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Published in:
Nanomedicine: Nanotechnology, Biology and Medicine

Link to article, DOI:
10.1016/j.nano.2017.08.013

Publication date:
2018

Document Version
Peer reviewed version

Link back to DTU Orbit

Citation (APA):
Liposome accumulation in irradiated tumors display important tumor and dose dependent differences

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Received 29 March 2016; accepted 21 August 2017

Abstract

Radiation therapy may affect several important parameters in the tumor microenvironment and thereby influence the accumulation of liposomes by the enhanced permeability and retention (EPR)-effect. Here we investigate the effect of single dose radiation therapy on liposome tumor accumulation by PET/CT imaging using radiolabeled liposomes. Head and neck cancer xenografts (FaDu) and syngenic colorectal (CT26) cancer models were investigated. Radiotherapy displayed opposite effects in the two models. FaDu tumors displayed increased mean accumulation of liposomes for radiation doses up to 10 Gy, whereas CT26 tumors displayed a tendency for decreased accumulation. Tumor hypoxia was found negatively correlated to microregional distribution of liposomes. However, liposome distribution in relation to hypoxia was improved at lower radiation doses. The study reveals that the heterogeneity in liposome tumor accumulation between tumors and different radiation protocols are important factors that need to be taken into consideration to achieve optimal effect of liposome based radio-sensitizer therapy.

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Key words: Liposome; PET; Radiotherapy; Radio-sensitizer; Hypoxia

External beam radiation therapy (RT) is a central part of the treatment regimen for more than half of all cancer patients. Liposomal drug delivery systems that carry radio-senzitizers to tumors can potentially improve therapeutic efficacy of RT without increasing loco-regional side effects in the irradiated region.\textsuperscript{1,2} Combining targeted RT and targeted drug delivery can therefore increase regional tumor control.\textsuperscript{3} Moreover, liposomes\textsuperscript{4} are flexible in regards to the selection of drugs that can be encapsulated, transported and released within tumors. Liposomes can therefore serve as optimal delivery systems for targeting radiosensitizers to malignant tissue.\textsuperscript{1,2} However, liposome accumulation in solid tumors has been demonstrated to depend on multiple factors, including interstitial pressure, tumor vasculature and perfusion.\textsuperscript{4,5} Liposome extravasation by the enhanced permeability and retention (EPR) effect is primarily driven by transvascular convection and their accumulation is...
Methods

Tumor model

FaDu (human head and neck cancer) xenografts were established by subcutaneous injection of \(-5 \times 10^6\) cells suspended in 100 \(\mu\)l of culture medium over the thigh/flank of 7 weeks old female NMRI nude mice. Tumors were allowed to grow for 12-14 days. CT26 (murine colon cancer) syngeneic tumors were established by subcutaneous injection of \(-3 \times 10^5\) cells suspended in 100 \(\mu\)l of culture medium over the thigh/flank of 6 weeks old female Balb/c mice. Tumors were allowed to grow for 18 days. The National Animal Experiments Inspectorate approved all study procedures.

Radiolabeled liposomes

Pegylated liposomes consisting of HSPC:CHOL:DSPE-PEG2k (56.5:38.2:5.3) were remote loaded with the PET isotope \(^{64}\text{Cu}^{2+}\). Briefly, 100 nm 50 mM pegylated liposomes entrapping 14 \(10 \text{mM} \text{DOTA}\) were prepared as previously described. Radiolabelling was achieved by adding a volume of liposomes to dried \(^{64}\text{CuCl}_2\) followed by incubation at 55 °C for 75 min. The loading efficiency was afterward evaluated by Thin Layer Chromatography (Radio-TLC) and Size Exclusion Chromatography (Radio-SEC), which showed a loading efficiency of \(>98\%\) for both techniques. The liposomes were prepared at either 3.3 mM or 6.6 mM lipid concentration and an activity concentration of 62.5 \(\text{MBq/ml}\) or 125 \(\text{MBq/ml}\) (activity at the time of injection) for the FaDu and CT26 tumors respectively. Each animal was dosed with a volume corresponding to 22 \(\mu\)mol/kg and an activity of \(-12.5 \text{MBq/animal}\).

Radiation therapy

Mice carrying FaDu xenografts were randomized into four treatment groups; non-irradiated controls (n = 11), 5 Gy (n = 10), 10 Gy (n = 10) and 20 Gy (n = 11). Mice carrying CT26 tumors were randomized into four treatment groups; non-irradiated controls (n = 8), 2 Gy (n = 8), 5 Gy (n = 8) and 10 Gy (n = 8). Radiation therapy was delivered as a single fraction at a dose-rate of 1 Gy/min (320 kV, 12.5 mA) using a small animal irradiator (X-rad320, pXi, CT, USA). Mice were irradiated in a dedicated fixation device securing that only the tumor bearing leg was exposed to irradiation and the remaining body shielded.

MicroPET/CT imaging

PET/CT imaging was performed on an Inveon\textsuperscript{\textregistered} small animal PET/CT system (Siemens Medical Systems, PA, USA) approximately 24 h after completion of RT. Mice were anesthetized by inhalation anesthesia (\(-3\%\) sevoflurane) and \(^{64}\text{Cu}\)-liposomes injected into a tail vein. \(^{64}\text{Cu}\)-liposomes were allowed to distribute for 1 h before commencing a 5-min PET scan (1-h scan) followed by a corresponding CT scan. A similar PET/CT scan (15 min acquisition) was performed after a distribution period of 24 h (24-h scan). Emission data were corrected for dead time and decay and attenuation correction was performed based on the corresponding CT scan. PET scans were reconstructed using a maximum a posteriori (MAP) reconstruction algorithm (0.815 × 0.815 × 0.796 mm). Image analysis was performed using Inveon\textsuperscript{\textregistered} software (Siemens Medical Systems, PA, USA). 3D regions of interest (ROIs) were manually constructed and decay corrected data (%injected dose per gram tissue (%ID/g)) reported.

Immunohistochemistry CD31, cell density and necrosis

Immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded 4 \(\mu\)m tumor sections that were stained with H&E for histological evaluation and with CD31 antibodies for tumor blood vessels. CD31 staining was performed by...
heating sections at 60 °C (1 h) followed by deparaffinization
in xylene and rehydration. Antigen retrieval was performed by
microwave-based antigen retrieval. Endogeneous peroxidase
was blocked using peroxidase blocking reagent (Dako, Glostrup,
Denmark) for 8 min and sections blocked in 2% BSA for (10
min). Sections were incubated with primary CD31 antibody
(1:100) in 2% BSA (1.5 h/room temperature) followed by incubation with secondary biotinylated EnVision
FLEX™ (40 min) (Dako, Glostrup, Denmark). Tissue sections
were stained with DAB (10 min) and counterstained with
hematoxylin. Between all steps sections were rinsed in PBS.
Slides were mounted for electronic slide scanning (Axio scan,
Carl Zeiss, Germany) (pixel size 0.022 × 0.022 μm). Tumor
necrosis was evaluated using the Advanced Weka segmentation
plug-in for Fiji (ImageJ). The degree of necrosis in sections was
determined by drawing ROIs in necrotic, background/artifacts
and viable tumor region and transferring these to the trainable
classifier to determine necrotic and viable areas.

Ten regions were selected on CD31 stained sections and sent
for analysis of microvessel density by automated segmentation
algorithm for analysis of microvessels in immunostained
histological tumor sections (CancerImageMaNalysis: http://
www.caiman.org.uk).20,21 The regions were additionally trans-
ferred to Fiji (ImageJ) for determination of nuclear density. In
short, color deconvolution was performed to yield a separate
hematoxylin image and the nuclei density determined by
excluding fragments and artifacts by automated exclusion of
structures below a cut-off size of (50 pixels²).

64Cu-liposome autoradiography and hypoxia immunohisto-
chemistry

For analysis of intratumor distribution of liposomes and
hypoxia FaDu tumors (controls) were intravenously injected
with the radiolabeled liposomes and these were allowed to
distribute for 24 h before sacrificing and bleeding mice. To
further study the influence of radiation therapy on intratumor
hypoxia and liposome distribution, tumors from and two CT26
Q3 tumors from each group were subjected to autoradiography and
hypoxia immunohistochemistry (24 h distribution period for
liposomes). For hypoxia immunohistochemistry the exogenous
hypoxia marker Pimonidazole (60 mg (kg animal)⁻¹ in PBS),
was administered by intraperitoneal injection two hours before
sacrifice. After sacrificing and bleeding animals, tumors were
snap frozen and cryosectioned (8 μm) in cutting media. Sections
separated by at least 400 μm were thaw mounted on Superfrost
Plus microscopy slides. Seventeen sections from eight different
FaDu tumors and five sections from included CT26 tumors were
evaluated. Intratumoral distribution of 64Cu-liposomes was
determined by exposing tumor sections to phosphor imaging
screens for approximately 18 h (−20 °C). Phosphor screen was
read using a phosphor imaging system (Cyclone Plus, Perkin
Elmer, MA, USA) and semi-quantitative luminescence images
(pixel size 0.04 × 0.04 mm) were obtained.

Tumor sections were fixed in acetone (4 °C/10 min). Tissue
peroxidase was quenched using peroxidase blocker (Dako,
Glostrup, Denmark) and non-specific binding blocked using 2%
BSA. Pimonidazole immunohistochemistry was performed
using mouse monoclonal anti-pimonidazole antibody (Hypoxygen-robe, MA, USA) diluted in 2% BSA (1:600) (1 h) followed by
Secondary biotinylated anti-mouse antibody (40 min) (Envision
Flex, Dako, Glostrup, Denmark). Antibody binding was visualized using DAB and sections were counterstained with
hematoxylin (H) and slide scanned as described above.

ROIs, including viable tumor regions and excluding necrotic
tumor regions and artifacts were manually drawn (Fiji, ImageJ, NIH,
MD, USA). Automated DAB-H color deconvolution and manual
thresholding of pimonidazole IHC staining followed by image
binarization was performed in Fiji software. Autoradiography
images were co-registered to the corresponding pimonidazole
Pimonidazole values on rescaled image (autoradiography
resolution) represent mean level pimonidazole positive pixels
on the constructed binarized image. 64Cu-liposome autoradiog-
raphy pixels were categorized into four activity levels (0-0.25,
0.26-0.50, 0.51-0.75 and 0.76-1.0) relative to the individual slide
single pixel maximum. The corresponding mean pimonidazole
pixel values for the four 64Cu-Liposome categories were determined for each slide and bar-plots constructed.

Statistical analysis

Prism 7 (GraphPad Software, La Jolla, CA, USA) was used for
all statistical analysis. One-way or two-way ANOVA
analysis and Holm–Sidak multiple comparison test were applied
for comparisons of groups. All data are reported as mean ± SEM
(standard error of mean) unless otherwise stated and a P-value
<0.05 considered statistically significant.

Results

64Cu-liposome PET/CT after radiation therapy

Radiation therapy was successfully delivered to all mice 24 h
before administration of radiolabeled liposomes. The treatment
schedule was chosen to ensure that the acute effect of irradiation
was activity during the period of liposome distribution. To evaluate the effects of the different radiation doses we extracted
tumor activity levels of 64Cu-liposome PET data from the 24 h
corresponding PET/CT images from the 1-h and 24-h PET/CT
scans. Two PET scans were performed to extract information on
accumulation, as intravascular liposome activity is expected to
dominate the 1-h PET scan and liposomes that have extravasated
through fenestrated tumor blood vessels the 24-h scan. PET/CT
images from the 24-h scans from each treatment group are
illustrated in Figure 1, A-H.

FaDu tumors displayed no significant 64Cu-liposome activity
difference between controls and treatment groups at the 1-h PET/
CT (Figure 2, A and B). At the 24-h PET/CT FaDu tumors receiving 5 Gy and 10 Gy had significantly higher mean 64Cu-liposome activity compared to the control group, while no statistical difference was observed for the mean activity of the 20 Gy treatment group (Figure 2, C). For the comparison of the 24-h
maximum activity, only the 5 Gy treatment group was statistically higher than the control group (Figure 2, D). The 20

The influence of radiation on liposome accumulation was further investigated in the syngenic CT26 tumors. Following the higher radiosensitivity in comparison to FaDu tumors, an irradiation schedule of 2 Gy, 5 Gy and 10 Gy was chosen. Interestingly, for the CT26 tumors an inverse correlation between radiation dose and liposome accumulation was observed. There was significantly higher mean activity of liposomes in the control group compared to all treatment groups at the 1-h PET scan (Figure 2, F). The control group also displayed the highest maximum activity of $^{64}$Cu-liposomes at the 1-h scan although this was not significant in comparison to irradiated groups (Figure 2, F). These observations could indicate that a high level of damage was induced to intratumoral blood vessels that limit intravascular liposome blood activity. Opposite to the observations in FaDu tumors, the irradiated CT26 groups displayed lower activity levels in comparison to controls. This was however only statistically significant for the controls in comparison to the 5 Gy irradiated group (Figure 2, G and H). Based on the conflicting results of the liposome uptake in the two included tumor models we evaluated the effect of radiation dose on tumor parameters that are expected to influence liposome accumulation.

**Micro vessels, nuclear density and necrosis**

The levels of intratumoral necrosis, nuclear density and micro vessels were investigated on stained tumor sections. For the FaDu tumor we observed a higher level of intratumoral necrosis primarily in the central parts of the tumors whereas less and more scattered distribution of necrosis was observed for the CT26 tumors. FaDu non-irradiated controls displayed a mean intratumoral necrosis level of 21.6% (± 4.5) while CT26 tumors only displayed 11.0% (± 1.3). For both tumor types the level of intratumoral necrosis increased with higher doses of radiation, except for the comparison of the 5 Gy FaDu group and controls. However, only the 20 Gy FaDu group and the 5 Gy and 10 Gy CT26 groups and corresponding controls were significantly different (Figure 3, A and D). As liposome accumulation is not expected to occur in devascularized non-vital necrotic regions this could explain the observed lower activity in comparison to controls for the 20 Gy FaDu group and the irradiated groups of CT26 tumors.

Nuclear density was found to decrease with increasing radiation dose. The cell density in the treatment groups all, except for the 5 Gy FaDu group, displayed significantly lower cellular density compared to control groups for both tumor types (Figure 3, B and E). The lower cell density is expected to decrease interstitial pressure in tumors and therefore facilitate an easier extravasation of liposomes. However, this was correlated to an increased the overall $^{64}$Cu-liposome accumulation. Additionally, cell density could potentially be counteracted by pressure changes stimulated by radiation-induced inflammation, apoptosis, necrosis and acute microvessel damage.

The micro vessel density (MVD) was investigated to identify if blood vessel density could explain the observed liposome activity differences. The MVD displayed no significant difference between FaDu groups (Figure 3, C). Interestingly, the CT26 control group displayed significantly higher MVD than all irradiated groups (Figure 3, F). The higher mean $^{64}$Cu-liposome activity at the 1-h PET could potentially be explained by the higher microvessel density. However, for the 24-h scan this did not result in significantly higher activity, whereas the FaDu tumors displayed significantly higher activity levels for the 5 Gy and 10 Gy groups.

**Microregional distribution of $^{64}$Cu-liposomes and pimonidazole**

To investigate the potential of liposomal drug delivery system to improve therapeutic control of radio-resistant hypoxic tumor regions we compared the accumulation of radiolabeled liposomes to pimonidazole hypoxia immunohistochemistry. $^{64}$Cu-liposome autoradiographies were compared to of pimonidazole immunohistochemistry for non-irradiated FaDu tumor sections. The co-registration process and resizing of images allowed us to include seventeen sections in the analysis. The microregional pixel-to-pixel comparison of pimonidazole values and corresponding categorized $^{64}$Cu-liposome activity level identified that hypoxia decreases significantly with increasing (within slide) $^{64}$Cu-liposome activity (Figure 4, A-D). This observation is important for liposome based radiosensitizer therapy, as they may have limited access to important hypoxic regions at least for the liposomes under investigation. Following the observed influence of radiation cellular density and vascular function the influence of dose on pimonidazole positive fraction was investigated in CT26 tumors. We observed a significantly
lower fraction of pimonidazole positive pixel in the 2 Gy irradiated group, no difference for the 5 Gy group and a significantly increased positive fraction in the 10 Gy group relative to controls (Figure 4, E). To determine if the observed changes in tumor oxygenation could influence the distribution patterns of liposomes relative to microregional hypoxia a comparison of $^{64}$Cu-liposome activity level and pimonidazole was performed. The control group displayed an inverse correlation that was comparable to that of non-irradiated FaDu tumors (Figure 4, F). However, the 2 Gy and 5 Gy-irradiated groups displayed an almost similar level of hypoxia in the different levels of $^{64}$Cu-liposome activity, which could indicate that these dose ranges can potentially both decrease levels of hypoxia and improve liposome accumulation in regards to hypoxic areas. This must of course be weighed against the overall accumulation of $^{64}$Cu-liposomes.

**Discussion**

The therapeutic combination of tumor targeting liposome-encapsulated radiosensitizers and radiation therapy holds great clinical potential following the dual tumor targeting properties. Notwithstanding this potential, the direct link between the parameters of central importance for liposome accumulation and the effects of radiation therapy makes the determination of optimal timing of radiation and dose and liposome administration important.

The two cancer models yielded opposite results in respect to liposomes accumulation. Whereas radiation improved accumulation in FaDu xenografts after 24-h (5 Gy and 10 Gy groups), the CT26 tumors displayed an insignificant decrease in liposome accumulation after radiation. These observations are interesting in respect to the study in human KB cancer xenografts where no effect, negative or positive, on liposome uptake was observed for radiation doses from 5 to 20 Gy evaluated invasively from 1 to 96 h after irradiation. Both cancer models displayed an increase in intratumoral necrosis and decreased cell density following irradiation, both of which were most significant for the CT26 tumors. Interestingly, MVD was found to respond very differently to irradiation between the models. Irradiation significantly decreased MVD in CT26 while FaDu tumors did not display changes or patterns in relation to radiation. This was also illustrated by the mean $^{64}$Cu-liposome activity between groups after a circulation period of only 1-h. In clinical head and neck squamous cell carcinomas a decrease in MVD was correlated to an improved response and overall survival. In light of this, our results indicate that the FaDu tumors represent a more radio-resistant tumor and that adjuvant liposomal radiosensitizer therapy could be beneficial, at least from a dose accumulation perspective for tumors maintaining a high MVD during irradiation. Based on the differences in liposomes accumulation and the histological analysis, accumulation appeared directly dependent on a high MVD. This observation is in agreement with previous publications identifying, blood flow as the rate limiting step for liposome extravasation in tumors with a high vascular permeability. However, irradiation can decrease nuclear density and damage vascular structures to potentially increase liposome accumulation by lowering IFP and facilitating transvascular extravasation. No direct measures for IFP in addition to nuclear density were performed but in a previous report on irradiation of colon carcinoma xenografts single fractions of 10 Gy significantly lowered IFP in tumor. From our results, the differences between MVD response and comparable decrease in nuclear density between FaDu and CT26 tumors indicate that the MVD is the most important parameter.
influence liposome accumulation. Compatible results were obtained for non-small cell lung cancer patients receiving adjuvant liposomal doxorubicin to fractionated radiotherapy where MVD was associated with increased accumulation and therapeutic efficacy. However, the association of MVD to hypoxia could also influence this observation as discussed below. Interestingly, the increased liposome accumulation for irradiated FaDu tumors could also result from a decreased IFP which may improve tumor perfusion by alleviating pressure dependent collapse of intratumoral vessels. Importantly, the optimal timing of liposomal drug administration in relation to fractionated radiation remains to be determined and the reported negative impact of RT five days after irradiation indicates that timing is central for optimization of liposome accumulation. Based on our observations improving liposome accumulation is a balance between maintaining functional blood vessels and improving intratumoral blood flow as discussed in recent literature. However, the heterogeneous response of different tumor models, in regards to these parameters, highlights the value of directly quantitative PET imaging using radiolabeled liposomes.

Single doses of (≥10 Gy) RT induce high levels of vascular damage that leads to secondary cell death when areas become deprived of oxygen and nutrients. On the other hand, fractionated low dose irradiation of tumors has been associated with improved perfusion and reoxygenation. The tumor sections evaluated from the 2 Gy and 5 Gy CT26 groups displayed less hypoxia across all levels of liposome activity, which is in line with reports on early reoxygenation after low dose irradiation. This indicates that the low dose irradiation, at least for the CT26 tumors, improves vascular perfusion and tumor oxygenation and provides the basis for a more homogeneous distribution of liposomes. The effect of radiation therapy can therefore potentially also improve liposome penetration and the potential of targeting liposomes that suffers from inability to reach their target if trapped in the perivascular regions. Considering the importance of hypoxia and its intricate link to vascularization, optimized radiation schedules can potentially improve the distribution of liposomes in radioresistant hypoxic region. Liposomal doxorubicin has been reported to increase radiosensitivity in hypoxic prostate cancer xenografts in one study where clamping of the tumor-bearing leg was used to induce hypoxia during RT. However, liposomes were administered prior to clamping and the study therefore provides no evidence that doxorubicin reaches regions of perfusion and diffusion limited hypoxia, but highlights the potential of liposomal chemoradiotherapy.

Liposomal doxorubicin and cisplatin, injected 16 h before irradiation, increased the therapeutic efficacy for 4.5 Gy single dose and 9 Gy/3 fractions but not a single dose of 9 Gy radiotherapy in KB head and neck cancer xenografts. No benefit was observed from dosing liposomes as a single compared to multiple injections of the same dose and the authors were not able to determine if the effects observed were truly radiosensitizing or additive, which highlights the importance of timing to achieve a supra-additive effect chemoradiotherapy.

**Conclusion**

The present study was conducted using a radiolabeled liposome imaging system that provided quantitative data on...
luminal accumulation as a function of RT. The study identifies that RT may influence the EPR effect and liposome accumulation in a tumor and dose dependent manner. This observation emphasizes that the $^{64}$Cu-liposome PET imaging system may provide a functional tool to identify patients and treatment combinations and kinetics that may benefit from liposomal drug delivery in relation to radiation therapy. Future studies of liposomal drug delivery systems for radiosensitizers focusing on the correlation between liposome accumulation in tumor tissue as a function of RT and the therapeutic effect induced are highly warranted.

### References


