washing and resuspension with PBS, viability was evaluated in a Bürker hematocytometer after the addition of trypan blue and the suspension was adjusted to 98% viability with a density of  $2 \times 10^6$  viable cells/100  $\mu\text{l}$  suspension by recentrifugation and resuspension.

Surgical anesthesia of the rats was induced with vaporized ether followed by intramuscular injection of pentobarbital at 20 mg/kg (Nembutal®, Pharmazeutische Handelsgesellschaft, Garbsen, Germany) followed by intramuscular administration of ketamine at 40 mg/kg (Ketanest®, Parke Davis & Company, Berlin, Germany). The tumor cells were injected into the left liver lobe.

**MR imaging.** The tumor size and position were determined by MRI using a 1.5 Tesla Magnetom (Siemens Co.). A T1-weighted spin-echo sequence was used with a slice thickness of 5 mm, a repetition time of 350 ms, an echo time of 15 min and a total measuring time of 3 min.

**Docetaxel SUV-PEG liposomes.** Docetaxel was encapsulated in SUV-PEG liposomes composed of hydrated soy phosphatidylcholine (HSCP, 50 mg/ml; Nattermann Phospholipid GmbH, Cologne, Germany), cholesterol (CH, 24.8 mg/ml; Merck, Darmstadt, Germany), dicetylphosphate (DCP) (Serva, Heidelberg, Germany) and polyethylene glycol (MPEG-DSPE 3000, 5.4 mg/ml; Sygena, Liestal, Switzerland), molecular ratio (1:1:0.1:0:1). The lipids were dissolved in chloroform (in a round-bottom flask) and subsequently a lipid film was created by evaporating the solvent under vacuum (rotation evaporator). The lipid film was dispersed at room temperature by adding docetaxel (20 mg) dissolved in phosphate-buffered saline (PBS), pH 7.4, and by subsequent shaking as described in earlier work (24). Subsequent intermittent application of ultrasound (10x4 min) to the multilayer liposome suspension led to the development of SUV. Separation of the non-encapsulated docetaxel component was dispensed with in this experimental approach and the cytostatic agent concentration was determined by HPLC. The size of the vesicles was determined by quasi-elastic light scattering in a Coulter counter N 4MD (Coulter Electronics, Hialeah, FL, USA). The liposomes measured  $113 \text{ nm} \pm 36 \text{ nm}$ .

**Experimental treatments.** The animals were randomized into the experimental groups when the tumors reached a size of 0.8-1.3 cm. The treatments are detailed in Table I. In all the rats undergoing systemic treatment (groups I and II), the cytostatic agent was applied *via* a vein. Rats randomized into the regional treatment groups were submitted to general anesthesia with Rompun® and Ketanest® for the implantation of a port system (Intraport, Braun-Melsungen, Germany) into the hepatic artery *via* the gastroduodenal artery. In groups I-VI, 5 animals each were killed 15, 30, 60, 90, 120, 240 minutes and 24 hours after therapy was started, and the docetaxel concentrations in the different organs were determined by HPLC.

Table I. *Experimental treatments.*

Group I	(n=45): 15 mg/kg docetaxel, systemic ( <i>i.v.</i> )
Group II	(n=45): 15 mg/kg docetaxel-PEG liposomes, systemic ( <i>i.v.</i> )
Group III	(n=45): 15 mg/kg docetaxel, regional HAI
Group IV	(n=45): 15 mg/kg docetaxel plus DSM, regional HAI
Group V	(n=45): 15 mg/kg docetaxel-PEG liposomes, regional HAI
Group VI	(n=45): 15 mg/kg docetaxel-PEG liposomes plus DSM, regional HAI

HAI; hepatic arterial infusion. (*i.a.*); DSM; degradable starch microspheres (Spherex® PharmaCept Berlin, Germany).

**HPLC.** The determination of in the docetaxel concentration various samples was carried out using a modification of the HPLC procedures described by Garg and Ackland (29). The HPLC system consisted of an Agilent series 1100 Chemstation and Agilent 1100 VWD absorbance detector. Sample separation was performed on a Phenomenex LUNA C18 column (5 m, 250 mm×4.6 mm) (Phenomenex, USA) with a guard column (Phenomenex C18, 4.0 mm×3.0 mm). The mobile phase consisting of acetonitrile–methanol–ammonium acetate buffer (pH 5; 0.02 M) (20:47.5:32.5, v/v/v) was prepared daily and degassed by sonication in an ultrasonic bath before use. The column temperature was 30°C and the flow rate was 1 ml/min. The UV absorbance of the sample (20 µl) was measured at the wavelength of 230 nm. Data collection and processing were performed using Agilent series 1100 Chemstation software.

**Statistics.** The mean values±SD of the docetaxel concentration were calculated for each group. The differences in the docetaxel concentration between the groups were investigated using the global Kruskal-Wallis test. The *p*-values were adjusted for multiple comparison according to Bonferroni. A probability value of <0.05 was considered significant.

## Results

The *in vitro* study demonstrated that docetaxel was effective against the CC-531 cells (Figure 1).

All four regional treatment groups (III, IV, V, VI) demonstrated significantly higher tumor docetaxel concentrations (*p*<0.01) than the systemic treatment groups (I, II) (Table II, Figure 3). After administering 15 mg/kg of docetaxel *i.v.*, the area under the curve (AUC) measured at the time-points from 15 min–24 h was 11.5 µg/g. In comparison to group I, the docetaxel concentration (AUC 15 min - 24 h) increased 3.5-fold after *i.v.* administration of docetaxel-PEG liposomes (40.3 µg/g); *i.a.* administration of docetaxel increased the application 11-fold to 125.65 µg/g; a further 17-fold increase in concentration was seen after *i.a.* administration of docetaxel combined with DSM (198.2 µg/g) and after *i.a.* administration of docetaxel-PEG liposomes, the concentration increased 26-fold to 299 µg/g. The highest concentrations were measured after administering docetaxel-PEG liposomes combined with DSM (38-fold increase to 437 µg/g). Docetaxel was still detected in the tumor tissue 24 h after administration in treatment groups V and VI (AUC 15

min–24 h) (Table II and Figure 6). These differences were significant (*p*<0.01). The following concentration increases were observed when comparing the docetaxel concentrations in the liver parenchyma with that of group I (18.9 µg/g): 1.8-fold with docetaxel-PEG liposomes *i.v.* (33.2 µg/g); 10-fold with docetaxel *i.a.* (188 µg/g); 8-fold with 5-docetaxel/DSM *i.a.* (144 µg/g); 13-fold with docetaxel-PEG liposomes *i.a.* (250 µg/g); 11-fold with docetaxel-PEG liposomes/DSM *i.a.* (208 µg/g). These differences were significant (*p*<0.01) (Table II and Figure 4).

Liposome encapsulation and DSM changed the pharmacokinetics of docetaxel. A prolonged higher concentration of docetaxel was demonstrated in the groups treated with docetaxel liposomes and in combination with DSM (Figures 3, 5 and 6).

## Discussion

The use of CC-531 colon carcinoma cells in tumor metastasis models has been evaluated in several studies (30, 31). Although docetaxel is a commonly used in the treatment of advanced ovarian and breast cancer with liver metastases, it is not used very often against liver metastases from colorectal cancer. This may be because it has low hepatic extraction compared to floxuridine (95-99% for floxuridine compared to 30-40% for docetaxel) (32).

However the *in vitro* evaluation of docetaxel on the CC-531 cells (originating from the colon) showed that the drug was effective.

High cytostatic agent concentrations in the tumor tissue are known to be the key to effective therapy. Some authors assume that increasing the concentrations of active substances counteracts resistance. A concentration increase by a factor of 10 resulted in doubling of the response rate (33).

In the present study, compared to *i.v.* non-encapsulated administration, the docetaxel tumor concentration increased 3.5-fold after *i.v.* administration of docetaxel-PEG liposomes and 17-fold after *i.a.* administration of the non-encapsulated agent combined with DSM, while *i.a.* application of combined docetaxel-PEG liposomes and DSM led to a 38-fold increase of the AUC. Thus the regional *i.a.* administration led to pronounced accumulation of cytostatic agent in the tumor.

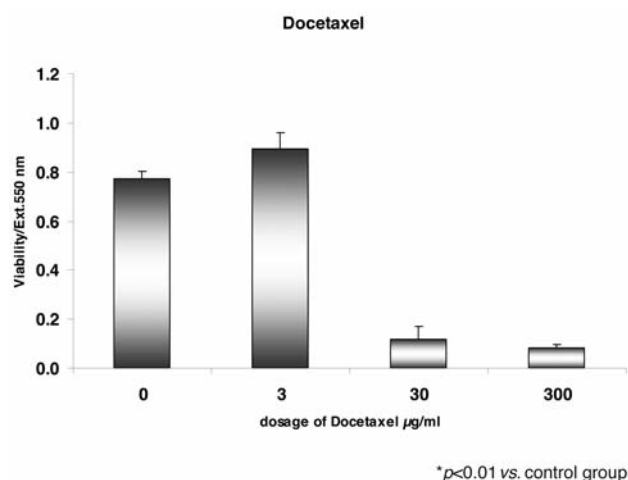


Figure 1. Viability of CC-531 cells after 72 h treatment with docetaxel.

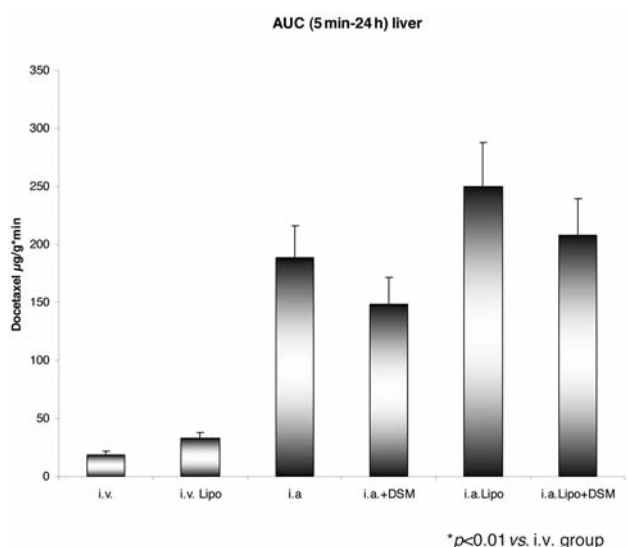


Figure 2. Liver parenchymal concentration (AUC) of docetaxel in the different therapy groups. Lipo, docetaxel-PEG liposomes; DSM, degradable starch microspheres.

An added advantage of regional application has been achieved by reducing the blood flow, for approximately 20 min with DSM (34, 35). Furthermore, the DSM had a target effect on the tumor.

Additionally, clinical studies with PEG (stealth) liposomes have reported reduced toxicity with prolongation of the plasma half-life with liposome-encapsulated cisplatin, doxorubicin and docetaxel (36, 37). This was also observed in the present experiments, but was considerably increased by DSM. Markedly increased tumor concentrations, 3- to 20-fold compared to *i.v.* administration of the non-encapsulated

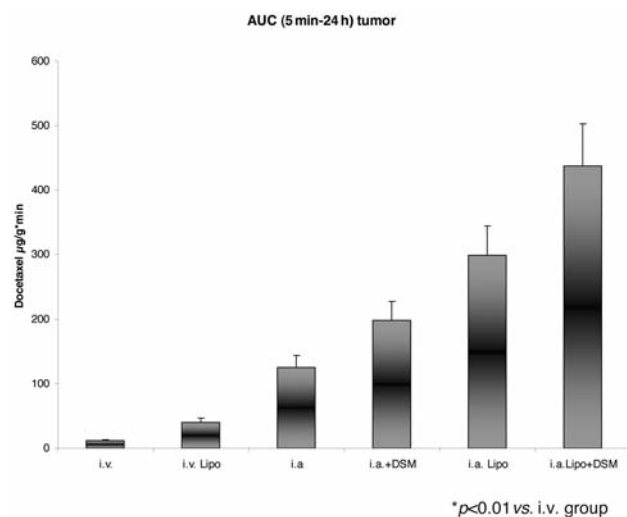


Figure 3. Docetaxel tumor concentration (AUC) in the different therapy groups. Lipo, docetaxel-PEG liposomes; DSM, degradable starch microspheres.

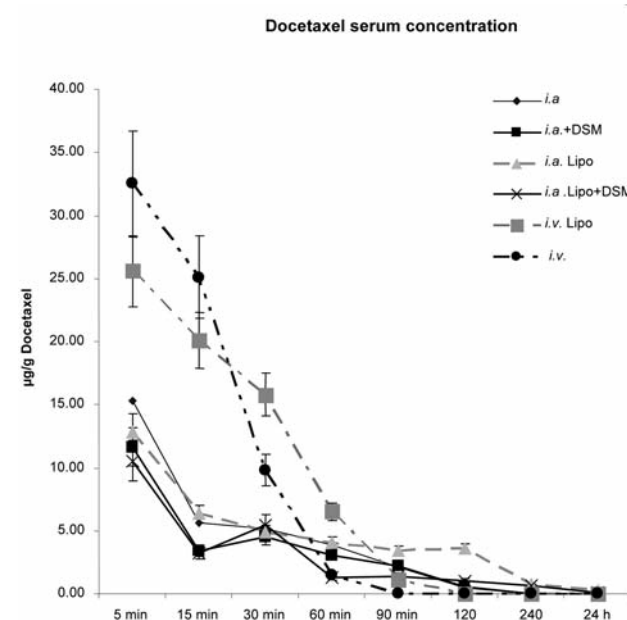


Figure 4. Concentration time course in serum after application of 15 mg/kg of docetaxel in the 6 groups.

agent, have been reported after *i.v.* administration of PEG-cytostatic liposomes in experimental and clinical pharmacokinetic studies (38, 39).

This was in agreement with the present results where the tumor concentration increased 3.5-fold after the *i.v.* administration of liposomal docetaxel compared to the *i.v.* administration of the non-encapsulated agent. The relatively selective tumor accumulation might be explained by the



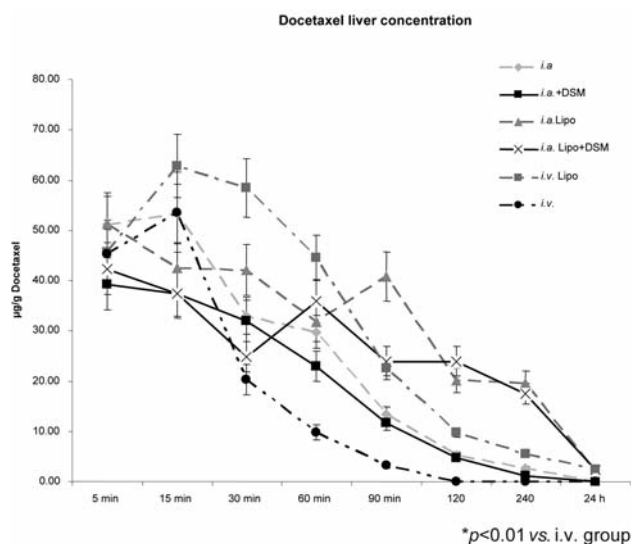


Figure 5. Concentration time course in the liver after application of 15 mg/kg of docetaxel in the 6 groups.

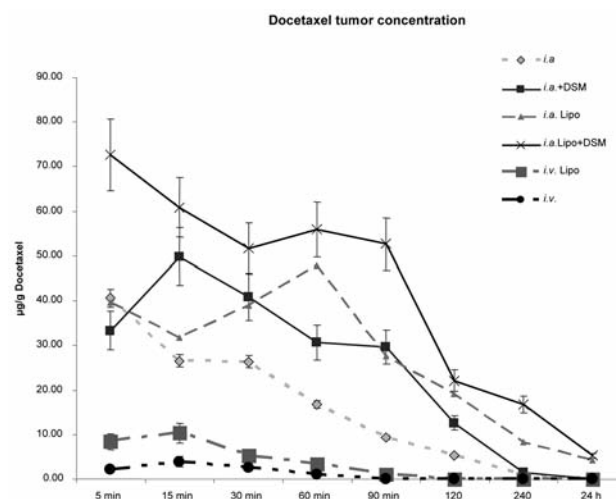


Figure 6. Concentration time course in the tumor after application of 15 mg/kg of docetaxel in the 6 groups.

enhanced permeability retention effect, since tumor vessels have defects with endothelial gaps of up to 100 nm. Globulin and vesicular structures can accumulate in such gaps. These polymeric conjugates release their drugs intracellularly *via* endocytosis (40, 41). Intravital microscopic examinations have supported this hypothesis. According to these examinations, PEG liposomes accumulate in the tumor interstitium and move in an intracellular direction due to increased vascular permeability (42, 43)

The affinity of liposomes to RES organs might explain the high concentrations in the liver parenchyma when applying liposomal docetaxel (44, 45).

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