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***In-vivo* fluorescence lifetime imaging for monitoring the efficacy of the cancer treatment**

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Abstract

Purpose—Advances in tumor biology created a foundation for targeted therapy aimed at inactivation of specific molecular mechanisms responsible for cell malignancy. In this paper, we used *in-vivo* fluorescence lifetime imaging with HER2 targeted fluorescent probes as an alternative imaging method to investigate the efficacy of targeted therapy with 17-DMAG (an HSP90 inhibitor) on tumors with high expression of HER2 receptors.

Experimental Design—HER2-specific Affibody, conjugated to Alexafluor 750, was injected into nude mice, bearing HER2-positive tumor xenograft. The fluorescence lifetime was measured before treatment and monitored after the probe injections at 12 hours after the last treatment dose, when the response to the 17-DMAG therapy was the most pronounced as well as a week after the last treatment when the tumors grew back almost to their pre-treatment size.

Results—Imaging results showed significant difference between the fluorescence lifetimes at the tumor and the contralateral site (~0.13ns) in the control group (before treatment) and 7 days after the last treatment when the tumors grew back to their pretreatment dimensions. However, at the time frame that the treatment had its maximum effect (12 hours after the last treatment) the difference between the fluorescence lifetime at the tumor and contralateral site decreased to 0.03ns.

Conclusions—The results showed a good correlation between fluorescence lifetime and the efficacy of the treatment. These findings show that *in-vivo* fluorescence lifetime imaging can be used as a promising molecular imaging tool for monitoring the treatment outcome in preclinical models and potentially in patients.

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Keywords

In-vivo fluorescence lifetime imaging; Near-infrared optical imaging; Targeted fluorescent probe; Cancer treatment; Human epidermal growth factor receptor 2

Introduction

Detection of specific oncogenic biomarkers is one of the most important requirements for choosing the proper type of targeted cancer therapy. Development of the drugs, targeting specific tumor receptors, such as monoclonal antibodies (mAb), has opened exciting opportunities to selectively attack the cancer-causing biomarkers, inactivate molecular mechanisms responsible for cell malignancy, and deliver the toxin specifically to the malignant cells (1-5). Recent advances in therapeutic mAb show that their efficacy depends strongly on the expression of tumor-specific biomarkers. Meanwhile, currently, the effectiveness of therapy is generally assessed either by *ex-vivo* tests of biopsied tissues, anatomic (e.g. CT, MRI) or metabolic (FDG-PET) comparison of disease progression before and after a complete therapeutic cycle. There is a huge demand for monitoring the effect of the drug on cancer biomarkers during the therapeutic cycles to evaluate the patient response to the therapy and optimize the course of the treatment, especially at its early stages.

Current diagnostic gold standards for detection of specific cancer biomarkers are all based on *exvivo* methods, such as immunohistochemistry (IHC) and fluorescent *in situ* hybridization (FISH). All these semi-quantitative methods are invasive and require biopsies from the patients (6). Inherently, diagnostic value of biopsies is limited by the heterogeneity of the malignant lesion (see, for example, the case of Human Epidermal Growth Factor 2 (HER2) distribution for N87 tumor in Fig. 1). In addition, the number of times that the biopsy can be taken during the therapeutic cycle is small; therefore it can hardly be used to monitor the therapeutic outcome. The general goal is to replace current methods with a minimally invasive imaging modality, reducing the time between imaging and diagnosis, and facilitate analysis of treatment progression in the clinic with portable and accessible systems. Molecular imaging, using targeted probes (7-11), can be used to assess the status of specific cancer receptors *in-vivo* and differentiate the malignant lesions based on their molecular specifications. This can be realized either with positron emission tomography (PET) (12, 13) or optical imaging (14-16). Though optical modality is limited by tissue penetration depth (in near infrared (NIR) region up to several cm), it is a minimally invasive, low cost modality without ionizing radiation. It is best suited for clinical applications such as non-invasive imaging of breast, head/neck, and skin cancer or finding the tumor and its margins during the surgery (17); and it can be extended for different oncogenic receptors (16). Optical imaging can benefit from using different colors of fluorescence proteins (10, 11) and/or exogenous targeted fluorescent probes (18) simultaneously to observe different receptors in cancer cells. High quantum yield NIR fluorescent dyes can be conjugated with high affinity targeted molecular probes (19, 20) and combined with fluorescence imaging and mathematical models for data analysis to create a platform for a new “image and treat paradigm”. In pre-clinical studies (small animal models), NIR imaging, using biomarker-

specific probes, can be applied for screening and testing the efficacy of different therapies/drugs to treat various cancers (15).

Due to the leaky vascularization of the tumor and its Enhanced Permeability and Retention (EPR) effect, using fluorescence intensity alone has limitations (21, 22). It is difficult to establish that the detected fluorescence intensity originates from the bound targeted fluorescent agents to specific cancer receptor or it comes from accumulated free fluorophores inside the tumor due to its EPR effect and leaky vascularization. On the other hand, even though fluorescence intensity based imaging methods have been successfully applied for analysis of some biomarkers *in vivo* (23-26), to be quantitative they require sequences of images at different time points to separate bound and unbound fluorophores and use pharmacokinetics of fluorescence intensity to take into account the accumulation and washout of the probe from blood circulation.

Fluorescence lifetime imaging is an alternative non-invasive *in-vivo* imaging approach, based on the time-resolved fluorescence data collection after a short excitation laser impulse. It maps the exponential decay rates (i.e. lifetime) of the fluorescence signal (27, 28). Fluorescence lifetime imaging can take advantage of environmental sensitivity of fluorescent contrast agents (e.g. to temperature or pH) and/or their interaction with other molecules (e.g. binding to cancer cell receptors) without dependence on dye concentration in tissues. This phenomenon is unique in optical imaging and can provide significant benefit relative to intensity-based methods including both optical and nuclear imaging. Here we pursue the latest possibility to relate the fluorescence lifetime with binding of the HER2-specific fluorescent contrast agent to HER2 receptors. From only one time point measurement after probe injection (which is more convenient for patients in clinic), fluorescence lifetime can evaluate the fraction of bound to total fluorescent probes in the tumor *in vivo* to separate high and low HER2 expression tumors (29) and follow the efficacy of targeted therapy. In addition, using other types of probes (e.g. pH sensitive dye), it can provide valuable information on cancer cell environment and characteristics of its abnormalities (30, 31).

In this study, we investigated the feasibility of fluorescence lifetime imaging to monitor *in-vivo* expression of the HER2 receptor in the breast carcinoma (mouse model) during the course of treatment. Amplification of HER2 gene and overexpression of HER2 receptor has been diagnosed in approximately 20% of invasive breast cancer cases (32). It is one of the most important factors in poor prognosis and resistance to traditional chemotherapy treatments in cancer patients (32, 33).

In our previous study (29), we showed that fluorescence lifetime can be used for a preliminary assessment of HER2 expression in tumor. We have observed *in-vivo* that binding of the monomer Affibody-based fluorescent probe to HER2 receptors significantly reduces the fluorescence lifetime of the probe in the mouse model of human tumors. The results showed considerable differences in the fluorescence lifetimes between the tumor area and the contralateral site, when and only when, the binding of optical probe to the tumor receptors could occur (HER2-specific Affibody (His₆-Z_{HER2}:GS-Cys)-DyLight 750 conjugate assessing the tumor xenograft with high HER2 expression).

To the contrary, no change in fluorescence lifetime was observed in the cases where the optical probes had no affinity to the HER2 receptors either because the tumor did not have HER2 receptors (HER2 negative) or the Affibody probe was non-HER2-specific (Affibody (His₆-Z_{Taq}:GS-Cys)-DyLight 750 assessing the same tumor xenograft with high HER2 expression).

In this study, we investigated the relation between the efficacy of the HER2-targeting treatment and the fluorescence lifetime. In these experiments, we used a HER2-Affibody fused to albumin binding domain (ABD) that has been described before (26). This dimmer Affibody probe shows high affinity to the receptor and stays longer in the blood. The results show that the difference between the fluorescence lifetime at the tumor and contralateral site has a good correlation with the treatment outcome and it can be used as early indicator of HER2 downregulation due to the treatment.

Materials and Methods

HER2 targeted contrast agent and therapeutic agent

In these studies, the HER2-specific Affibody® molecule with albumin binding domain, ABD-(Z_{HER2:342})₂-Cys, was used which was kindly provided by Affibody Co. through our Cooperative Research and Development Agreement (CRADA). The ABD-HER2-Affibody was conjugated with AlexaFluor 750 with maleimide linker (Invitrogen, Eugene, OR). Detail of the conjugation process can be found in (26). 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) (InvivoGen, San Diego, CA), which is known as a heat shock protein 90 (HSP90) inhibitor, was used as therapeutic agent. It is known from the literature (34-36) that this drug (like a similar inhibitor 17-AAG) results in HER2 degradation due to HSP90 inhibition.

Animal model

BT-474 human breast carcinoma xenograft (ATCC; Manassas, VA), characterized by very high levels of HER2 expression, and Human glioblastoma U-251 MG cells (were kindly provided by Dr. Kevin Camphausen, ROB, NCI, Bethesda, MD), characterized by no-HER2 expression were chosen for our studies. Both cell lines were tested with Western blots in order to confirm the HER2 protein expression levels.

We prepared working cell banks (usually around 10 vials) from each cell vial with the use of recommended culture media. The cell lines were used within 6 months after received from cell banks. Quality control and authentication procedures that were conducted with ATCC includes post freeze viability, growth properties, morphology, mycoplasma using the Hoechst DNA stain (indirect method) and agar culture (direct method). They also performed the species determination test using isoenzyme assay for interspecies and STR analysis (unique DNA profile).

The cells were grown in RPMI culture media supplemented with 10% fetal bovine serum (FBS) and Pen/Strep (10,000 U penicillin, 10 mg streptomycin) at 37°C and 5% CO₂ in a humidified incubator. Solution of 0.05% trypsin and 0.02% EDTA was used for cells detachment.

Eight million cells were injected in 0.1 mL of 50% Matrigel (BD Biosciences, Bedford, MA) into the right forelimb of five to eight weeks old athymic nude mice (nu/nu genotype, BALB/c, NCR, NCI-Frederick, Frederick, MD). Growth of BT-474 cell lines was facilitated by s.c. implantation of estrogen pellets (0.72 mg, 90 days release, Innovative Research of America, Sarasota, FL) 24 hours prior to cells injection. The study was approved by National Institutes of Health Animal Care and Use Committee (Approval ID: ROB117). In this preliminary study, we used subcutaneous tumors at mice forelimb instead of orthotropic tumor (37, 38) to image the tumor and contralateral site without the influence of the background fluorescence of other organs like kidney, liver and bladder.

Five mice were randomly assigned to one of three groups: (i) control, before the treatment; (ii) imaging started 12 hours after completion of the last treatment; and (iii) imaging started 7 days after completion of the last treatment. Tumor volume was measured periodically with calipers. Intraperitoneal (i.p.) injection of 17-DMAG was initiated, when mean tumor volume had reached approximately 400 mm³. To monitor the effects of HSP90 inhibition, two groups of mice were treated with four doses of 17-DMAG (40 mg/kg) 24 h apart.

***In-vivo* time-resolved near infrared fluorescence imaging**

Fluorescence intensity and lifetime was quantified using our NIR fluorescence small-animal imager (39). Briefly, the system is based on a time-domain technique, where an advanced time-correlated single-photon counting device is used in conjunction with a high-speed repetition-rate tunable laser to detect individual photons. It contains a photomultiplier tube, used as a detector, a temperature-controlled scanning stage with an electrocardiogram and temperature monitoring device for small animals, and a scanning head. The scanning head consists of multimode optical fibers that are used to deliver light from an excitation source to the region of interest (ROI) and an emitted fluorescence signal back to detectors. The imager has a laser source for fluorescence excitation ($\lambda = 750$ nm), an emission filter ($\lambda = 780$ nm) for fluorescence detection. The imager scans in a raster pattern over the tissue surfaces to produce a real-time, two-dimensional image of the ROI. A cooled, charge-coupled device (CCD) camera is used to locate the tumor and guide the scanner to the ROI by measuring the fluorescence intensity distribution over the mouse body.

Before imaging, the mouse was anesthetized by isoflurane inhalation. The mouse was placed on a temperature-controlled stage and two sets of images from the tumor region and contralateral site were captured before injection. Then, 10 μ g of the ABD-HER2-Affibody conjugated to Alexafluor750 was injected through the tail vein. Images were captured continuously every 3 hours.

Lifetime estimates

To calculate the fluorescence lifetime we approximated the time-resolved decay of the fluorescent signal with one-exponent assumption. We have calculated the fluorescence lifetime, assuming that the effect of the impulse response function (IRF) of the system is negligible (See supplementary information – Fig. S1a). To substantiate this approximation, we performed a numerical simulation of the data collection, combining the IRF exponential decay rate (210ps) of our system and expected minimum lifetime of the fluorescent imaging

probe (600ps). The exponential decay rate of the convolution of the IRF with the fluorescence signal was 0.6017 ns which has only 0.28% error compared to the actual fluorescence lifetime (See supplementary information – Fig. S1b). Since slope calculation was faster and more robust, for lifetime estimates we calculated the exponential decay rate by regression of the linear part of the signal (in logarithmic scale).

Each pixel covers the intensity of 2x2 (mm²), which is much smaller than the size of the tumor. The fluorescence intensity and lifetime at tumor site and contralateral site were averaged over 6 pixels and 16 pixels, respectively to reduce the effect of noise.

Results

The fluorescence signal at the tumor and contralateral sites were captured continuously every 3 hours for the first 16 hours after injection of ABD-HER2-Affibody conjugated to AlexaFluor 750. We observed very stable values of the fluorescence lifetime at the contralateral site, in all three groups, (before, 12 hours and 7 days after the last treatment) and over the 16 hours monitoring, while significant variations in observed fluorescence intensity between different mice and during this time range were present (due to probe washout).

As an example, Figures 2a and 2b show the lifetime at the contralateral site for two mice before the treatment. Even though, the amplitudes of the two signals were very different (fluorescence intensity in mouse #1 is 240% higher than mouse #2); their lifetime difference was less than $\pm 1.9\%$ at different time points.

Figures 2c and 2d show the average amplitude and fluorescence lifetime at the contralateral site for five mice before, 12 hours and 7 days after the last treatment. These results also demonstrate that the fluorescence lifetime at the contralateral site was very consistent among different mice and different time points, even though corresponding fluorescence intensities were very different.

The fluorescence lifetime was measured at the tumor and contralateral sites of each mouse at different time points. Figure 3a shows the average fluorescence lifetime at the tumor site as a function of time after the probe injection. Comparing these data with the data from contralateral site one could see that for the control group of mice (before treatment) and the mice, imaged 7 days after treatment, there is a significant difference in the lifetimes between tumor and contralateral site ~ 0.13 ns, which stays the same after 3 hours post probe injection (Fig. 3b). Meanwhile, for the group of mice that was observed soon after the therapy (fluorescence probe injected 12 hours after the last injection of the drug) the average difference between fluorescence lifetime at the tumor and contralateral sites proved to be much lower slowly increasing from 0.03 ns at 3 hours after injection to 0.065 ns at 14 hours (Fig. 3b). Figure 4 shows the fluorescence intensity and lifetime images at the tumor and contralateral site of one mouse in each group at different time points.

We also measured the tumor volume before each imaging cycle (Fig. 5). The effect of treatment was maximal (tumor volume reduced ~ 2 times) at 12 hours after the last treatment

with 17-DMAG, while a week later, tumor came back to almost pre-treatment dimensions, as can be seen in Figure 5.

To substantiate the relationship between the binding of HER2-specific Affibody probe to HER2 receptors and difference in fluorescence lifetimes at the tumor and contralateral site, we have applied the HER2-specific ABD-Affibody to non-HER2 expressing tumors (U-251 MG) in mice.

Our analysis of the time-resolved data, obtained from mice with non-HER2 expressing tumors (U-251 MG), injected with HER2-specific ABD-Affibody, shows that if the probe does not bind to tumor's HER2 receptors (for the lack of them), the difference in the fluorescence lifetimes between tumor and contralateral sites is negligible, (<4%, 2%, 1.1% and 1.1% at 3, 6, 10 and 16 hours after injection, respectively), while for HER2 positive tumor imaged with the same probe this difference was considerable (>15% at all measurement time points), see Figure 6. These results are consistent with our earlier findings (ref(29)) for monomer Affibody probes that such difference is observed when and only when binding of the probe to HER2 receptors can occur (the difference is negligible in the absence of receptors or no specificity of the probe to HER2).

Discussion

HSP90 chaperone is responsible for the properly folding of subset of proteins, including HER2, which are essential for cell signaling. Therefore, disruption of cell proliferation and apoptosis in tumors through HSP90 inhibitors (e.g. 17-DMAG) is realized by a simultaneous inhibition of multiple pathways critical for tumor survival, including the HER2 pathway (40, 41). In relation to HER2 proteins, HSP90 not only controls the stability of the nascent form of HER2 in its maturation process in the endoplasmic reticulum, but also regulates its mature form during its residency at the plasma membrane and maintain it in a state capable for activation (42). Tumor cells demonstrate higher HSP90 activity and lesions can accumulate more HSP90 inhibitors than normal tissues through their leaky vascularization.

It is known from the literature that application of HSP90-inhibiting drugs results in considerable downregulation of HER2 receptors soon after the course of therapy. For HER2-positive tumors (similar to mouse xenografts of human breast carcinoma BT-474, studied in this work) it was shown, using both PET and *ex-vivo* methods, that HER2 overexpression was considerably reduced (~ 3 times) at ~12 hours after the last 17-DMAG treatment (35). On the other hand, in the paper (34), PET was used to follow variations in HER2 expression in mouse model after treatment with 17-AAG drug (another derivative of Geldamycin, HSP90 inhibitory component of 17-DMAG). Authors observed significant downregulation of HER2 (~3 times) at ~20 hours after the therapy was completed, while at later times (5 days) HER2 overexpression rapidly recovered, returning to pre-treatment level at 12 days after the therapy.

Comparison of the results, obtained after injection of ABD-HER2-specific Affibody in mice with non-HER2 expressing tumor (U-251 MG) and mice with high HER2 expression tumor (BT-474), presented in Figure 6, indicates that the difference in fluorescence lifetimes at the

tumor and contralateral site in BT-474 tumors is due to binding of the fluorescent ligands by the corresponding HER2 receptor and not other microenvironment (e.g. pH) or optical differences that are common in tumors (e.g. U-251 MG that does not express HER2 receptors). Regarding the effect of optical properties of the tumor on our measured fluorescence lifetime, most tumors including U-251 MG and BT-474 tumors have higher absorption compared to the normal tissue (contralateral site) due to their leaky vascularization and, probably, higher scattering coefficient due to increased collagen concentration, however, no significant difference was observed in fluorescence lifetimes at the tumor and contralateral site in the control experiment with U-251 MG xenograft (Fig. 6). Also, tumor depth and size cannot be the major factor in fluorescence lifetime variations since in our experiments both HER2-positive (BT-474) and HER2-negative (U-251 MG) tumors had similar dimensions.

Based on these results and our previous findings on relationship between fluorescence lifetime and binding of HER2-specific probe to corresponding receptors (29), we believe that the differences between the fluorescence lifetimes at the tumor and contralateral site, observed for mice with BT-474 xenografts after treatment with 17-DMAG, results from the strong downregulation of HER2 receptors soon after the therapy (i.e., less binding sites for the HER2-specific probe) and the corresponding changes in the fraction of bound to total fluorescent probes in the tumor. Immediately after treatment, the fraction of bound to total fluorophores inside the tumor changed considerably, resulted in noticeable increase in the average fluorescence lifetime. Subsequent tumor and HER2 expression recovery a week later caused gradual restoration of the original level of binding ratio of HER2-targeting probe in the tumor and corresponding return to pre-treatment values of the fluorescence lifetime.

Even though the Affibody probe internalization can potentially change fluorescence lifetimes for pH sensitive dyes, according to our tests, HER2 specific Affibody-Alexafluor750 conjugate, is not pH sensitive. Considering the effect of tumor volume and depth on the fluorescence lifetime difference observed between the tumor and contralateral sites, the results (See supplementary information – Figs. S2a and S2b) showed very similar fluorescent lifetimes in each group (before, 12h and 7days after treatment), while their volume were significantly different (e.g. twice difference in before treatment group). Therefore, the fluorescence lifetime changes observed at 12h after the last treatment cannot be caused by the change in the tumor volume. On the other hand, regarding the potential probe delivery problem at 12 hours after the last treatment, the higher and increasing fluorescence intensity after the probe injection at the tumor relative to the contralateral site (See supplementary information – Fig. S3) indicates that the Affibody probes were delivered and accumulated inside the tumor.

It should be noted that in all these experiments the fluorescence lifetime of ABD-HER2-Affibody-based probe at the contralateral site is practically constant (0.837 ± 0.016 ns), while the variations in the fluorescence intensity are quite significant both within different mice groups and at different measurement time points for an individual mouse (Fig. 2). Therefore, the measurement from the contralateral site can be used, as a reference, to evaluate variations of the fluorescence lifetime at the tumor site. Low variations in the fluorescence

lifetime at the contralateral site after the therapy also indicate that the effect of free 17-DMAG molecules in the circulation was not significant on fluorescence lifetime.

In conclusion, based on our observation, fluorescence lifetime imaging with a targeted fluorescent probe can potentially be used for real-time non-invasive *in-vivo* monitoring of the efficacy of the targeted therapy. Even though fluorescence lifetime imaging is an advanced modality compared to fluorescence intensity imaging and requires a more sophisticated instrumentation, however, the price of its main components, i.e., fast lasers (ps lasers) and fast detectors (e.g. PMT's) has been dropped significantly over the last years and gradually more time-resolved systems become commercially available, e.g., eXplore Opix (GE Medical Systems, London, ON) and ART Advanced Technologies (43). By moving these technologies to clinic, mass production of the systems can further decrease their cost.

Fluorescence lifetime imaging, based on evaluating the fraction of the bound and unbound fluorophores inside the tumor, can be used as an alternative *in vivo* imaging approach to characterize tumors, separate high and low HER2 expression tumors (29) and monitor the efficacy of targeted therapies from only one time point measurement after probe injection to avoid complications related to pharmacokinetics measurements of the probe. This can make fluorescence lifetime imaging a promising molecular imaging tool for treatment monitoring in preclinical models and potentially in patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Currently, the assessment of downregulation of oncogenic receptors in response to targeted therapy is limited by *ex-vivo* tests of biopsied tissues, anatomic or metabolic comparison of disease progression before and after a complete therapeutic cycle. Optimization of the treatment would greatly benefit from a real-time *in-vivo* assessment of the expression of the targeted biomarkers during the treatment and especially at the early stages of the therapy.

In this study, we showed the correlation between fluorescence lifetime and efficacy of the treatment by 17-DMAG using *in-vivo* near infrared fluorescence imaging. Though the optical modality is limited by tissue penetration depth (up to several cm), it is a minimally invasive low-cost modality without ionizing radiation. It is best suited for clinical applications such as breast, head/neck, and skin cancer and can be extended for different oncogenic receptors. Another application is in pre-clinical studies for screening and testing the efficacy of different therapies/drugs to treat various cancers.

Figure 1. Immunohistochemistry image of N87 tumor shows heterogeneity of HER2 receptors at the different locations inside the tumor.

Figure 2. Fluorescence variations at the contralateral site during 16 hours after injection of ABD-HER2-specific Affibody probe (two mice with BT-474 xenografts) before treatment with 17-DMAG: (a) Intensity (b) Lifetime. Fluorescence variations at the contralateral site during 16 hours after injection of ABD-HER2-specific Affibody probe (averaged of 5 mice with BT-474 xenografts, divided in three subsamples, i.e., before, 12 hours and 7 days after the last treatment with 17-DMAG) (c) Intensity (d) Lifetime

Figure 3.

- (a) The average fluorescence lifetime at the tumor site for five mice at different time points after injection of ABD-HER2-specific Affibody probe; before, 12 hours and one week after the last treatment with 17-DMAG
- (b) The difference between the fluorescence lifetimes at the tumor and contralateral site for the same mice of part (a).

Figure 4. Fluorescence intensity and lifetimes at different time points at the tumor site (a) before, (c) 12 hours, (e) one week after the last treatment with 17-DMAG and at the contralateral site (b) before, (d) 12 hours, (f) one week after the last treatment with 17-DMAG.

Figure 5.
The average tumor volume in 5 mice with BT-474 tumor as a function of time after the therapy

Figure 6.

In vivo fluorescence lifetimes of tumor and contralateral site of the xenograft mouse with no HER2 expressing human tumor model (U-251 MG) and high HER2 expressing human tumor (BT-474), after injection of the ABD-HER2-specific Affibody® conjugated to AlexaFluor750.