Hepatic Arterial Infusion in the Treatment of Liver Metastases with PEG Liposomes in Combination with Degradable Starch Microspheres (DSM) Increases Tumor 5-FU Concentration. An Animal Study in CC-531 Liver Tumor-bearing Rats

U. POHLEN, R. RESZKA*, H.J. BUHR and G. BERGER

Department of Surgery, Benjamin Franklin Campus, Charité-Universitätsmedizin Berlin, Germany

Abstract. *The regional application of cytostatics in liver metastases leads to increased concentrations in the tumor tissue. The effect of flow retardation by temporary occlusion and drug targeting with liposome encapsulation (PEG liposomes) on tumor 5-fluorouracil (5-FU) concentrations was investigated. Materials and Methods: Tumor-bearing rats were submitted to i.v. or intraarterial (i.a.) therapy with liposome-encapsulated or non-encapsulated 5-FU. The i.a. groups were additionally treated with or without Spherex® degradable starch microspheres (DSM). The tumor 5-FU concentrations were determined by high-performance liquid chromatography (HPLC) as area under the curve (AUC). Results: A comparison with i.v. in administered 5-FU yielded the following increases tumor concentrations: 5-FU-PEG liposomes i.v. 27-fold, 5-FU i.a. 19-fold, 5-FU i.a. + DSM 1760-fold, 5-FU-PEG liposomes i.a. 110-fold, 5-FU-PEG liposomes i.a. + DSM 7665-fold. Conculsion: Liver intratumoral 5-FU concentration increases to >7,500 times that following i.v. administration by a combination of regional administration via the hepatic artery with temporary embolization by DSM and drug targeting by liposome-encapsulated 5-FU.*

The level of therapeutic agent in tumor tissue is the decisive parameter for successful chemotherapy (1, 2). The response has been shown to double when the tumor drug concentration is increased by a factor of 10 (3). A promising approach is regional chemotherapy with intraarterial (*i.a.*) administration of the cytostatic agent into the target region. The administration of degradable starch microspheres (DSM) has been shown to slow down the blood flow in the unaffected residual liver in favor of the liver tumor. Moreover, the blood flow rate reduction was accompanied by a concomitant increase of the cytostatic agent tumor contact time (4, 5). An increase of the cytostatic agent concentration was also achieved by using liposomes as a drug carrier (6, 7). Liposome-encapsulated cytostatic agents were shown to be therapeutically more effective in experimental tumors, since they were able to overcome both systemic toxicity and drug resistance (8-10). Furthermore, a number of authors (11-13) including ourselves (14) 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 (15). They are defined as vesicular structures consisting mainly of amphiphilic, biologically degradable 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 the liposomes. Small unilamellar vesicles (SUV), reversed-phase-evaporated vesicles (REV) and multilamellar vesicles (MLV) liposomes are used in cytostatic encapsulation. However, Papahadjopoulos *et al*. (16) have demonstrated that modifying the SUV liposome membrane by adding polyethylene glycol (PEG) markedly reduced the interaction of the vesicles with stationary macrophages in the liver and spleen after *i.v.* administration (16). This increased the circulation half-time of so-called stealth liposomes. The best tumor accumulation was achieved with SUV-PEG liposomes when super-paramagnetic iron oxide particles were incorporated into PEG

*Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany.

Correspondence to: Dr. U. Pohlen, Charité-Universitätsmedizin Berlin, Chirurgische Klinik, Campus Benjamin Franklin, Hindenburgdamm 30, 12200 Berlin, Germany. Tel: +49 308445-0, Fax: +49 3084452740, e-mail: uwe.pohlen@og.ortenau-klinikum.de

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liposomes as a contrast medium, and accumulation in the tumor was examined by magnetic resonance imaging (MRI) (14).

The aim of the present study was to assess 5-fluorouracil (5-FU) concentrations in various tissues and in liver tumors, and compare PEG-5-FU liposomes to non-encapsulated 5-FU in systemic or regional administration with or without DSM.

Materials and Methods

5-FU SUV-PEG liposomes. 5-FU (Medac, Wedel, Germany; 10 mg/ml) was encapsulated in SUV-PEG liposomes of hydrated soy phosphatidylcholine (HSCP, 50 mg/ml; Nattermann Phosphilipid 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 to 1:0.1). The lipids were dissolved in chloroform (in a round-bottom flask) and a lipid film was created by evaporating the solvent under vacuum (rotation evaporator). The lipid film was dispersed at room temperature by adding 5-FU (50 mg/ml) dissolved in phosphate buffer (PBS), pH 7.4, and by subsequent shaking. Subsequent intermittent application of ultrasound (10×4 min) to the multilayer liposome suspension led to the development of small SUV. The separation of the non-encapsulated 5-FU component was dispensed with in this experimental approach and the cytostatic agent concentration was determined by HPLC. The size of these vesicles was estimated on the basis of quasi-elastic light scattering in a Coulter counter N 4MD (Coulter Electronics, Hialeah, FL, USA). The liposomes measured 113 nm±36 nm.

Experimental animals. The experimental animals were 210 WAG-RIJ rats (breeder: Charles Ribber, Extertal, Germany). At the start, the animals were 80-125 days old and weighed 180-250 g. The animals were housed individually in rooms maintained at 21˚± 1˚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 CC531 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 cell line was cultivated at 37˚C with 5% 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). After 3 days, the 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 in PBS, the viability was evaluated in a Bürker hematocytometer after adding trypan blue to a sample suspension. The suspension was adjusted to 98% viability at a density of 2×10^6 viable cells/100 μl suspension by recentrifugation and resuspension.

Surgical anesthesia of the rats was induced with vaporized ether followed by intramuscular injection of pentobarbital 20 mg/kg (Nembutal®; Pharmazeutische Handelsgesellschaft, Garbsen, Germany) followed by intramuscular administration of 40 mg/kg ketamine (Ketanest®; Parke Davis & Company, Berlin, Germany). The tumor cells (2×10^6) were injected into the left liver lobe.

Table I. *Experimental treatments*.

Group I	(n=25): 10 mg 5-FU, systemic (*i.v.*)
Group II	(n=25): 10 mg 5-FU-PEG liposomes, systemic (*i.v.*)
Group III	(n=30): 10 mg 5-FU, regional HAI
Group IV	(n=35): 10 mg 5-FU plus DSM, regional HAI
Group V	(n=35): 10 mg 5-FU-PEG liposomes, HAI
Group VI	(n=45): 10 mg 5-FU-PEG liposomes plus DSM, regional HAI

DSM: Degradable starch microspheres (Spherex® PharmaCept Berlin, Germany). HAI: hepatic arterial infusion (i.a.).

MR imaging. The tumor size and position was determined by MRI using a 1.5 Tesla Magnetom (Siemens, Karlsruhe, Germany). 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.

Preparation of the experimental animals. The animals were randomized into the experimental groups when the tumors reached a size of 1.5-2 cm. In all the rats undergoing systemic therapy (groups I and II), the cytostatic agent was applied *via* the tail vein. The rats randomized into the regional therapy groups (groups III to VI) 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.

Determination of 5-FU concentration by HPLC. A new procedure to determine the 5-FU concentration in the various organs by HPLC was adopted (17). Blood samples were centrifuged to obtain serum and the individual organs (tumor, liver, spleen, kidneys, stomach, pancreas, peritoneum and lymph nodes) were homogenized. After adding 5-bromouracil (Sigma, Deideshofen, Germany) as an internal standard, the proteins in the serum and homogenates were precipitated by 10% HCl and centrifuged.

Ten micro liters of supernatant were injected into the HPLC device which consisted of an HPLC pump (Gyntek, High Precision Pump, Model 300C), a UV-Vis spectrophotometric detector (Shimadzu, SPD-6AV) and an autosampler (LKB, bromma 2157). An ODS hypersil column, 5 μm, 250×4.6 mm (VDS Optilap) was used as HPLC column. Data transmission was conducted by D2500 cromoto-integrators (Merck-Hitachi, Darmstadt, Germany). The flow of the mobile phase was 1.0 ml/min, the solvent mixture contained 3% methanol + 0.05% acetic acid and water (Merck, Darmstadt). A wavelength of 254 nm was used and the HPLC was performed at room temperature.

The experimental treatments for each group are shown in Table I. In groups I and II, 5 animals each were killed 15, 30, 60, 90 or 120 minutes after therapy was started, and the 5-FU concentrations in the different organs were determined by HPLC. An additional time-point (240 min) was selected in group III, two additional time-points (240 and 480 min) in groups IV and V, and four additional time points (240 and 480 min, 12 h and 24 h).

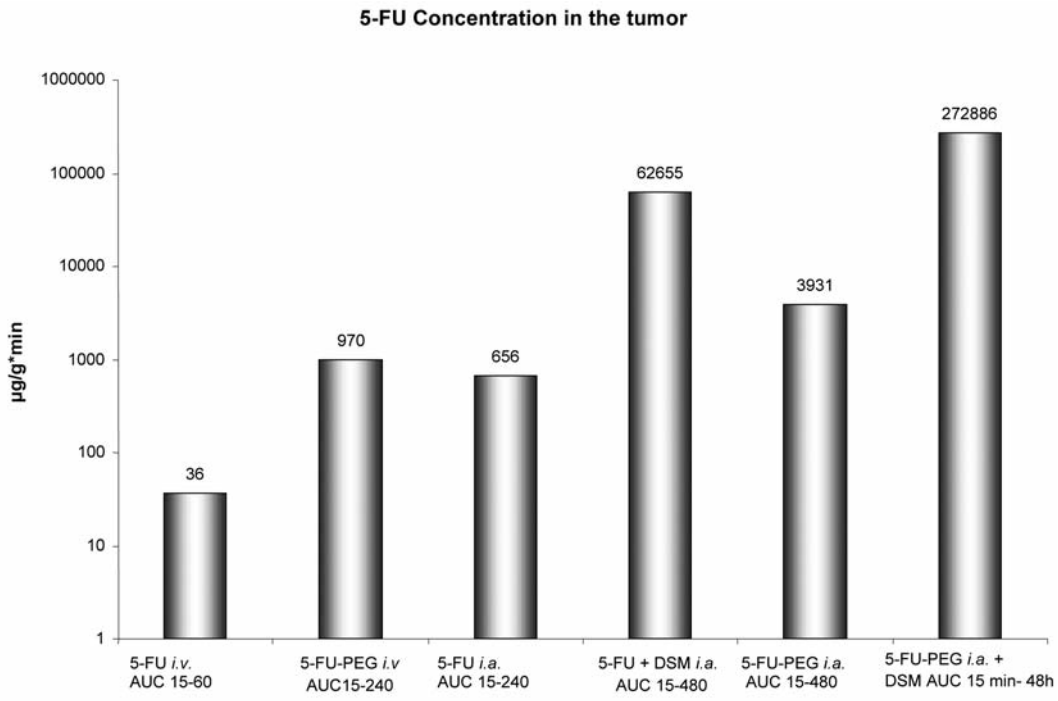

Figure 1. *Logarithm of 5-FU tumor concentration (AUC) in the different therapy groups. 5-FU-PEG: 5-FU-PEG liposomes, DSM: degradable starch microspheres.*

Table II. *Area under the curve (AUC) of 5-FU in various tissues and serum.*

	Group I 5-FU *i.v.*	Group II 5-FU-PEG *i.v.*	Group III 5-FU *i.a.*	Group IV 5-FU + DSM *i.a.*	Group V 5-FU-PEG *i.a.*	Group VI 5-FU-PEG *i.a.* + DSM
	AUC 15-60 min µg/g	AUC 15-240 min µg/g	AUC 15-240 min µg/g	AUC 15-480 min µg/g	AUC 15-480 min µg/g	AUC 15-24 h µg/g
Tumor	35.6	970.2	655.5	62655.0	3931.0	272886.0
Liver	366.9	4861.9	1704.2	27822.0	11562.0	35842.0
Kidneys	870.0	3886.0	1538.0	3188.0	7504	4774.0
Spleen	690.0	3723.0	1367.0	911.0	7620	2934.0
Stomach	627.7	3082.0	1843.0	4680.0	8312	5068.0
Peritoneum	521.0	4892.0	1019.0	1109.0	6754	6394.0
Pancreas	553.0	1532.0	875.0	3816.0	9773	20415.0
Serum	850.0	1129.0	861.0	1258.0	1448	1053.0

Statistics. The mean values±SD of the 5-FU concentration in the control and therapy groups, were calculated for each group. The difference in the 5-FU concentrations between the groups were determined using the global Kruskal-Wallis test. *P*-values were adjusted for multiple comparison according to Bonferroni. A probability value of <0.05 was considered significant.

Results

Overall the regional therapy groups (III, IV, V, VI) demonstrated significantly higher 5-FU concentrations ($p<0.01$) than the systemic therapy groups (I, II). In group I (10 mg of 5-FU *i.v.*) the 5-FU AUC in the tumor tissue measured at the time-points from 15-60 min was 35.6 µg/g (5-FU was no longer detected at later time points) (Table II, Figure 1). The tumor 5-FU concentration compared to group I increased 27-fold after *i.v.* administration of 5-FU-PEG liposomes (AUC group II, 15-240 min 970.2 µg/g). The *i.a.* administration of 5-FU increased the tumor concentration 19-fold to 655.5 µg/g (group III), 1760-fold with 5-FU combined with DSM (AUC group IV 15-480 min, 62655 µg/g) and with 5-FU-PEG liposomes 110-fold to 3931 µg/g (AUC group V 15-480 min). The highest tumor concentrations were

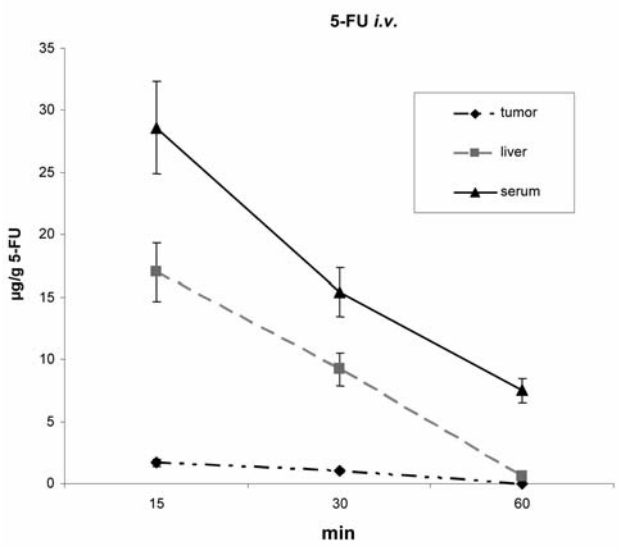

Figure 2. *Concentration time course after i.v. administration of 10 mg of 5-FU.*

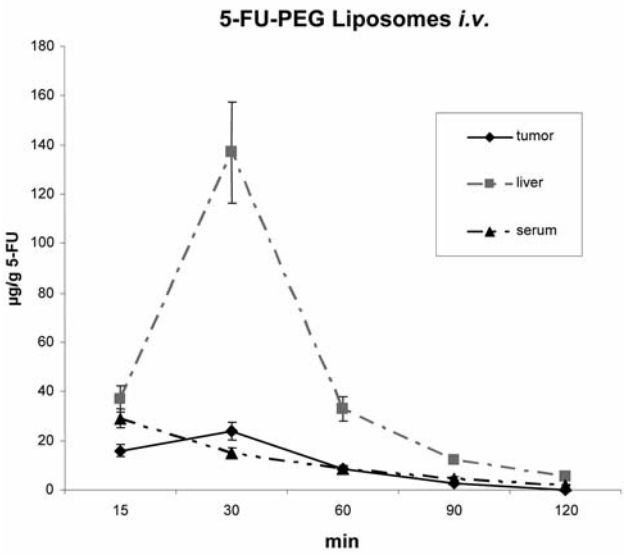

Figure 3. *Concentration time course after i.v. administration of 10 mg of 5-FU-PEG liposomes.*

measured after the administration of 5-FU-PEG liposomes combined with DSM (7665-fold increase to 272886 µg/g (group VI)). In this group alone, 5-FU was still detected in the tumor tissue 24 h after administration (AUC 15 min-24 h) (Figures 1-4). These differences were significant (p<0.01-0.0001). The following concentration increases were observed when the 5-FU concentrations in the liver parenchyma of group II to VI were compared with that in group I (366.9 µg/g), 13-fold in 5-FU-PEG liposomes *i.v.* (4861.9 µg/g), 5-fold in 5-FU *i.a.* (1704.2 µg/g), 76-fold in 5-FU/DSM *i.a.* (27822 µg/g), 32-fold in 5-FU-PEG liposomes *i.a.* (11562 µg/g) and 98-fold in 5-FU-PEG liposomes/DSM *i.a.* (35842 µg/g) (Table II and Figure 2). These differences were significant (p<0.01).

Liposome encapsulation changed the pharmacokinetics of 5-FU. When non-encapsulated 5-FU was administered *i.v.*, the maximal tumor concentration was reached after 15 min compared to 30 min after *i.v.* administration of liposomal 5-FU (Figures 2 and 3). Maximal tumor concentrations were reached 2 h after infusing 5-FU-PEG liposomes and DSM into the hepatic artery (Figure 4).

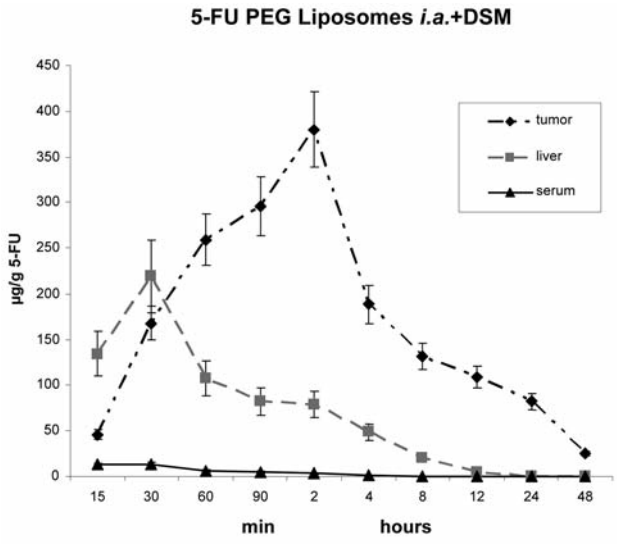

Figure 4. *Concentration time course after hepatic arterial infusion (HAI) of 10 mg 5-FU-PEG liposomes combined with degradable starch microspheres (DSM).*

Discussion

Compared to the tumor 5-FU concentration after non-encapsulated *i.v.* administration, the tumor 5-FU concentration was increased 27-fold after *i.v.* administration of 5-FU-PEG liposomes and 1760-fold after *i.a.* administration of the non-encapsulated form combined with DSM, while the locoregional *i.a.* administration of combined 5-FU-PEG liposomes and DSM led to a 7665-fold increase of the concentration time curve (AUC). Furthermore, the pharmacokinetics of 5-FU were changed so that the concentration maximum after *i.v.* administration of non-encapsulated 5-FU was reached after 15 min, after *i.v.* administration of liposomal 5-FU after 30 min and after hepatic arterial infusion (HAI) of liposomal 5-FU and DSM after 2 h. Regional *i.a.* administration led to cytostatic

agent accumulation in the tumor. A number of experimental and clinical studies on the pharmacokinetics of regionally administered 5-FU have also found increased concentration in the tumor tissue (18-24).

An added advantage of regional administration can be gained by reducing the blood flow. DSM slow down the blood flow for approximately 20 min. Furthermore, DSM have a target effect on the tumor (25, 26).

Clinical studies with PEG liposomes, also called stealth liposomes, have reported reduced patient toxicity with prolongation of the plasma half-life in liposome-encapsulated cytostatic agents (27, 28). The prolonged accumulation of 5-FU was also observed in the present experiments and was considerably increased by the addition of DSM. Markedly increased tumor concentrations after *i.v.* administration of PEG liposomal cytostatics of 6- to 30-fold compared to *i.v.* administration of the non-encapsulated cytostatics have also been reported in experimental and clinical pharmacokinetic studies (29-31). This was in agreement with our results in which the tumor concentration increased 6-fold after *i.v.* administration of liposomal compared to non-encapsulated 5-FU. Additional reports have also shown that active substances encapsulated in stealth liposomes resulted in better accumulation in tumor tissue than the non-encapsulated substances or other liposomal preparations (30, 32).

The relatively selective tumor accumulation might be explained by the 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. Polymeric conjugates could then release their drugs intracellularly *via* endocytosis (32, 33). Intravital microscopic examinations have supported this hypothesis, suggesting that PEG liposomes accumulate in the tumor interstitium and move in an intracellular direction due to increased vascular permeability (34, 35). The affinity of liposomes to RES organs explains the high concentrations in the liver parenchyma when applying liposomal 5-FU (36, 37).

Conclusion

The combined regional applications of 5-FU *via* the hepatic artery with temporary embolization by DSM and drug targeting by liposome-encapsulated 5-FU increases the intratumoral concentration 7665-fold compared to *i.v.* application.

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