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Drug-Eluting Beads Loaded with Anti-Angiogenic Agents for Chemoembolization: *In Vitro* Sunitinib Loading, Release and *In Vivo* Pharmacokinetics in an Animal Model

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Abstract

Purpose: The combination of embolic beads with a multi-targeted tyrosine kinase inhibitor that inhibits tumor vessel growth is suggested as an alternative and improvement to the current standard doxorubicin-eluting beads for use in transarterial chemoembolization. This study demonstrates the in vitro loading and release kinetics of sunitinib using commercially available embolization microspheres, and evaluates the in vitro biological efficacy on cell cultures and the resulting in vivo pharmacokinetic profiles in an animal model.

Materials and Methods: DC Bead microspheres, 70-150 µm and 100-300 µm (Biocompatibles Ltd., Farnham, United Kingdom), were loaded by immersion in sunitinib solution. Drug release was measured in saline in a USP-approved flow-through apparatus and quantified by spectrophotometry. Activity after release was confirmed in cell culture. For pharmacokinetics and in vivo toxicity evaluation, New-Zealand white rabbits received sunitinib either by intra-arterial injection of 100-300 µm sized beads or per os. Drug concentrations in the plasma and liver tissue were assessed by liquid chromatography–tandem mass spectrometry.

Results: Sunitinib loading on beads was close to complete and homogeneous. A total release of 80% in saline was measured, with similar fast release profiles for both sphere sizes. After embolization, drug plasma levels remained below the therapeutic threshold (< 50 ng/ml), but high concentrations at 6 h (14.9 µg/g) and 24 h (3.4 µg/g) were found in the liver tissue.

Conclusions: DC Bead microspheres of two sizes were efficiently loaded with sunitinib and displayed a fast and almost complete release in saline. High liver drug concentrations and low systemic levels indicated the potential of sunitinib-eluting beads for use in embolization.

Keywords: Hepatocellular carcinoma, transarterial chemoembolization, drug-eluting beads, anti-angiogenic agent, sunitinib

Abbreviations: DEBs: drug-eluting beads, HUVECs: human umbilical vein endothelial cells, USP: United States Pharmacopeia, VEGF: vascular endothelial growth factor, VEGFR2: vascular endothelial growth factor receptor 2
1 Introduction

Embolization techniques are widely used to treat various types of hypervascular liver tumors. Recent studies have discussed the potential benefit of drug eluting beads (DEBs) over conventional transarterial chemoembolization (1, 2). These two options differ by the use of embolic agent, the time span and the amount of drug delivered to the tumor. However, this therapeutic approach suffers from two main drawbacks: first doxorubicin might not be the ideal drug for this application (3-5), second the ischemia induced by the embolization procedure contributes to the formation of new vessel sprouts, particularly in the periphery of the tumor (6-8).

The combination of the (doxorubicin-loaded) spheres with systemic anti-angiogenic therapy has already been reported (9-11), and several phase II and III trials with oral sunitinib (SATURNE) or sorafenib are under way or completed (12, 13). Sunitinib malate (SU11248) is available as an oral formulation from Pfizer (New York, New York) and was approved by the U.S. Food and Drug Administration in 2006 for the treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor (14). This drug acts as a potent inhibitor of several tyrosine kinases, mainly vascular endothelial growth factor (VEGF) and platelet-derived growth factor receptors (15).

Its lack of specificity results in numerous side effects, including fatigue, nausea and diarrhea (16-19). Therefore, the local delivery of sunitinib via DEB could be a reasonable means to reduce systemic toxicity compared to oral treatment while maintaining local anti-tumoral efficacy.

In the first part of this investigation, sunitinib loading and release kinetics of small-sized beads and the size effects related to drug loading were examined in vitro, resulting in an understanding of the affinity and interaction of the active principle with its carrier.

In the second part of this study, the efficacy of sunitinib-eluting beads on an endothelial cell line and several cancer cell lines compared to unloaded beads and free sunitinib was investigated.

In the third section, we addressed the in vivo pharmacokinetics and tolerance of 100-300 µm sunitinib-eluting beads in healthy New Zealand white rabbits, comparing sunitinib concentrations after given time points in the tissue and in the systemic circulation after local and oral
administration. Toxicity was examined by monitoring the liver enzyme activity and general animal status.

2 Materials and Methods

2.1 Materials

DC Bead samples of 70-150 μm and 100-300 μm diameter range (Biocompatibles Ltd., Farnham, UK) were selected for the study.

Sunitinib was purchased as base from LC Laboratories, Woburn, MA, USA. Other chemicals were of analytical grade and were used as received.

2.2 Methods

2.2.1 DEBs Characterization

**Loading of Sunitinib into Beads.** The beads were loaded by incubation in different amounts of a sunitinib stock solution (10 mg/ml), which was prepared according to a proprietary protocol (20).

The amount of drug loading was determined indirectly by measuring the residual unloaded drug in the supernatant of the beads suspension using UV-Vis spectrophotometry at 430 nm (HP 8453, Agilent Technologies AG, Basel, Switzerland).

**Release of Sunitinib from Beads.** The flow-through United States Pharmacopeia method IV using a Sotax CE 6 (Sotax, Allschwil, Switzerland) served to quantify the drug released from sunitinib-eluting beads in saline.

The loading and release profiles were compared using the similarity factor f₂, following FDA guidelines for dissolution profile comparison (21-23).

**Microsphere Morphometry.** Beads were imaged with a Zeiss Axiovert 200 microscope (Carl Zeiss, Feldbach, Switzerland). The size was measured using the open source image analysis software (ImageJ 1.38 software; NIH, Bethesda, MD, USA), analyzing an average of 150 microspheres in a
monolayer. Statistical analysis was performed using a two-sided Student’s t-test, at a significance level $P = 0.001$. Details of DEB characterization can be found in Appendix A (Section 7 Supplementary Material).

### 2.2.2 Proof of Biological Efficacy in a Cell Culture Model

Cells were exposed to control beads, sunitinib-eluting microspheres and free sunitinib to study the ligand-receptor interactions and cell viability.

Sunitinib-eluting beads, size 70-150 μm, were prepared under sterile conditions at a concentration of 10 µmol/l/well (≈4 µg/ml, 40 mg beads/well).

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza Ltd, Visp, Switzerland and were cultured in endothelial growth medium (EGM; Lonza Ltd). Additionally, 786-0 and Caki-1 (kidney), LS174T and SW480 (colon), MDA-MB-231 (breast) and A549 (NSCLC) were purchased from the American Type Culture Collection and were cultured in Dulbecco’s Modified Eagles’ medium with 10% fetal calf serum, containing 4.5 g/l glucose, L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Buchs, Switzerland). Cells were plated on the upper side of transwells in triplicate at a density of $10^4$ cells/well. Control beads, sunitinib-eluting beads or sunitinib solution was added to the lower part of the transwell.

**Phosphorylation of VEGFR2.** After 1 h of treatment and stimulation with VEGF (catalog number 100-20; Pepro-Tech France, Neuilly-Sur-Seine, France) for 30 min, the HUVECs were washed once with phosphate-buffered saline and lysed in RIPA Lysis Buffer (Santa Cruz Biotechnology, Inc, Heidelberg, Germany) containing 1 mmol/l sodium orthovanadate and protease inhibitor cocktail (Sigma-Aldrich). An equal amount of protein (20 µg) was separated on 4-12% polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Zug, Switzerland). Membranes were blocked with the Odyssey blocking buffer (LI-COR Biosciences, Lincoln, Nebraska) and immunoblotted with primary antibodies (rabbit antihuman VEGF #2479, rabbit antihuman phosphorylated vascular endothelial growth factor receptor 2 [pVEGFR2] #4991, Cell signaling Technology, Inc, Danvers, Massachusetts), followed by infrared secondary antibodies (Alexa fluor 680 goat antirabbit #A21109, Invitrogen AG, Basel, Switzerland). Bands from
immunoreactive proteins were visualized by an Odyssey infrared imaging system (LI-COR Biosciences).

**HUVEC Migration.** HUVEC migration to the lower surface of the filter in the transwells was determined by counting the cells under light microscopy in three high-power fields. For this, transwells were fixed in 2% paraformaldehyde and stained with 0.5% crystal violet 3 h after the addition of the control, sunitinib-eluting beads or sunitinib.

**Proliferation and Survival of Cell Lines.** All cell types were evaluated by cell counting using a Neubauer hemocytometer and by a Cell Death Detection ELISA plus kit (Roche Diagnostics, Basel, Switzerland) after 24 h of treatment.

**Cell Growth in Three-Dimensional Colonies.** The capability of the cells to grow in three-dimensional colonies was examined by mixing five hundred 786-0 or Caki-1 cells into Matrigel (BD Biosciences, Allschwil, Switzerland) and plating them on 12-well plates. After 24 h, control beads, sunitinib beads or sunitinib were added to the wells. The number of colonies was counted after 10 days of culture.

### 2.2.3 Preclinical *In Vivo* Studies

All *in vivo* experiments were carried out at the Centre de Recherche en Imagerie Interventionnelle CR2i in Jouy-en-Josas, France. They were approved by the institutional animal care and use committee of the Center and were conducted according to European Community rules of animal care (Directive EC 86/609).

**Experimental Protocol.** Healthy New Zealand white rabbits (*n* = 15; 3.2 - 3.8 kg) were separated into 3 groups. Group 1 and group 2 received 0.2 ml of sunitinib-eluting beads in the hepatic artery. The animals in group 1 (*n*=4) were sacrificed 6 h after embolization. Animals in group 2 (*n*=7) were sacrificed one day after embolization. The third group (*n*=4) received a single dose of sunitinib (6 mg) per os, which should result in therapeutic plasma concentrations (*c*<sub>max</sub>) of 45-55 ng/ml, according to previous pharmacological studies (24, 25). In this third group, 2 animals were sacrificed at 6 h, and 2 animals were sacrificed at 24 h.
**Preparation of Sunitinib-Loaded Beads and Oral Solution.** Syringes prefilled with sunitinib-loaded 100-300 μm sized beads (6 mg sunitinib per 0.2 ml beads per syringe) were prepared under aseptic laminar air-flow conditions for pharmacokinetic studies in rabbits (details can be found in Appendix B [Section 7 Supplementary Material]). The oral sunitinib solution (1 mg/ml) was prepared by diluting the stock solution (10 mg/ml) with pre-filtered glucose 5% (w/v).

**Transarterial Chemoembolization Procedure.** Embolization procedures were performed under general anesthesia with isoflurane. Access to the right common femoral artery was obtained via surgical cutdown, where a 4 F vascular sheath (Radifocus®, Terumo, Leuwen, Belgium) was introduced. A 2.1/1.7 F, 45° tip microcatheter (Echelon, ev3, Paris, France) was then advanced and used to engage the celiac trunk followed by common hepatic artery under fluoroscopy. Common hepatic arteriography (Omnipaque 300, GE Healthcare, Aulnay-sous-Bois, France) was performed to demonstrate the arterial anatomy. Sunitinib loaded beads were injected through the microcatheter.

Injections were performed as followed: beads were suspended in a total volume of 1 ml and slowly infused by hand in the common hepatic artery, over a period of 20-30 minutes under careful real time fluoroscopy to prevent non-target embolization. Immediately after completion of embolization procedures post-anesthesia care was provided and the animals were monitored.

**Dosage of Sunitinib after Administration.** Liver enzymes were measured immediately before embolization or oral administration of the drug and at 6 h in all groups and again at 24 h in group 2 and in 2 animals in group 3. Sunitinib plasma levels were measured immediately before and at the end of the embolization procedure, as well as 1, 2, 3, 4, 5 and 6 h after administration and after 24 h for group 2. Plasma levels were determined in only 3 out of 4 animals of group 3 for technical reasons. Whole blood samples were collected into potassium ethylenediaminetetraacetic acid tubes and centrifuged. Plasma samples were stored at -20°C until analysis by liquid chromatography-tandem mass spectrometry (26). Measurements of sunitinib were performed in 4 tissue samples per animal to limit sampling errors (2 in the right lobe and 2 in the left lobe), using the same mass spectrometry method after sacrifice.
3 Results

3.1 Loading of Sunitinib into Beads

Beads showed close to complete loading with sunitinib (100% for 70-150 µm and 97% for 100-300 µm sized beads) after a two-hour incubation under agitation and at a loading concentration of 30 mg sunitinib/g beads. Experiments with 100-300 µm beads showed a maximal drug loading capacity of 32 mg drug/g beads, though at an inferior loading efficiency (Figure 1). Thus, all of the following tests were carried out at a concentration of 30 mg sunitinib per gram of beads.

Profile comparison of the fraction of sunitinib loaded into 70-150 µm and 100-300 µm sized spheres showed initially different profiles until t = 30 min (similarity factor $f_2 = 47$), converging to complete drug loading with an $f_2 = 51$ for longer incubation periods, indicating profile equivalence of the loading for the two different bead sizes.

![Loading profiles of 70-150 µm (□, n=4) and 100-300 µm (■, n=3) beads with a maximal loading of 30 mg sunitinib/g beads and 100-300 µm sized beads loaded with 60 (▲, n=3) and 90 (▲, n=3) mg sunitinib/g beads.](image)

3.2 Bead Size Changes with Sunitinib Loading

The microsphere spherical shape was preserved without aggregation or particle damage during loading and after release (Figure 2). Loaded beads were colored an intense orange due to drug absorption. Beads lost this coloration upon elution by ionic exchange in isotonic NaCl solution.
However, Figure 2C shows an inhomogeneous bead discoloration in saline even after several days under flow, which occurs when aggregates form and shield the inner beads from the elution medium. Beads regained the initial blue appearance without any yellow traces upon ethanol addition, thus validating the ethanol wash as an internal standard for 100% drug release.

At 30 mg sunitinib/g beads loading, both sphere sizes shrunk significantly ($P < 0.001$) by 17% (70-150 µm) and 15% (100-300 µm) and in volume by 44% (70-150 µm) and 39% (100-300 µm). Initial mean diameters of $126 \pm 17$ µm and $184 \pm 66$ µm decreased to $104 \pm 14$ µm and $156 \pm 50$ µm, respectively. Figure E 1 A–D (Section 7 Supplementary Material) elucidates the shift in bead size and the bead size distribution. This phenomenon was found to be reversible after the release of the drug with diameters of $131 \pm 17$ µm vs. $210 \pm 73$ µm in saline and $140 \pm 20$ vs. $231 \pm 81$ in saline/30% ethanol (V/V).

![Image](image.jpg)

Figure 2. 100-300 µm beads (A) in saline, (B) after loading (30 mg sunitinib/g beads), (C) after elution in NaCl 0.9% and (D) after elution in NaCl 0.9%/ethanol 30% in a flow-through apparatus. The scale bar indicates 200 µm. (E) Flow-through release profiles of 70-150 µm (□, n=5) and 100-300 µm (■, n=3) sunitinib-eluting beads showed equivalence for the two bead sizes. Error bars display standard deviation.
3.3 Release of Sunitinib from Loaded Beads

Similar release profiles for 70-150 μm and 100-300 μm sized spheres were obtained (Figure 2E). The drug release half time $t_{50%}$ was 1.1 h for 70-150 μm beads and 1.6 h for 100-300 μm beads, whereas the difference in $t_{75%}$ was greater (13 h vs 8 h, respectively).

Sink conditions were kept throughout the whole experiment, attaining maximum concentrations of approximately 27.5 mg/l during drug elution, far below the drug solubility of 25 g/L (27).

Profile comparisons showed similarity with $f_2 = 81$, and no significant differences were found between 70-150 μm and 100-300 μm sized beads. Values within the first 3 h of release were more distinct with $f_2 = 67$, albeit still indicating a similar initial release profile.

Final release values plateaued at 81% (70-150 μm beads) and 82% (100-300 μm beads). A two-tailed unpaired Student’s t-test for the average release of the two bead sizes at 1.5, 2 and 3 days when the plateau was reached revealed $P = 0.662$, 0.546 and 0.594, respectively, indicating similar fractions of sunitinib were released. Overall, sunitinib was released from beads of both sizes in saline in a fast manner and to a large extent.

3.4 Biological Efficacy in Cell Culture

3.4.1 Effects of Sunitinib-Eluting Beads on Endothelial Cell Proliferation, Survival and Migration

To assess the activity of the anti-angiogenic agent sunitinib, the effects of this drug on human umbilical vein endothelial cells (HUVECs) as an indicator of tumor progression were investigated.

As expected, VEGF increased the phosphorylation of VEGFR2, a growth factor receptor present on endothelial cells, in the presence of control beads. In contrast, sunitinib-eluting beads and sunitinib reduced the baseline phosphorylation of VEGFR2 and blocked VEGF-induced VEGFR2 phosphorylation (Figure E 3 [Section 7 Supplementary Material]).

A nonparametric, paired Friedmann test validated the significant effects of sunitinib on HUVEC proliferation ($P = 0.0040$), apoptosis ($P = 0.0137$) and migration ($P = 0.0162$). Herein, VEGF addition
did not significantly affect the activity of sunitinib on the cells, as the nonparametric Wilcoxon test revealed with $P > 0.9999$ in the three assays. The mean cell numbers (in the proliferation and migration assays) and the mean DNA enrichment factors (in the apoptosis assay) after treatment with sunitinib-eluting beads or free sunitinib were outside of the 95% confidence interval (CI) of the controls, both with and without VEGF addition. This comparison takes into account the standard deviation of the results and reinforces the hypothesis that sunitinib has a significant effect on HUVECs.

To summarize, VEGF-induced HUVEC responses were inhibited by sunitinib-eluting beads (Figure 3 A-C, Figure E 2 [Section 7 Supplementary Material]).
Figure 3. Effects of sunitinib-eluting beads on (A) HUVEC proliferation, (B) HUVEC apoptosis, (C) HUVEC migration. (A) and (B) Endothelial cells were treated with control beads, sunitinib-eluting beads or sunitinib in the presence (black columns) or not (white columns) of VEGF. After 24 h, endothelial cell proliferation was determined by cell counting (± SD) and endothelial cell apoptosis was measured by quantifying DNA fragmentation following the manufacturer’s instructions (absorbance of the treated cells/absorbance of the control cells). (C) The migration of endothelial cell stimulated (black columns) or not (white columns) with VEGF was quantified in presence of control beads, sunitinib-eluting beads or sunitinib. Significance (*) indicates that the mean cell numbers (A, C) and the mean DNA enrichment factors (B) after treatment with sunitinib-eluting beads or free sunitinib were outside of the 95% confidence interval of the respective controls. VEGF-induced endothelial cell responses were inhibited by sunitinib-eluting beads. HPF = high-power field.

3.4.2 Effects of Sunitinib-Eluting Beads on Cancer Cell Proliferation, Survival and Three-Dimensional Growth

The proliferation assay revealed that sunitinib-eluting beads reduced the proliferation of the renal cancer cell lines 786-0 and Caki-1, LS174T (colon), MDA-MB-231 (breast) and A549 (NSCLC) to a similar extent as sunitinib when compared with control beads (Figure 4, P = 0.009 in nonparametric Friedmann test). No toxicity was observed with the control beads alone. Following treatment with sunitinib-eluting beads or free sunitinib, the mean amounts of cell proliferation for the 786-0 and A-549 cells were located outside of the 95% CI of the control, indicating that these cells showed the most significant reduction in proliferation.

In contrast, sunitinib-eluting beads or free sunitinib did not affect the cancer cell survival. Moreover, they showed no effect on the SW480 colon cancer cell line in either the proliferation assay or the survival assay. Finally, sunitinib-eluting beads also reduced the growth of 786-0 and Caki-1 cells in Matrigel as observed by a reduction of the number of colonies formed in Matrigel (Figure E 3 [Section 7 Supplementary Material]).
Figure 4. Sunitinib-eluting beads (black columns) and free sunitinib (shaded columns) affected (A) cancer cell proliferation, but not (B) apoptosis compared to control beads (white columns). After 24 h, cell proliferation was determined by cell counting and endothelial cell apoptosis was measured by quantifying DNA fragmentation following the manufacturer’s instructions (absorbance of the treated cells/absorbance of the control cells). Significance (*) indicates that the means (± SD) after treatment with sunitinib-eluting beads or free sunitinib were outside of the 95% confidence interval of the respective controls.

3.5 Preclinical In Vivo Studies with Healthy New Zealand White Rabbits

Transarterial Chemoembolization Procedure. A total of eleven hepatic artery catheterizations were performed. Three animals in group 2 died from catheterization-related complications (gastric ischemia and perforation), during the 24 h observation period. The injection of the beads was technically not feasible in one animal in this group. Finally, four animals were analysed in group 1 and the three animals that reached the scheduled time of sacrifice in group 2.

Injection of 100-300 μm beads loaded with sunitinib in the common hepatic artery of rabbits resulted in an expected elevation of transaminases (alanine transaminase ALT, aspartate transaminase AST). Alkaline phosphatase (ALP), another biomarker for liver cytolysis, showed a 3-fold increase from the baseline to 54±54 UI/l.

Toxicity. Injection of 100-300 μm beads loaded with sunitinib in the common hepatic artery of rabbits resulted in an expected elevation of transaminases (alanine transaminase ALT, aspartate transaminase AST, Table E 1 [Section 7 Supplementary Material]). Alkaline phosphatase (ALP), another biomarker for liver cytolysis, showed a 3-fold increase from the baseline to 54±54 UI/l.
Bilirubin levels were never detected to be higher than 10 µmol/l after embolization or oral administration of sunitinib and were therefore below the sensitivity limit of the method.

Areas of necrosis were noted at the surface of some livers during necropsy and liver harvesting.

Pharmacokinetics. The tissue concentration of sunitinib in the harvested livers was found to be high at 14.9 ± 4.7 µg/g (n=4) and 3.4 ± 0.8 µg/g of tissue (n=3) at 6 and 24 h, respectively, when the beads were administered intra-arterially. Lower, but still high, drug levels were achieved by the peroral route (4.2 ± 0.6 µg/g after 6 h (n=2) and 2.6 ± 2.6 µg/g after 24 h (n=2)). Embolization of the hepatic artery with sunitinib-eluting beads resulted in a local concentration 3.5 times higher after 6 h and 1.3 times higher after 24 h than after oral administration.

In both administration routes, the plasmatic sunitinib levels remained below the theoretical therapeutic threshold of 50 ng/ml, with slightly higher levels after intra-arterial administration (Figure 5). The low 24 h value (p.o.) of 7 ng/ml was determined in an animal whose plasma levels accounted only for 4 ng/ml at 6 h.

Figure 5. Plasmatic sunitinib levels after oral administration (●) of a single dose of 6 mg of sunitinib and after embolization of the hepatic artery (■) with sunitinib-eluting beads were below the therapeutic threshold of 50 ng/ml.
4 Discussion

The bead consists of poly(vinyl alcohol)-based hydrogel linked to sulfonate groups, resulting in a negatively charged structure (28, 29) that loads the protonated sunitinib with good performance. In accordance with Lewis’ investigations, which did not show a significant difference in the loading kinetics for the smallest tested bead size ranges of 100 to 500 µm (28), the $f_2$ factor comparison revealed similar loading curves for the tested bead sizes.

As observed for doxorubicin-loaded beads, sunitinib-loaded beads undergo size changes. A similar shrinking was found without major changes in the size distribution profile of the beads. Bead shrinking may have implications in vivo because more distal arterioles can be blocked by smaller beads. Padia et al. (30) have recently favored the utilization of smaller (100-300 µm) beads in hepatocellular carcinoma because fewer side effects and a more complete response was observed when compared with 300-500 µm beads.

A similar, relatively fast drug release of more than 80% in approximately 3 h was shown for both sizes in saline. In the absence of salts, negligible drug elution occurs (29, 31), as observed during eight-week extraction assays in 5% glucose, which served as an isotonic storage medium in the syringes for the preclinical studies.

Like sunitinib, two other drugs of clinical interest, doxorubicin and irinotecan, can be efficiently loaded into beads, but these drugs display distinct features in terms of elution extent and kinetics (32). In contrast to the complete or almost complete irinotecan and sunitinib elution (100% and 82%, respectively), doxorubicin was partially retained in the beads under saline flow (28% of drug eluted), which could be attributed to stronger ionic interactions with sulfonates or to doxorubicin in situ gelation (33). Drug physicochemical properties such as solubility and polarity and sterical ligand hindrance govern binding affinity and, in turn, release kinetics in vitro as well as in vivo.

Chow et al. (18) stated 20 to 40 mg/kg p.o. as a minimal daily dose to reach the therapeutic in vivo concentrations of 50-100 ng/ml in mice plasma to inhibit VEGFR2 and PDGFR phosphorylation over 12 h. With our regimen of approximately 2 mg/kg administered intra-arterially as a single dose, we
determined the concentration at the target liver tissue to be above this limit and thereby probably in toxic ranges. In humans, 25-50 mg of sunitinib malate are recommended daily doses for GIST, metastatic RCC and pancreatic neuroendocrine tumors (34), corresponding to 19-37 mg of pure sunitinib drug per day or only 0.3-0.5 mg/kg/day for a person of 70 kg.

Due to the first-pass effect, sunitinib is metabolized by CYP450 enzymes in the liver, which is in concordance with a reported accumulation of sunitinib in the tissue (34) and may explain high tissue levels also with oral administration.

Comparing the relatively rapid release kinetics obtained \textit{in vitro} ($t_{50\%}=1.6$ h) with the retention of the drug in the tissue for at least 6 h points towards sunitinib binding to the surrounding liver tissue. This proposed mechanism is supported by the fact that sunitinib is 95% human plasma protein-bound (18).

The tissue concentrations fell after 6 h, but plasma concentrations continued to increase until 24 h, suggesting that elimination was ongoing in the liver while sunitinib was still transported into the blood circulation for both routes of administration. The drug should be present long enough to cover the early proliferative response to the ischemic effect after the embolization procedure, which is known to peak at day 1 and then gradually decrease (35). In comparison, the Sutent® monography (34) reported a $t_{\text{max}}$ between 6 and 12 h after capsule (sunitinib malate) intake and Speed et al. (36) determined an elimination half time of 51 h in humans after an oral dose of 50 mg.

Higher plasmatic levels were expected after oral administration compared to a local delivery. The \textit{in vitro-in vivo} correlation suggested that the drug was accumulating in plasma rather than being eliminated. A direct plotting of the measured plasma concentrations against the cumulative released sunitinib concentration revealed a tendency towards a linear correlation with a coefficient $R^2 = 0.95$ (i.a.) and 0.84 (p.o.) until 6 h. This linearity declined at 24 h. The measured plasma concentrations after intra-arterial injection after 24 h exceeded the linear prediction, but remained below therapeutic levels. Presumably, the relatively high administered concentrations led to persistence of the drug in the blood circulation. Superior intra-tissular and plasmatic drug concentrations below therapeutic levels after embolization when compared with an oral administration of the same dose suggested a higher bioavailability from the bead-drug combination. Additionally, no prolonged tissue exposure to the drug was observed when compared with oral uptake. It is still controversial if an extended release is desirable to avoid multiple
administrations and plasma peaks or if a permanent exposure to the anticancer drug creates unwanted tumor cell resistance.

Regarding toxicity of sunitinib-eluting beads, transaminase levels, which indicate acute liver injury and cell disruption, increased following the embolization procedure of the hepatic artery, whereas ALP levels augmented only slightly. In contrast, sunitinib given perorally did not result in a change in liver enzymes, indicating that there was no drug-related change in enzyme levels. Recently, a randomized, unblinded phase III trial with oral sunitinib 37.5 mg/d vs. sorafenib 400 mg twice /d has revealed inferior overall survival and more frequent adverse events with sunitinib and was therefore discontinued (19), although sunitinib doses had been lowered compared to earlier phase II trials (37, 38). These issues might be overcome by local drug targeting, for which appropriate doses need to be determined.

Tumor cells are capable of recruiting adjacent supportive cells, enhancing the proliferation of these cells to form vessel walls (15). This effect on the surrounding tissue is hampered by sunitinib and other anti-angiogenic agents. As angiogenesis is not typical for a specific type of cancer, the effects of anti-angiogenic agents are seen in various tumor cell lines and tumor types (18, 25).

The cellular assays in this study confirmed that sunitinib was efficiently released from the spheres and had comparable activity as sunitinib in solution. For primary endothelial cells, the cytotoxic response was based on the mitogenic VEGFR inhibition, which is the major effect of sunitinib. However, the cellular target is less obvious for some cancer cell lines. Because apoptosis was not induced and only cytostatic effects at a concentration of 10 µmol/l were seen, the drug activity in this study must be based on anti-angiogenic activity by VEGF and PDGF receptor inhibition (25).

The study’s scope did not cover the evaluation of the antitumoral effect of sunitinib-eluting beads, this combination is currently assessed in a VX2 tumor model. Although no hepatocellular carcinoma cell lines were tested, we used cell lines that are likely to develop metastases to the liver. Measurement of sunitinib concentrations by liquid chromatography-tandem mass spectrometry in liver samples took into account sunitinib both bound to the tissue and to the beads embedded in
the samples. *In vitro* results suggest that 6 hours and 24 h after injection, most of the drug (73% and 81%, respectively) is released.

To conclude, the VEGFR antagonist sunitinib could be efficiently combined with DC Bead microspheres, consequently qualifying sunitinib as a potential drug candidate for the treatment of hypervascular tumors that suppresses ischemia-triggered angiogenesis and tumor recurrence.

### 5 Acknowledgment

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6 References


7 Supplementary Material

A

B
Figure E 1. Size distribution of 70-150 µm beads (A) before and (B) after sunitinib loading and 100-300 µm beads (C) before and (D) after sunitinib loading. A similar shrinking was found without major changes in the size distribution profile of the beads.
Figure E2. Sunitinib-eluting beads as well as sunitinib reduced baseline phosphorylation of VEGFR2 and blocked VEGF-induced VEGFR2 phosphorylation. Endothelial cells were exposed to control beads, sunitinib-eluting beads or sunitinib for 1 h. Cells were subsequently stimulated with VEGF for 30 minutes. Cell lysates were prepared and analysed for the phosphorylation of VEGFR2 and the total amount of VEGFR2 by Western blot. pVEGFR2 = phosphorylated vascular endothelial growth factor receptor 2.

Figure E3. Effects of control beads (white columns), sunitinib-eluting beads (black columns) and free sunitinib (shaded columns) on three-dimensional growth in Matrigel. Results are represented as the mean number of colonies ± SD counted in five fields (microscope magnification: 100 x) of three independent experiments. Cell growth in three-dimensional gel matrix, where cell sensitivity to anti-cancer agents may change, allowed for an in vivo-like environment where sunitinib inhibited the formation of colonies for the two RCC lines. A nonparametric Friedmann test could not indicate significance (P = 0.1944 for 786-O, P = 0.0556 for Caki-1 three-dimensional growth) due to its low power resulting from the small sample size. Significance (*) indicates that the mean cell numbers after treatment with sunitinib-eluting beads or free sunitinib were outside of the 95% confidence interval of the respective controls.
Table E 1. Mean Serum Alanine Transaminase, Aspartate Transaminase, and Alkaline Phosphatase before and 6 Hours and 24 Hours after Embolization of the Hepatic Artery with Sunitinib-Eluting Beads and after Peroral Administration.

<table>
<thead>
<tr>
<th></th>
<th>Intraarterial Administration [UI/l]</th>
<th>Peroral Administration [UI/l]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline (n=7)</td>
<td>6 h (n=7)</td>
</tr>
<tr>
<td>ALT</td>
<td>90 ± 42</td>
<td>186 ± 62</td>
</tr>
<tr>
<td>AST</td>
<td>82 ± 49</td>
<td>831 ± 364</td>
</tr>
<tr>
<td>ALP</td>
<td>17 ± 13</td>
<td>24 ± 13</td>
</tr>
</tbody>
</table>

ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase.

7.1 Appendix A: DEBs Characterization

Loading of Sunitinib into Beads. A sunitinib stock solution (10 mg/ml) was prepared according to a proprietary protocol (19) by acidifying the sunitinib base in 0.1N hydrochloric acid in a 1.1 molar excess to solubilize the drug. Then, a 5% (w/v) glucose solution was added.

The storage saline solution was removed from the vials. Defined volumes of the bead suspensions were incubated with sunitinib solution. Maximum sunitinib loading capacity was tested by adding different volumes of the stock solution. The incubation lasted for 2 h under agitation at ambient temperature.

The loading curves were obtained by a depletion method, i.e., sampling the supernatant at predetermined time points, quantifying sunitinib using UV-Vis spectrophotometry at 430 nm (HP 8453, Agilent Technologies AG, Basel, Switzerland) and subtracting the content from the original known amount of the added drug.

The loading profiles were compared using the similarity factor $f_2$, following FDA guidelines for dissolution profile comparison (20-22). Similar curves show $f_2$ values > 50 in the case of equivalency within a 10% mean profile difference. Loading is based on a similar ion-exchange mechanism as release, which justifies the use of $f_2$ for the comparison of drug binding kinetics.

Release of Sunitinib from Beads. The flow-through United States Pharmacopeia method IV using a Sotax CE 6 (Sotax, Allschwil, Switzerland) with 6 parallel cells served to quantify the drug released from sunitinib-eluting beads. Each cell was filled with 100 mg of drug-loaded packed beads suspended in equal amounts of 5% glucose.
Each chamber was connected to a reservoir of 200 ml of saline (NaCl 0.9% in purified water, MilliQ academic, Millipore, Zug, Switzerland) by a closed loop, ensuring sink conditions, and a spectrophotometer (HP 8453, Agilent Technologies AG, Basel, Switzerland). The flow rate was set at 5 ml/min (CY 6 pump, Sotax). Chambers with loaded beads were kept at a temperature of 37°C.

As an internal standard of total drug dissolution and release from the beads, ethanol was added to the Sotax apparatus when the dissolution plateau was reached, to achieve a final concentration of 30% V/V. Absorbance at 3 h after ethanol addition was chosen as a reference. We analyzed the obtained release profiles in terms of the time necessary to reach 50% and 75% of the maximum drug release and compared the profiles by means of the similarity factor $f_2$.

**Microsphere Morphometry.** Beads were imaged with a Zeiss Axiovert 200 microscope (Carl Zeiss, Feldbach, Switzerland). Coloration, homogeneity of loading and size alterations were observed visually. Attention was paid to the white field adjustment to ensure true color rendering. The size was measured using the open source image analysis software (ImageJ 1.38 software; NIH, Bethesda, MD, USA), analyzing an average of 150 microspheres in a monolayer. Statistical analysis was performed using a two-sided Student’s t-test, at a significance level $P = 0.001$.

### 7.2 Appendix B: Preparation of Sunitinib-Loaded Beads and Oral Solution

Syringes prefilled with sunitinib-loaded 100-300 μm sized beads were prepared under aseptic laminar air-flow conditions for pharmacokinetic studies in rabbits.

All consumables were autoclaved according European Pharmacopeia requirements. Sunitinib stock solution and 5% glucose solution were filter sterilized. Sunitinib-loaded beads, obtained as described in Appendix A, were resuspended in 5% glucose, aliquoted volumetrically and distributed in the syringes, resulting in a drug content of 6 mg per 0.2 ml beads per syringe. Beads were allowed to sediment to remove excess supernatant, and the syringes were capped and packed under sterile conditions. Blank beads were aliquoted similarly.

The oral sunitinib solution (1 mg/ml) was prepared by diluting the stock solution (10 mg/ml) with pre-filtered glucose 5% (w/v).