New cancer therapies are increasingly molecular-pathway specific. The evaluation of these novel therapies would be greatly facilitated by the development of noninvasive methods to assess multiple tumor cellular and molecular parameters. Using fluorescent probes specific for HER2/neu (AF750-trastuzumab) and apoptosis (Cy5.5-Annexin), we demonstrate a multichannel near infrared molecular imaging approach that yields accurate and early assessment of treatment susceptibility, drug target inhibition and tumor response during HER2-targeted therapy of orthotopic human mammary carcinomas in mice with trastuzumab (Herceptin). This combined approach detects both partial treatment response (tumor growth inhibition without regression) as well as therapeutic resistance before alterations in tumor growth are apparent. Partially responsive tumors exhibit increased Annexin signal when trastuzumab is combined with a cytotoxic agent (paclitaxel), which predicts subsequent tumor regression and suggests that imaging can guide therapy optimization. This multiparametric imaging approach has great potential in the clinical setting for determining patient eligibility, adequate drug dosing and early biological response of molecularly-targeted cancer therapies.

Key words: breast cancer; trastuzumab; molecular imaging; antibody therapy; HER2/neu; apoptosis

The ability to image tumor susceptibility, drug target inhibition and early treatment response requires an imaging modality capable of simultaneous assessment of multiple physiologic parameters. Among the different imaging modalities, near-infrared fluorescence (NIRF) optical imaging is particularly promising through its use of multiple molecularly-specific exogenously-administered fluorescent probes with discrete excitation and emission spectra. Also, NIR light (650–900 nm) exhibits relatively low absorption by water and hemoglobin, making it possible to image tumors deep within tissues. This technology is readily translatable to humans, as diffuse near-infrared optical imaging in clinical studies has already demonstrated the ability to distinguish benign from malignant breast lesions.

The purpose of the current study was to determine whether a multiparametric NIRF imaging approach could provide accurate noninvasive evaluation of a molecularly targeted cancer therapy. HER2/neu is a receptor tyrosine kinase that is overexpressed in ~25% of invasive human breast cancers, with overexpression linked to worse clinical prognosis. We chose to image HER2-targeted therapy in human breast cancer with trastuzumab (Herceptin), a humanized monoclonal antibody targeting the HER2/neu extracellular domain. This therapeutic agent has been FDA approved for treatment of patients with HER2/neu-overexpressing breast cancers. We demonstrate accurate noninvasive assessment of (i) susceptibility to trastuzumab therapy, (ii) therapeutic HER2 inhibition and (iii) early tumor response to therapy, using NIR fluorphore-conjugated molecular probes that fluoresce at different wavelengths and are specific for HER2/neu and apoptosis. This imaging method detects early partial therapeutic response and therapeutic resistance before alteration in tumor growth is detectable, providing an opportunity to modify therapy to optimize tumor response. Such treatment-altering information would not be obtainable using single imaging probes alone. This multiparametric approach is generalizable to other antibody-based molecular cancer therapies and represents a significant advance in the ability of molecular imaging to guide cancer treatment.

Material and methods

Imaging probe synthesis

An optical imaging probe for detecting HER2 was generated by combining the humanized anti-HER2/neu antibody trastuzumab (Genentech, San Francisco, CA) with the near-infrared dyes cyanine 5.5 (Cy5.5) or Alexa Fluor 750 (AF750; Molecular Probes, Carlsbad, CA) followed by column purification (Sephadex G50; Amersham, Piscatway, NJ). The final molar ratio of fluorochrome to antibody was verified at 1:1 by spectrophotometric analysis (to determine dye concentration) and the Bio-Rad bicinchoninic acid method (to determine protein concentration). An optical probe for detecting apoptosis was generated by conjugating Annexin V with...
Cy5.5 with an equimolar ratio of dye to protein as described previously. Briefly, recombinant annexin V (Theseus Imaging, Boston, MA) was dialyzed against sodium bicarbonate (0.1 M, pH 8.0) before use. Equimolar amounts of Annexin V and Cy5.5 dye were reacted and subsequently purified over a BioGel P6 column (Bio-Rad, Hercules, CA).

**Tumor lines and rodents**

The 9L human glioma cell line was maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. The BT-474 human mammary carcinoma cell line was maintained in complete DMEM/F12 medium supplemented with human insulin (3.6 × 10⁻⁷ mg/mL). MCF-7 and SKBR-3 human mammary carcinoma cell lines were maintained in complete McCoy’s medium. Trastuzumab-resistant SKBR-3 (SKBR-3 R1), which maintain HER2 overexpression but are therapeutically resistant to the effects of trastuzumab, were a kind gift from R. Nahta and F. Esteva. For use in intravitral laser scanning microscopy, tumor cell lines were stably transfected with an expression vector encoding green fluorescent protein (pAcGFP-N1; Clontech, Mountain View, CA), using the SuperFect transfection reagent according to the manufacturer’s protocol (Qiagen, Valencia, CA). 6–8 week old female C57BL/6J nude mice were purchased from The Jackson Laboratory. Mice were housed and maintained under aseptic conditions according to guidelines set by the Institutional Animal Care and Use Committee.

**In vitro experiments**

For in vitro assessment of probe binding, tumor cells were incubated with varying concentrations of HER2 probe for 15 min at 37°C and then washed twice in Hank’s balanced salt solution. AF750 fluorescence was assessed by flow cytometry (FacsCalibur, Becton Dickinson, Franklin Lakes, NJ). For competitive inhibition of probe binding, 100× molar excess of unlabeled trastuzumab was added to the cells concomitantly with the probe. For in vivo assessment of trastuzumab effects on cell growth, 10⁶ tumor cells were cultured in the presence or absence of 10 μg/mL trastuzumab for 72 hr. For combination therapy with trastuzumab and paclitaxel, cells were cultured for 48 hr with trastuzumab alone, followed by 24 hr with trastuzumab plus 100 nM paclitaxel (Nova-Plus). Cell counts were obtained by counting trypan-blue-excluding cells on a hemocytometer. Cell cycle analysis was performed by propidium iodide staining. Briefly, cells were fixed in 70% ethanol, washed in PBS and then incubated with 50 μg/mL propidium iodide, 1 mg/mL RNaseA (Sigma, St. Louis, MO) and 0.1% Tween-20 for 15 min at 37°C prior to analysis by flow cytometry. Apoptosis was assessed by both Annexin V (ApoAlert, Becton Dickinson, Franklin Lakes, NJ) and TUNEL methods (ApopTag, Chemicon, Temecula, CA) according to the manufacturer’s protocols.

**In vivo tumor growth and trastuzumab treatment**

Tumor cells in culture were detached with protease-free PBS-EDTA solution (Versene, Invitrogen), then combined with 100 μL of Matrigel (Collaborative Research, Bedford, MA) and implanted orthotopically into the mammary glands of female C57BL/6 nude mice (Jackson laboratories). 10 × 10⁶ BT-474, SKBR-3 and MCF-7 cells, or 1 × 10⁶ 9L cells, were implanted per tumor. For mice bearing BT-474 or MCF7 tumors, a 0.72 mg (60-day release) 17β-estradiol pellet (Innovative Research, Southfield, MI) was implanted subcutaneously at the time of tumor cell inoculation. Tumors were measured bidirectionally using calipers, and tumor volume was calculated by approximating a spheroid. Trastuzumab therapy consisted of intraperitoneal administration of 100 mg/kg of trastuzumab diluted in sterile water (15 nmole per dose) twice per week once tumors reached 50–100 mm³ in size. Purified whole human IgG (Jackson ImmunoResearch) was injected to control tumor-bearing mice at the same dose and schedule. For combination therapy, paclitaxel was administered intraperitoneally at 10 mg/kg twice weekly (days 0 and 3 of each 7-day cycle) beginning on the day of trastuzumab treatment initiation.

**Whole animal multiparametric near-infrared fluorescent imaging**

Whole animal epifluorescence imaging was performed as described previously using a multichannel whole animal optical imaging system (BonoSai, Siemens Medical Solutions, Malvern, PA) optimized for Cy5.5 (675/894 nm) and AF750 (752/779 nm) excitation/emission wavelengths. HER2 imaging was performed 48 hr following intravenous administration of 0.4 nmole of HER2 probe. Apoptosis imaging was performed 2 hr following intravenous administration of 1 nmole of Annexin V-Cy5.5. Quantitative measurements were performed on the acquired epifluorescent images using a circular region of interest (ROI) placed over each tumor and reported using image analysis software (Syngo, Siemens Medical Solutions). For each tumor, standard fluorescence values were calculated by normalizing ROI fluorescence intensity to dermal fluorescence assessed from an ROI contralateral to the tumor.

**Intravitral fluorescent microscopy**

Tumor cells were implanted in dorsal skinfold window chambers according to a previously described protocol.17 Tumor cells were implanted in a total volume of 20 μL of buffered saline. 7–10 days after implantation, intravitral laser scanning microscopy was performed using a 4-laser scanning microscope system as previously described.18 Excitation lasers and band-pass filter settings were optimized for GFP (488/505–525 nm), Cy5.5 (633/660–730 nm) and AF750 (748/770 nm) excitation/emission. HER2 and Annexin probes were administered intravenously and imaged as per the protocol described earlier for imaging of orthotopic tumors. All image stacks were acquired using identical laser power and photo multiplier tube settings. All images were recorded and stored as proprietary multilayer 16-bit Tagged Image File Format files, and then displayed at identical window level settings.

**Histological analysis**

Tumor-bearing mice were sacrificed 72 hrs following trastuzumab therapy and tumors were harvested and snap-frozen in optimal cutting temperature compound (Tissue-Tek). 10-μm thick frozen sections were cut on a cryostat. All fluorescent microscopy was performed on a Nikon Eclipse TE2000 microscope equipped with lasers and filters for Cy5.5 and AF750 fluorescence (Omega Optical) and a CCD camera (CoolSnapHQ-M; Photometrics, Tucson, AZ). Light microscopy was performed on a Nikon E400 microscope equipped with a color CoolSnap CCD camera. Cy5.5-Annexin V probe binding was assessed by imaging freshly cut tumor sections. Adjacent tumor sections were stained for apoptosis by the TUNEL method (ApopTag) and for routine hematoxylin and eosin.

**Statistical analysis**

Assessment of statistical significance was performed using standard 1-way Analysis of Variance to compute the p-values using Octave (standard Unix software).

**Results**

**Specific binding of HER2 imaging probe in vitro and in vivo**

A near-infrared fluorescent imaging probe for detecting HER2/neu (HER2 probe) was generated by attaching the near-infrared fluorochromes Cy5.5 or AF750 to trastuzumab (see Material and methods). The HER2 imaging probe initially was tested for in vitro binding to human cancer cells with differing HER2 expression. These included the non-HER2-expressing 9L glioma line, the MCF7 breast cancer line expressing normal mammary HER2/neu levels (1 copy of the HER2/neu locus), and the BT-474 and SKBR-3 breast cancer lines overexpressing HER2/neu (4-8-fold gene amplification).14,39 The HER2 probe demonstrated greater than 10-fold increased binding to BT-474 and SKBR-3 cells compared with 9L cells (Fig. 1a; p < 0.005). Probe binding
to SKBR-3 and BT-474 cells was ~4-fold higher than that shown by HER2/neu-normal MCF7 cells, comparable to the 4–8-fold HER2/neu amplification exhibited by these lines.19 Probe binding was competitively inhibited by addition of 100× molar excess of unlabeled trastuzumab (data not shown). Fluorescence microscopy confirmed increased probe binding to HER2/neu-overexpressing cell lines and demonstrated probe localization to the cell membrane (Figs. 1b and 1c).

**Noninvasive imaging of HER2 inhibition and tumor response following trastuzumab treatment**

To image early tumor response, we synthesized a second imaging probe (Cy5.5-Annexin) to detect externalized phospholipids on apoptotic cells according to a previously described method.15 The fluorophore attached to the apoptosis probe (Cy5.5) has discrete excitation and emission spectra relative to the HER2 probe dye (AF750), allowing multiparametric imaging with 2 molecular probes. Multichannel imaging with the 2 probes was initially performed by intravital scanning fluorescent microscopy of HER2-overexpressing SKBR-3 tumors implanted in dorsal skin chambers. Initial imaging of untreated tumors showed avid HER2 probe binding without significant Annexin probe accumulation (Figs. 2a and 2d). To examine the effect of treatment, HER2 probe was given 18 hr following trastuzumab administration. Following treatment, HER2 probe binding was decreased (Fig. 2e), with subsequent appearance of clusters of Annexin-positive cells (Fig. 2f). The persistent low-level HER2 probe binding exhibited by the tumors was confirmed by histologic analysis of SKBR-3 tumors during trastuzumab treatment. Representative images from untreated SKBR-3 (a, d) and trastuzumab treated SKBR-3 (b, e) and SKBR-3 R1 (c, f) tumors are shown. The top row shows Annexin signal (green) and the bottom row shows HER2 signal (red). Note the reduction in HER2 signal in both SKBR-3 and SKBR-3 R1 tumors following treatment. Scale bar equals 500 μm. (g–i) Histologic analysis of SKBR-3 tumors during trastuzumab treatment. (g) Representative high-powered field of a treated tumor showing Annexin probe administration demonstrates probe binding to tumor cells. Adjacent sections stained for TUNEL (h) and routine hematoxylin and eosin (i) confirm that Annexin probe binds to apoptotic tumor cells. Scale bar equals 500 μm.
Annexin-positive cells additionally confirmed that these were tumor cells. As a control for tumor response, we also tested SKBR-3 R1 tumors, derived from a subpopulation of SKBR-3 cells that are resistant to trastuzumab treatment despite maintaining HER2/neu overexpression. In vitro studies confirmed that SKBR-3 R1 cells demonstrate comparable HER2 probe binding to wild-type cells (mean fluorescence intensity R1 cells 105 ± 5 F.U. compared with W.T. 107 ± 4 F.U. by flow cytometry). In vivo, trastuzumab-treated SKBR-3 R1 tumors did not exhibit significant Annexin positivity (Fig. 2c) despite successful HER2 inhibition (Fig. 2f).

We next performed multichannel whole animal imaging on established orthotopic tumors. HER2-overexpressing SKBR-3, BT-474 and SKBR-3 R1 tumors all demonstrated high HER2 probe binding and no significant Annexin probe binding (Figs. 3i and 3a–3b). Trastuzumab treatment was associated with 55, 78, and 63% decrease in HER2 probe signal in SKBR-3, BT-474 and SKBR-3 R1 tumors, respectively, 18 hr after treatment initiation (Fig. 3i). Trastuzumab treatment of SKBR-3 tumors also produced a 2.3-fold increase in Annexin signal following trastuzumab treatment (Figs. 3f and 3j) that was significantly higher than that observed in treated SKBR-3 R1 tumors (Figs. 3f and 3j; p < 0.01). SKBR-3 tumors treated with equivalent doses of nonspecific human Ig did not exhibit increased Annexin signal (Fig. 3j), indicating that the observed Annexin induction was specific to trastuzumab treatment. No significant increase in Annexin signal was observed in BT-474 tumors following trastuzumab therapy. Histologic analysis of trastuzumab-treated SKBR-3 tumors demonstrated that the tumor regions binding the Annexin-Cy5.5 probe (Fig. 2g) also exhibited nuclear TUNEL positivity (Fig. 2h), confirming that the Annexin probe binds apoptotic tumor regions in vivo.

**Trastuzumab treatment produces apoptosis in SKBR-3 cells but only G1 arrest in BT-474 cells**

Multichannel NIRF imaging demonstrated increased Annexin signal in trastuzumab-treated SKBR-3 but not BT-474 tumors despite comparable HER2 inhibition, which led us to ask whether trastuzumab treatment has differing effects on the 2 tumor cell lines. We examined trastuzumab effects on cell growth in vitro. In cell counting studies, trastuzumab inhibited BT-474 growth by 82% compared with untreated cells (Fig. 4a). Its effect on
Figure 4 – Trastuzumab effects on tumor cell growth and death. (a) Effect of trastuzumab treatment on cell number. Tumor cells were cultured in the absence (gray bars) or presence (black bars) of trastuzumab and quantitated as described in “Material and methods.” Each cell population started with 1 million cancer cells. The experiment was performed in triplicate and error bars indicate standard deviation. Asterisks denote statistical significance ($p < 0.005$). (b) Cell cycle analysis by propidium iodide staining of tumor cells cultured in the absence (blue histograms) or presence (red histograms) of trastuzumab. Numbers indicate the percent reduction in proliferating cell fraction ($G_2 + M$) following trastuzumab treatment. (c) Representative flow cytometry histograms following FITC-Annexin V staining of trastuzumab treated MCF7 (green), BT-474 (blue) and SKBR-3 cells (red) to detect apoptosis. (d) Comparison of Annexin V (gray bars) and TUNEL (black bars) methods for detecting apoptosis in trastuzumab treated tumor cells. The experiments were performed in triplicate and error bars indicate standard deviation. Asterisks denote statistical significance ($p < 0.05$).
SKBR-3 cells was more dramatic—after treatment, there was lost of 14% of tumor cells relative to the onset of treatment. In contrast, trastuzumab treatment did not significantly affect the growth of MCF7 or 9L cells (Fig. 4a), consistent with its specificity for HER2-overexpressing tumor cells. The effect of trastuzumab on tumor cell cycle progression was then assessed by flow cytometric analysis following propidium iodide staining. Treatment of BT-474 cells produced a 47% decrease in the fraction of cell in S + G2/M phases, consistent with relative G1 arrest (Fig. 4b). Trastuzumab treated SKBR-3 cells exhibited only a 23% decrease in the proliferative index, which was insufficient to explain the cytotoxic effect observed in the cell counting study and suggested that trastuzumab was causing SKBR-3 cell death. Flow cytometric assessment of apoptosis by Annexin V staining demonstrated 10% apoptosis of SKBR-3 cells following 72 hr of trastuzumab treatment (Figs. 4c–4d). In contrast, no significant apoptosis was observed following treatment of BT-474, MCF7, or 9L cells. This selective susceptibility of SKBR-3 cells to trastuzumab-induced apoptosis was confirmed by the TUNEL method (Fig. 4d). Trastuzumab did not produce significant apoptosis in SKBR-3 R1 cells by either Annexin or TUNEL methods. The in vitro results show that trastuzumab is cytotoxic to SKBR-3 cells but only cytostatic toward BT-474 cells, which explains the disparate effects observed in vivo by Annexin probe imaging.

**Multichannel imaging predicts tumor response to trastuzumab therapy**

To see whether the multichannel imaging data obtained during the first 48 hr following trastuzumab treatment initiation were predictive of subsequent tumor response, serial measurements of tumor size were performed on mice bearing orthotopic tumors in the presence or absence of trastuzumab administration. Trastuzumab was administered according to a dosing regimen (100 mg/kg intraperitoneally twice per week) known to inhibit tumor growth in vivo. Trastuzumab did not significantly affect the growth of 9L or MCF7 tumors (Figs. 5 and data not shown). SKBR-3 tumors, which exhibited increased Annexin signal following the initial trastuzumab dose, subsequently underwent tumor regression (Fig. 5), with a 39% decrease in average tumor volume from days 7–14 post-treatment initiation (26.9 mm³ vs. 43.9 mm³). BT-474 tumors underwent growth inhibition, with tumor growth slowed by 70% after 14 days of treatment compared with untreated controls. Importantly, treated BT-474 tumors did not regress, consistent with the cytostatic effect of trastuzumab observed in vitro and the lack of Annexin signal observed in vivo. Trastuzumab-resistant SKBR-3 R1 tumors did not respond to treatment, with treated tumors continuing to grow at nearly an identical rate as untreated SKBR-3 tumors, despite HER2 inhibition. This is consistent with lack of growth inhibition observed following trastuzumab treatment of SKBR-3 R1 cells in vitro (data not shown).

**Addition of paclitaxel (Taxol) to BT-474 treatment regimen increases annexin signal by NIRF imaging, which predicts subsequent tumor regression**

Our results show that NIRF Annexin imaging is able to predict tumor response to trastuzumab, with increased Annexin signal only observed in SKBR-3 tumors that subsequently underwent regression in response to treatment. Trastuzumab-resistant SKBR-3 R1 tumors exhibited very low Annexin signal (Fig. 3) on imaging and did not undergo regression despite treatment and growth inhibition. We next asked whether the imaging results could be used to guide treatment, focusing specifically on BT-474 tumors, which underwent tumor growth inhibition without regression. Prior studies had indicated that trastuzumab renders HER2-overexpressing tumor cells more susceptible to certain conventional chemotherapeutic agents, and we hypothesized that such a combination regimen could lead to an enhanced anti-tumor effect detectable by imaging.

Addition of the cytotoxic agent paclitaxel to the trastuzumab treatment of BT-474 cells in vitro induced 11% tumor cell apoptosis (Annexin staining, comparable to the degree of SKBR-3 apoptosis produced by trastuzumab monotherapy (Fig. 6a). The addition of paclitaxel to the treatment regimen of BT-474 tumors in vivo led to a 2-fold increase in Annexin signal by NIRF imaging (Figs. 6c and 6d), significantly higher (p < 0.01) than that exhibited during trastuzumab treatment alone (Figs. 6e and 6f). Finally, serial assessment of BT-474 tumor size during combination treatment demonstrated enhanced anti-tumor activity compared with trastuzumab treatment alone. As predicted by Annexin imaging, paclitaxel plus trastuzumab led to regression of BT-474 tumors, with a 48% decrease in mean tumor volume from the onset of treatment to day 14 post-treatment (Fig. 6g).

**Discussion**

Molecular imaging may provide important information regarding tumor physiology that can direct and evaluate cancer treatments. This study demonstrates the ability of multichannel NIRF imaging to provide early assessment of multiple important therapeutic parameters—namely, tumor treatment susceptibility, drug target inhibition and early tumor response. The ability to evaluate multiple tumor physiologic parameters is critical for clinical translation of molecular imaging. Cancer cells generally contain genetic alterations in multiple signaling pathways, and imaging therapeutic modulation of 1 molecular target may not be an accurate surrogate indicator of anti-tumor effect. In addition, the prospect of combination therapeutic regimens involving multiple molecularly-targeted agents necessitates a multiparametric imaging approach for determining efficacy. Conversely, lack of observed anti-tumor effect during therapy may be the result of either inadequate drug delivery/dosing or tumor resistance, and multiparametric imaging methods that can distinguish between these possibilities early during treatment would be important for deciding between drug dose escalation and cessation. The noninvasive
nature of molecular imaging makes it ideally suited for serial dynamic assessment of treatment response. The alternative would be serial biopsy, which is more technically challenging, less well-tolerated by patients and more susceptible to tissue sampling error.

Imaging agents derived from the HER2/neu-specific humanized monoclonal antibody trastuzumab (Herceptin) have previously demonstrated binding to HER2/neu-overexpressing tumors in preclinical models, and have been used as a surrogate marker in a defined system to image response to heat shock protein inhibition. The current study extends this paradigm into in vivo optical imaging by conjugating trastuzumab with 2 distinct NIR fluorochromes. NIRF imaging with these HER2/neu-specific probes was sensitive enough to distinguish HER2-normal from HER2-overexpressing mammary carcinomas in vitro and in vivo, which correlated with tumor susceptibility to trastuzumab therapy and subsequent changes in tumor growth. The distinction between HER2-normal from HER2-overexpressing tumors is clinically relevant as an index of susceptibility to trastuzumab as well as anthracycline-based chemotherapy regimens. It is important to note that the dose of HER2 probe administered (0.4 nmoles/20 g mouse) is extremely low and approaches the tracer doses used for clinical nuclear medicine studies. The imaging dose of HER2 probe did not induce apoptosis in SKBR-3 tumors (data not shown), showing that the NIRF probe allows accurate assessment of receptor expression without the confounding effect of tracer anti-tumor activity.

Serial tumor imaging with the HER2 probe demonstrated significantly reduced tumor probe binding less than 1 day following

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**Figure 6** – Using multichannel imaging to optimize tumor treatment. (a) In vitro flow cytometric apoptosis assay of BT-474 cells using both Annexin and TUNEL methods. Cells were treated with trastuzumab and paclitaxel, either alone or in combination. Asterisk indicates statistical significance (p < 0.05). (b–e) Multichannel imaging of representative untreated (b, c) or combination paclitaxel/trastuzumab treated (d, e) BT-474 tumors. (f) Summary of BT-474 tumor Annexin probe binding before and after treatment with trastuzumab, either alone or in combination with paclitaxel. Values indicate the relative increase in Annexin signal in tumors after treatment compared with pretreatment imaging. Asterisks indicate statistical significance (p < 0.01). (g) Effect of trastuzumab treatment on tumor size. Tumor volumes are plotted for established trastuzumab-treated (green), trastuzumab/paclitaxel-treated (red), or size-matched control (blue) BT-474 tumors. The black arrow indicates the time-point, at which tumors were imaged for tumor response (Annexin probe) during treatment (day 2).
initiation of trastuzumab therapy, showing that the probe can be used as an early indicator of successful drug target inhibition. Previously, the determination of trastuzumab efficacy in human tumors was inferred from therapeutic alteration of either overall tumor growth or levels of downstream molecules assessed by immunohistochemical analysis of excised tumor tissue. HER2 imaging with a NIRF probe provides multiple advantages, including its ability to provide serial determination of HER2 levels in patients, while trastuzumab dose escalation is underway. Additionally, HER2 imaging uniquely provides a direct assessment of trastuzumab primary therapeutic efficacy. The ability to assess the kinetics and magnitude of HER2 inhibition would allow optimization of trastuzumab dosing and scheduling on an individual patient basis to maximize therapeutic effect, while minimizing drug toxicity. The observed decrease in tumor HER2 probe binding likely reflects competitive inhibition by trastuzumab rather than decreased HER2 expression, given the short time interval (16 hr) between administration of trastuzumab and probe.

The second imaging probe was selective for apoptotic cells and consisted of a NIR fluorophore-conjugated version of the phosphatidylserine binding protein Annexin V, a standard cellular marker of apoptosis. Annexin-based imaging probes are a promising new method for noninvasive detection of apoptosis in a number of disease settings, including tumors undergoing cytotoxic chemotherapy (reviewed in Refs. 27,28). Our data demonstrate the utility of Annexin-based imaging for detecting apoptosis in orthotopic tumors treated with a molecularly-targeted agent. In this study, the differential trastuzumab effects on SKBR-3 and BT-474 tumor cells proved the specificity of Annexin probe binding. Trastuzumab treatment of BT-474 cells in vitro led to G1 growth arrest without significant apoptosis; similarly, trastuzumab treated BT-474 tumors did not exhibit significant Annexin probe binding in vivo despite HER2 inhibition and tumor growth retardation. In contrast, trastuzumab treatment of SKBR-3 tumor cells in vitro produced apoptosis that was concordant with the increased Annexin probe signal exhibited by trastuzumab-treated SKBR-3 tumors in vivo. Histological analysis of trastuzumab-treated SKBR-3 tumors confirmed that the areas of Annexin probe binding corresponded to apoptotic regions. The differential responses observed between BT-474 and SKBR-3 cells are consistent with previous work examining the effects of trastuzumab on multiple HER2-overexpressing breast cancer lines29,30 and likely are a reflection of intrinsic differences in PI3K/Akt signaling between the 2 cell lines.27

This imaging approach is able to discern differences in tumor trastuzumab response soon after initiation of treatment, before differences in overall tumor growth are apparent. For example, trastuzumab-resistant SKBR-3 R1 tumors, which maintain HER2 overexpression but possess downstream genetic alterations, including decreased p27 expression conferring therapeutic resistance,31 did not exhibit increased Annexin signal despite adequate HER2 inhibition on early imaging. These tumors subsequently demonstrated continued tumor growth despite treatment, confirming the resistant phenotype. BT-474 tumors also demonstrated HER2 inhibition without significant Annexin signal on early treatment imaging, which in this case predicted partial tumor response evidenced by inhibition of tumor growth without tumor regression. The addition of paclitaxel to the BT-474 treatment regimen produced increased tumor cell apoptosis in vivo and increased Annexin signal on early treatment imaging, which predicted enhanced anti-tumor activity compared with trastuzumab treatment alone. This demonstrates the great potential of multichannel near-infrared optical imaging for providing an early opportunity to modify and optimize tumor treatment regimens. Pretherapy HER2 imaging of mammary carcinomas would be used to screen for HER2/new overexpression and trastuzumab susceptibility. Early imaging during tumor treatment would demonstrate adequate drug dosing (decreased HER2 probe binding) and predict tumor regression (increased Annexin signal). Lack of Annexin signal despite adequate HER2 inhibition would be an early sign of suboptimal tumor response and an indication for therapy augmentation with additional agents. Multiple such combination treatment regimens involving trastuzumab, including trastuzumab/paclitaxel, have been FDA-approved for treatment of HER2-positive breast cancer in patients.31,32 For tumors that exhibit persistently low Annexin signal despite maximal combination therapy, the decision would be made early to stop treatment and switch regimens altogether. This early evaluation of tumor response would obviate the need to wait for alterations in tumor growth, potentially saving patients weeks or months of nonfucitcious treatment depending on the tumor’s intrinsic growth rate. Imaging tumor apoptosis may be a more reliable index of tumor response than changes in tumor growth in the clinical setting, given the lack of control tumors in patients for growth rate comparison. Also, imaging tumor cell death is likely to be advantageous in certain malignancies, in which treatment response or malignant recurrence do not involve significant alterations in primary tumor size.33,34

A critical technique for demonstrating the in vivo specificity of binding of the optical probes in this study was intravalier laser scanning fluorescence microscopy of tumors. This is an ideal technique for following probe localization on a cellular level within tumors in situ. Serial imaging allows temporal resolution of probe egress from the circulation and, through the use of multichannel imaging with fluorescent tumor cell markers, confirms tumor cell binding specificity in vivo. This approach should be widely applicable for evaluating kinetics of molecular probe binding on a cellular level.

The promising results demonstrated here point to the possible translation of these concepts to the clinic, including the noninvasive in situ characterization of breast tumors, individualized treatment dosing in patients with breast cancer, as well as the simultaneous imaging of primary therapeutic efficacy and treatment response. The ability of near-infrared optical imaging to assess multiple tumor physiologic parameters simultaneously represents an important advance beyond current tumor imaging methods. Furthermore, this approach is easily generalizable to other monoclonal antibody cancer therapies, such as bevacizumab and cetuximab that are currently being used in the clinical setting. Thus, the method of using a small tracer dose of labeled antibody to assess the adequacy of therapeutic dosing of the same antibody to block the target receptor may see an increased use in the future. Similarly, the ability of NIRF optical imaging to assess multiple tumor parameters simultaneously could be of potential benefit in upcoming clinical trials evaluating combinations of molecularly targeted cancer agents.35

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