Harnessing competing endocytic pathways for overcoming the tumor-blood barrier: MRI and NIR imaging of bifunctional contrast media

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Introduction:

Targeting of therapeutics and contrast media to tumors is limited by unfavorable biodistribution. We previously reported the exclusion of intravenously administered biotin-BSA-GdDTPA to stroma tracks in ovarian tumor xenografts (1, 2). Within the stroma tracks, biotin-BSA-GdDTPA was effectively internalized by the myofibroblasts by caveolae mediated uptake, which could be suppressed by treatment with nystatin (1). Upon internalization, the material is sequestered within small intracellular vacuoles leading to suppressed MRI relaxivity, which is regained only after the material is redistributed in the cells with cell division (1, 2). The contrast material does not partition to the interstitial space within the tumor nodules (1). We aimed to alter the distribution of biotin-BSA-GdDTPA, and to enhance the partition of the contrast material to the tumor by addition of daidzein as a targeting ligand, recently reported to show high affinity to the ovarian carcinoma cells (3). The use of daidzein-BSA-GdDTPA/CyTE-777 (or FAM) as a bifunctional targeted contrast media revealed a novel mechanism regulating endocytosis by ovarian cancer cells, involving competition between receptor mediated internalization through binding of daidzein and caveolae mediated internalization via binding of albumin.

Materials and methods:

MRI measurements: MRI experiments were performed on a horizontal 4.7 T Bruker Biospec spectrometer using an actively radio-frequency decoupled 1.5 cm surface coil embedded in a Perspex board (for *in-vitro*) and a birdcage transmission coil (for *in-vivo)*. *In-vitro:* R1 measurements spin echo images were acquired at 8 different repetition times ranging between 2000 and 100 ms; 2 averages, field of view 4X4 cm, slice thickness 1 mm, matrix 128 X 128). *In-vivo*: T1 weighted 3D gradient-echo (GE) images, with pulse flip angles of 50,150,300, 500 and 700 were acquired to determined the R1 values. The acquisition parameters: TR 10ms; TE 3.561ms; 2 averages; field of view 4X4X2 cm; 128X128X128 pixels.

MRI in-vivo model: The tumor bearing mice were injected with BSA-GdDTPA or daidzein-BSA-GdDTPA. R1 was measured 24h, 48h and 72h after injection of the contrast material.

Fluorescence microscopy**:** MLS cells were incubated with Daidzein-BSA-FAM or BSA-FAM (200 µg/ml) or combination of both (Daidzein-BSA-FAM with BSA-ROX) in the presence or absence of a blocking dose of nystatin (50 µg/ml). The excess of fluorescent material was washed 3 times with PBS, and the cells were fixed with 4% PFA, then washed, and stained with DAPI and mounted. The images were monitored by two-photon and by confocal microscopy (2PM; Zeiss LSM 510 META NLO).

Results:

Uptake of daidzein-BSA-FAM by ovarian carcinoma cells: Binding and uptake of BSA-FAM and daidzein-BSA-FAM by MLS human epithelial ovarian carcinoma cells could be detected by two-photon and by confocal fluorescence microscopy, showing internalization of the fluorescent probes into intracellular vesicles. Suppression of caveolae mediated uptake by treatment of the cells with nystatin, resulted in slightly reduced uptake of BSA-FAM and in significantly enhanced internalization of daidzein-BSA-FAM by MLS cells. The distribution of contrast material inside the tumor was visualized by fluorescence microscopy in tumors isolated 24 after injection with daidzein-BSA-FAM or BSA-FAM. Daidzein-BSA-FAM was localized in the tumor cells areas, while BSA-FAM that was localized to tumor blood vessels.

In vivo MRI detection of targeted delivery of daidzein-BSA-CyTE-777 to ovarian carcinoma: Analog contrast media was synthesized to allow detection of targeted tumor delivery by MRI, by conjugation of GdDTPA to daidzein-BSA.MRI data acquired from MLS tumor bearing mice 24h after administration of daidzein-BSA-GdDTPA or BSA-GdDTPA (12 mg/200 µl), showed significantly higher accumulation of daidzein-BSA-GdDTPA in the tumor site as compared to vehicle-injected mice or mice injected with BSA-GdDTPA (Figure 1 B,C). Time course experiments showed specific localization and retention of targeted contrast agent (daidzein-BSA-GdDTPA) in the tumor site 24, 48 and 72h after injection as compared to controls or mice injected with non targeted contrast agent (BSA-GdDTPA). Statistically significant elevation of R_1 relaxation was visualized in tumors injected with daidzein-BSA-GdDTPA as compared to non injected animals or mice injected with BSA-GdDTPA.

Conclusions: We show presence of two competing endocytic pathways for uptake of targeted bifunctional daidzein-BSA MRI/NIR contrast media to human ovarian carcinoma cells. The ability to manipulate between this pathways by nystatin or BSA, augments both *in-vivo* and *in-vitro* the distribution and endocytosis of the contrast material to the cancer cells.

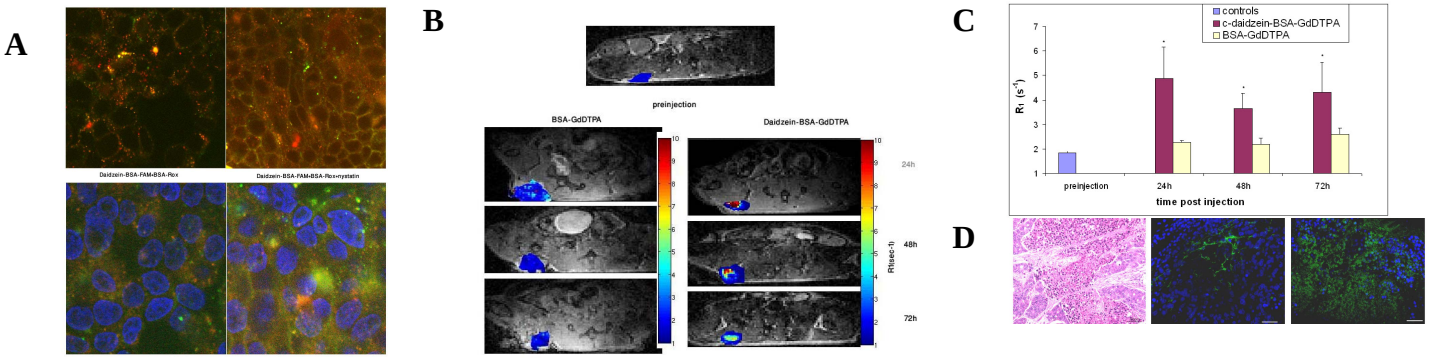

Figure 1: **Bifunctional targeted imaging of ovarian carcinoma. A) Specific binding and endocytosis of Daidzein-BSA-FAM by MLS ovarian carcinoma cells** (competition experiment- Daidzein-BSA-FAM and BSA-ROX uptake without nystatin (left) and with nystatin (right)). **B), C) Specific localization and retention of the contrast material inside the tumor:** MLS bearing mice were administered intravenously with 12mg BSA-GdDTPA (left) or daidzein-BSA-GdDTPA (right). T1 weighted MRI images and R1 maps were obtained 24, 48 and 72h after injection, and used for derivation of mean R1 values for the tumor. ROI analysis of MLS tumors of mice injected with BSA-GdDTPA (left) or daidzein-BSA-GdDTPA (right) and control (preinjection) in comparison of treatments. Student ttest $p<0.05$ (n=5-7). **D) Ex-vivo characterization of contrast material distribution inside the tumor**: H&E (left), BSA-FAM (middle), Daidzein-BSA-FAM (right).

References:

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