### Study of the Targeting Ability of Anticancer Drug-Loaded Polymeric Micelles to Metastatic Cancer

![Image](http://doi.org/10.15083/00006829)

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Metastasis is the main cause of death from cancer and current clinical therapies display inadequate activity against metastatic disease. In this way, efficient therapies against metastases should target the later stages of the metastatic cascade, including disseminated dormant cells, preangiogenic micrometastases and vascularized metastases, and therapies limiting the survival of the metastatic foci have the potential to achieve the greatest clinical benefit. In this way, targeted therapies by using nano-scaled drug carriers can provide selective delivery of bioactive payloads to the metastatic disease, being capable of achieving the utmost therapeutic impact.

Nanocarriers can selectively accumulate in solid tumors due to the enhanced permeability and retention (EPR) effect, that is, the increased permeability of the tumor blood vessels, and their enhanced retention as a result of the impaired lymphatic drainage. In this way, several studies have reported that nanocarriers can also accumulate in vascularized metastases. Nevertheless, even though the transition between preangiogenic metastases and vascularized metastases is an important target in the metastatic cascade, the ability of nanocarriers for reaching preangiogenic micrometastases is not clear. Because these preangiogenic micrometastases do not present leaky neovasculature, the accumulation of nanocarriers cannot be expected to occur by EPR effect. However, preangiogenic micrometastases may display inflammatory microenvironment induced by metastatic cells, metastasis-associated immune cells and stromal cells, which can produce vascular permeability. Thus, systemically administered nanocarriers may target these preangiogenic metastatic sites
by taking advantage of such inflammatory microenvironment.

Polymeric micelles have demonstrated unique advantages as nanocarriers for selective delivery of their cargo to solid tumors, including a biocompatible PEG shell, relatively small size, extended circulation in the bloodstream and augmented accumulation in tumors tissues. Accordingly, several drug-loaded micelles are under clinical evaluation, showing high efficacy and reduced side effects. Current thesis focuses on the targeting ability of polymeric micelles against vascularized and preangiogenic metastases.

Because liver is the most frequent organ for metastasis, the activity of the micelles was studied in bioluminescent syngeneic models of liver metastasis, which were prepared by inoculating bioluminescent murine colon adenocarcinoma C26 expressing luciferase (C26-luc) into the spleen of Balb/c mice. C26-luc cells were established by transfecting the luciferase gene through Lipofectamine. Moreover, since oxaliplatin is the first-line clinical treatment for colorectal metastatic cancer, polymeric micelles loading the parent complex of oxaliplatin, i.e. (1,2-diaminocyclohexane)platinum(II) (DACHPt), in their core were selected for this study. DACHPt-loaded micelles (DACHPt/m) have shown enhanced antitumor activity in several tumor models, and have recently proceeded to clinical evaluation. To prepare DACHPt/m, poly(ethylene glycol)-b-poly(glutamic acid) copolymer and DACHPt were mixed in water, self-assembling into core-shell micellar structures after the polymer-metal complex formation between the carboxylates in poly(glutamic acid) and the Pt ions.

The activity of the micelles was first confirmed in a model orthotopic colon cancer. Thus, $5 \times 10^4$ C26-luc cells were injected into the colonic wall of Balb/c mice to develop orthotopic colon cancer and the treatment started 5 days after inoculation. After 3 treatments of DACHPt/m at 4 mg/kg, oxaliplatin at 8 mg/kg or PBS at days 0, 2 and 4, DACHPt/m showed significant anticancer efficacy as compared with PBS or oxaliplatin ($P < 0.05$). Also, 24 h after injecting fluorescence-labeled DACHPt/m, colocalization of luminescence from C26-luc colon tumor and fluorescence from DACHPt/m was observed ex vivo.

To establish liver metastatic model of colon cancer, $5 \times 10^4$ C26-luc cells were injected into the spleens of Balb/c mice. Twenty-four hours post-inoculation, the spleens were removed to prevent splenic tumor formation. Stages of metastatic models were determined by histology. Metastatic model before day 6 was considered as
preangiogenic due to their relative small size (less than 300 µm), while model after day 6 was vascularized (larger than 300 µm). Thus, 9 day after inoculation, DACHPt/m at 3 mg/kg, oxaliplatin at 8 mg/kg or PBS at days 0, 3 and 6 were injected to treat the overt metastases. Also, DACHPt/m showed significant anticancer efficacy against this model (P < 0.05), and specific tumor accumulation of fluorescent-labeled micelles was observed ex vivo.

To treat preangiogenic metastases, DACHPt/m at 3 mg/kg, oxaliplatin at 4 mg/kg or PBS were injected 3 days after inoculation. Again, significant anticancer effect was observed for DACHPt/m (P < 0.05). Moreover, as it has been previously observed that the size of DACHPt/m affect the penetration and activity in hypopermeable tumors, DACHPt/m having 30- and 70-nm diameter were prepared and evaluated against preangiogenic metastases. Accordingly, 30- and 70-nm DACHPt/m presented similar anticancer efficacy and accumulating ability studies, suggesting that the targeting ability of DACHPt/m in this tumor model does not rely on their size. To observe DACHPt/m accumulation in high resolution, in vivo confocal laser scanning microscopy was used, allowing the detection of micrometastatic niches of approximately 200 µm, which includes C26 cells expressing green fluorescence protein (C26-GFP) and their microenvironment with morphology distinct from liver tissue. Real time video microscopy showed that fluorescent DACHPt/m circulated outside the metastatic niche at first, but then gradually penetrated into the niche. Eighteen hours later, specific accumulation was found in both cancer cells and surrounding microenvironment, supporting the enhanced activity of the micelles against these preangiogenic metastases.

The characteristics of the microenvironment of the metastatic niches were investigated. Firstly, COX-2 levels were evaluated in metastatic sites by injecting COX-2 probe, which is used as an inflammation marker. Four hours after injection, the metastatic microenvironment was distinctly marked by the COX-2 probe, indicating the inflammatory status of the metastatic niche. It has been reported for a similar metastatic model that Kupffer cells/macrophages and activated hepatic stellate cells (HSCs) were critical for angiogenesis and development of macrometastasis. Thus, the presence of Kupffer cells and activated HSCs was determined by immunohistochemistry of the micrometastases of C26-luc cells. Kupffer cells, which were marked with anti-CD68, and activated HSCs, which were labeled with anti-α-smooth muscle actin antibodies, were distributed within C26-luc metastasis (labeled with anti-cytokeratin 20 antibody). Because macrophages and activated HSCs can increase COX-2 levels in the tumor microenvironment, they may mediate the inflammatory reaction in preangiongenic metastatic niches. Moreover, in another independent experiment, 24 h after injection,
both COX-2 probe and fluorescent-labeled DACHPt/m were found to specifically co-localize within the metastatic niche. In the same mouse, metastatic niches without signal from COX-2 probe were also found. In those COX-2 negative niches, no accumulation of DACHPt/m was observed. Thus, the enhanced accumulation of DACHPt/m in the preangiogenic metastatic niches was hypothesized to be affected by COX-2-mediated inflammatory conditions.

To study the role of inflammation in the targeting of DACHPt/m to preangiogenic metastases, mice bearing C26-GFP metastases were pretreated with celecoxib, which is a selective COX-2 inhibitor, and the antitumor activity and accumulation of the micelles was studied. While the combination of celecoxib and oxaliplatin showed improved therapeutic effect, the efficacy of DACHPt/m was reduced after pretreatment with celecoxib. Moreover, from in vivo confocal microscopies of metastases pretreated with celecoxib, neither COX-2 probe nor fluorescent DACHPt/m accumulated in the micrometastases, even 16 h after injection. This preclusion of DACHPt/m accumulation and anticancer efficacy suggested that the targeting ability of DACHPt/m against preangiogenic metastases is associated with the inflammatory conditions of the metastatic niche.

Our findings proved that DACHPt/m were effective against both vascularized overt metastasis and preangiogenic liver metastasis. While the enhanced activity of the micelles against vascularized metastasis can be associated with the EPR effect, the ability of the micelles to target preangiogenic metastases was associated with the COX-2-related inflammatory conditions of the niches. To the best of our knowledge, this thesis represents the first demonstration of the targeting capability of systemically injected nanocarriers to preangiogenic metastasis. Such capability may offer a novel strategy for efficient diagnosis and therapy of metastases.