Imaging agents for *in vivo* molecular profiling of disseminated prostate cancer: Cellular processing of [^{111}In]-labeled CHX-A''DTPA-trastuzumab and anti-HER2 ABY-025 Affibody in prostate cancer cell lines

JENNIE MALMBERG[1], VLADIMIR TOLMACHEV[1,2] and ANNA ORLOVA[1]

Divisions of [1]Biomedical Radiation Sciences, and [2]Nuclear Medicine, Uppsala University, S-751 85 Uppsala, Sweden

Received September 20, 2010; Accepted February 4, 2011

DOI: 10.3892/etm.2011.217

Abstract. The treatment of disseminated prostate cancer remains a great challenge in current oncology practice. The proliferation of prostate cancer cells is testosterone-driven, but clonal selection during androgen deprivation therapy promotes the development of androgen-independent (hormone-refractory) cells, which become phenotypically dominant. Human epidermal growth factor receptor type 2 (HER2) is capable of activating the androgen receptor pathway, even in the absence of the ligand. The detection of phenotypic changes associated with the development of androgen independence may influence patient management, suggesting the initiation of a second-line therapy. This study aimed to establish the level of HER2 expression in a number of prostate cancer cell lines (LNCaP, PC3 and DU145) in order that they be used as models in further studies, and to evaluate the binding and cellular processing of [^{111}In]-labeled trastuzumab and the anti-HER2 synthetic Affibody molecule ABY-025 in these cell lines. The expression of HER2 was demonstrated and quantified in all three tested prostate cancer cell-lines. Studies on cellular processing demonstrated that internalization of both conjugates increased continuously during the whole incubation. The internalization rate was approximately equal for both monoclonal antibodies and Affibody molecules. In both cases, internalization was moderately rapid. Such features would definitely favor the use of radiometal labels for trastuzumab and, most likely, for affibody molecules. The level of HER2 expression in these cell lines is sufficient for *in vivo* molecular imaging.

Correspondence to: Dr Anna Orlova, Division of Biomedical Radiation Sciences, Rudbeck Laboratory, Uppsala University, S-751 85 Uppsala, Sweden
E-mail: anna.orlova@bms.uu.se

Key words: human epidermal growth factor receptor type 2, prostate cancer, trastuzumab, Affibody molecule, DU145, PC3, LNCaP, indium-111, *in vitro*, cellular processing

Introduction

The treatment of disseminated prostate cancer remains a great challenge in current oncology practice (1). The proliferation of prostate cancer is testosterone-driven, and androgen deprivation by surgical or chemical castration causes a remission lasting 1.5-3 years (2). However, a clonal selection during androgen deprivation therapy promotes the development of androgen-independent (hormone-refractory) cells, which become phenotypically dominant. The median survival of patients with androgen-independent prostate cancer is 12-24 months, depending on the treatment (3,4). Possible molecular pathways of androgen independence include a hypersensitive pathway, when more androgen receptors are produced to compensate for the low level of androgens, a promiscuous pathway, when androgen receptors are activated by non-androgenic steroids, and an outlaw pathway, when the androgen receptors are phosphorylated by either the mitogen-activated receptor tyrosine kinases (RTKs) or their downstream signaling kinases (5).

The detection of phenotypic changes associated with the development of androgen independence may influence patient management, suggesting the initiation of a second-line therapy. Moreover, accurate molecular phenotyping may suggest the most suitable molecular targets for a second-line therapy, making androgen-independent prostate cancer treatment more personalized. One challenge includes the heterogeneity of gene expression in metastases. In principle, biopsy-based methods enable the detection of a multitude of genes simultaneously. However, repeated sampling of several metastases is more than questionable in the clinical setting. The use of radionuclide molecular imaging would enable repeated imaging of the aberrant expression of different gene products in all metastases simultaneously. Moreover, the use of contemporary combined imaging devices (PET/CT or SPECT/CT) would provide anatomical landmarks for biochemical changes.

One candidate for imaging in disseminated prostate cancer is human epidermal growth factor receptor type 2 (HER2), a receptor tyrosine kinase that is involved in the outlaw pathway. HER2 is one of four members of the human epidermal growth factor receptor (EGFR or HER) family,

which also includes EGFR (ErbB1), HER3 (ErbB3) and HER4 (ErbB4). While HER2 is weakly expressed in normal adult tissue, several types of cancer overexpress HER2 in both primary tumors and their metastases. In prostate cancer, the progression towards androgen independence is characterized by a gradual increase in HER2 expression by tumor cells. The initial function of HER2 may be to permit prostate cancer cell survival in an androgen-depleted environment (6). Changes in the hormonal environment precipitate a cascade of events in gene expression and in the signaling network of the cell. This in turn provides a selective survival and growth advantage for HER-2-expressing subpopulations of cells, and accelerates the progression of the tumor towards androgen independence. This process also renders the cells more resistant to therapy (7,8). In the absence of androgen, the overexpression of HER2 activates the transcription of PSA. Unlike other kinases, HER2 is capable of activating the androgen receptor (AR) pathway, even in the absence of its ligand (9-11). This suggests that increased expression of HER2 may be a prostate-specific, rather than tumor-specific, mechanism of survival in an androgen-depleted environment (12).

HER2 is a molecular target for several anti-cancer drugs. Extensive studies have been dedicated to the development of radiolabeled imaging probes for the visualization of HER2, with the aim of identifying patients who may benefit from HER2-targeting therapy (13). One obvious potential targeting probe for the imaging of HER2-overexpression is trastuzumab, a humanized anti-HER2 monoclonal antibody. Trastuzumab is currently approved for the treatment of HER2-expressing breast cancer. The antibody is commercially available and clinical trials have demonstrated its safety. ^{111}In-DTPA-trastuzumab has been used to identify breast cancer patients responding to trastuzumab treatment (alone or in combination with chemotherapy) (14).

A promising alternative to radiolabeled anti-HER2 monoclonal antibodies may be Affibody molecules (15). Affibody molecules are proteins composed of a three-helix bundle based on the scaffold of one of the IgG-binding domains of Protein A. By randomizing thirteen of the amino acid residues in the helices 1 and 2, combinatorial Affibody libraries have been created for the selection of binders for a multitude of proteins (16). In contrast to the 150 kDa weight of an antibody, the molecular weight of Affibody scaffold is only 7 kDa, which provides rapid extravasation and penetration in the extracellular space of tumors. An Affibody molecule, $Z_{HER2:342,}$ with an affinity (dissociation constant, KD) to HER2 of 22 pM, has been developed (17). Several radiolabeled derivatives of $Z_{HER2:342}$ have demonstrated excellent targeting of HER2-expressing xenografts in murine models (15). A pilot clinical study confirmed that [^{111}In]- and [^{68}Ga]-labeled $Z_{HER2:342}$ can be used to successfully visualize HER2-expressing metastases (18). Recently, the Affibody scaffold was re-engineered in order to improve its properties and to provide a surface distinctly different from that of the bacterial parental scaffold (19). A DOTA-conjugated anti-HER2 Affibody molecule, ABY-025, which is based on a new scaffold, has demonstrated highly specific targeting of HER2-expressing ovarian carcinoma xenografts in mice (20). This makes ABY-025 a promising candidate for the imaging of HER2 expression in prostate cancer metastases.

This study aimed to establish the level of HER2 expression in a number of prostate cancer cell lines in order that they be used as models in further studies, and to evaluate the binding and cellular possessing of [^{111}In]-labeled trastuzumab and ABY-025 in these cell lines.

Materials and methods

Materials. Affibody molecule ABY-025 was provided by Affibody AB (Stockholm, Sweden) in a freeze-dried form. The monoclonal antibody trastuzumab (Herceptin®) was from Roche Pharma AG (Germany). Before use, trastuzumab was purified using the NAP-5 size exclusion column (Amersham Biosciences, Uppsala, Sweden), pre-equilibrated and eluted with MilliQ-water and freeze dried. Isothiocyanate-CHX-A''DTPA was purchased from Macrocyclics (Dallas, TX, USA).

Buffers including 0.1 M phosphate-buffered saline (PBS), pH 7.5, 0.07 M sodium borate, pH 9.3, and 0.2 M ammonium acetate, pH 5.5, were prepared using common methods from chemicals supplied by Merck (Darmstadt, Germany). High-quality Milli-Q© water (resistance >18 MΩ cm) was used to prepare the solutions. NAP-5 size-exclusion columns were from GE Healthcare (Uppsala, Sweden). Buffers used for conjugation and labeling were purified from metal contamination using Chelex 100 resin (Bio-Rad Laboratories, Richmond, CA, USA). [^{111}In]-indium chloride was purchased from Covidien (Hazelwood, MO, USA).

Cell lines. Three different prostate cancer cell lines were evaluated for HER2 expression, and their HER2-receptor levels were quantified. The cell lines, all originating from prostate cancer metastases, were DU-145 (brain metastasis), PC3 (bone metastasis) and LNCaP (lymph node metastasis). All cell lines were purchased from the American Type Culture Collection (ATCC).

The cells were cultivated in complete RPMI-medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamate, 100 IU/ml penicillin and 100 μg/ml streptomycin. For the LNCaP cells, the medium was supplemented with Na-pyruvate and HEPES. All reagents, including the medium and trypsine-EDTA, were from Biochrom KG (Berlin, Germany). Bottles and Petri dishes for cell cultivation were from Nunclon Surface (Roskilde, Denmark). The cells were incubated in a humidified incubator with 5% CO_2 at 37˚C, unless stated otherwise.

Instrumentation. Radioactivity was measured using an automated γ-counter with a 3-inch NaI(Tl) detector (1480 WIZARD; Wallac Oy, Turku, Finland). Indium-111 was measured using both photo peaks and the summation peak (energy setting from 140 to 507 keV). The distribution of radioactivity along the ITLC strips was measured on a Cyclone™ Storage Phosphor system (further referred to as Phosphorimager) and analyzed using OptiQuant™ image analysis software. Cells were counted using an electronic cell counter (Beckman Coulter).

Labeling chemistry. For labeling, ABY-025 was reconstituted in 0.2 M ammonium acetate buffer, pH 5.5, to a concentration of 1 mg/ml. For typical labeling, 30 μl of ABY-025 solu-

tion was mixed with 50 μl 0.2 M ammonium acetate buffer, pH 5.5, and 30-70 MBq of [^{111}In]-indium chloride (80-160 μl solution in 0.05 M HCl). The reaction mixture was incubated at 60˚C for 30 min and the radiochemical purity was evaluated using Tec-Control Chromatography 150-771 strips eluted with 0.2 M citric acid.

Trastuzumab was labeled with [^{111}In]-indium chloride using the CHX-A"DTPA chelator. Coupling (~4 chelators per antibody) was performed similarly to a previously described method (21). In brief, 1.4 mg purified antibody was reconstituted in 200 μl 0.07 M sodium borate buffer, pH 9.3, and a freshly prepared solution of isothiocyanate-CHX-A"DTPA (27 μl, 1 mg/ml in 0.07 M sodium borate, pH 9.3) was added. The mixture was incubated at 37˚C for 4 h, and then CHX-A"DTPA-trastuzumab was purified from unreacted chelator using the NAP-5 column, equilibrated and eluted with 0.2 M ammonium acetate, pH 5.5. The CHX-A"DTPA-trastuzumab was divided in aliquots of 100 μg in 64 μl 0.2 M ammonium acetate, pH 5.5, each, and stored frozen at -20˚C.

For typical labeling, an aliquot of CHX-A"DTPA-trastuzumab was mixed with 10 MBq [^{111}In]-indium chloride (12 μl solution in 0.05 M HCl). The mixture was incubated for 60 min at ambient temperature and analyzed using Tec-Control Chromatography 150-771 strips.

In vitro binding specificity test. Pre-cultivated cells from the DU-145, PC3 or LNCaP cell lines were incubated for 2 h with a 150 pM solution of [^{111}In]ABY-025 in medium. Simultaneously, another set of dishes was treated in the same way, but with the inclusion of a blocking amount of ABY-025 before the addition of the solution containing [^{111}In]ABY-025. The experiment was also performed with [^{111}In]CHX-A"DTPA-trastuzumab at a 1-nM concentration. Both experiments were performed in triplicate.

Subsequently, the incubation media were collected and the cell cultures were trypsinized with 0.5 ml trypsin-EDTA for 10 min at 37˚C. To each dish, 0.5 ml of medium was added, and the cells were resuspended. The cell suspension was also collected.

Medium and cell samples were measured for radioactivity content, and cell-associated radioactivity was calculated as follows: (Cell-associated radioactivity, CPM) x 100%/[(Cell-associated radioactivity, CPM) + (Radioactivity in media, CPM)]. The significance of the blocking was analyzed by the t-test.

Quantification of HER2 receptor expression in prostate cancer cell lines. DU-145, PC3 or LNCaP cells were incubated for 4 h at 4˚C with [^{111}In]CHX-A"DTPA-trastuzumab at concentrations of 0.2-33 nM in complete medium. For each data point, four dishes were used, including one pre-saturated with unlabeled trastuzumab at a 3-μM concentration. For each data point, a sample of the added solution was obtained for concentration calculations. After incubation, the medium was aspirated and the cells were washed once with cold serum-free medium. The cells were treated with trypsin for 10-15 min, and the cells in each dish were resuspended after the addition of 1 ml medium. The cell suspension (0.5 ml) was used for cell counting and for radioactivity measurements (1 ml).

Samples were measured for radioactivity content in an automated γ-counter, and data were analyzed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

Cellular binding and processing of radiolabeled conjugates. Internalization of [^{111}In]ABY-025 and [^{111}In]CHX-A"DTPA-trastuzumab was evaluated as described by Wållberg and Orlova (22). DU-145, PC3 or LNCaP cells were incubated with 0.1 nM of [^{111}In]ABY-025 or 1 nM [^{111}In]CHX-"DTPA-trastuzumab in complete medium at 37˚C. At designated times during the incubation (0.5, 1, 2, 3, 4, 8 and 24 h), one group of 3 dishes was analyzed for cell-associated radioactivity. The incubation medium was collected. Membrane-bound radioactivity was determined by acid wash. Cells were treated with 0.5 ml 4 M urea solution in a 0.1 M glycine buffer, pH 2.5, for 5 min on ice. The acid fraction was collected, and the cells were washed with an additional 0.5 ml acid solution, which was added to the acid fraction. Subsequently, the cells were lysed and collected for the measurement of internalized radioactivity. For lysis, 0.5 ml of 1 M sodium hydroxide solution was added, and the cells were incubated at 37˚C for at least 0.5 h. The basic solution was collected. Dishes were washed with an additional 0.5 ml basic solution, and the basic fractions were pooled.

The radioactivity content of the samples was measured using an automated γ-counter, and data were normalized to the maximum uptake.

Results

Conjugation and labeling chemistry. In agreement with previous results, [^{111}In]-labeling of ABY-025 provided a yield exceeding 95% with specific activities up to 7 GBq/μmol. Labeling of CHX-A"DTPA-trastuzumab was as efficient; all yields were >98%, and the maximum specific radioactivity of 26.6 GBq/μmol was obtained. Since the labeling yield was consistently >95%, no further purification was required. The labeled proteins were diluted with PBS for further experiments.

In vitro binding specificity. The results of the *in vitro* specificity tests for [^{111}In]CHX-A"DTPA-trastuzumab and [^{111}In] ABY-025 are presented in Fig. 1. A pre-saturation of HER2 receptors using non-labeled molecules reduced the cell-bound radioactivity of [^{111}In]CHX-A"DTPA-trastuzumab from 6.5±0.3 to 1.89±0.08% for LNCaP, from 3.31±0.07 to 1.06±0.51% for PC3, and from 2.72±0.14 to 0.92±0.08% for the DU145 prostate cancer cell line. Similarly, pre-saturation of HER2 with non-labeled Affibody molecule reduced the binding of [^{111}In] ABY-025 from 15.22±0.31 to 0.37±0.12%, from 11.00±1.39 to 0.42±0.36%, and from 5.59±0.37 to 0.88±0.09%, for the same cell lines, respectively. In all cases, the binding reduction was highly significant ($p<0.0005$). This demonstrated that the binding of all tested conjugates to prostate cancer cells was specific, and that all tested cell lines expressed HER2.

Quantification of HER2 receptor expression in prostate cancer cell lines. The HER2 expression level was quantified using saturation experiments with [^{111}In]CHX-A"DTPA-trastuzumab. The results of a typical saturation experiment are presented in Fig. 2. All cell lines demonstrated moderate, yet detectable, expression levels. Quantitative data concerning HER2 expression are shown in Table I.

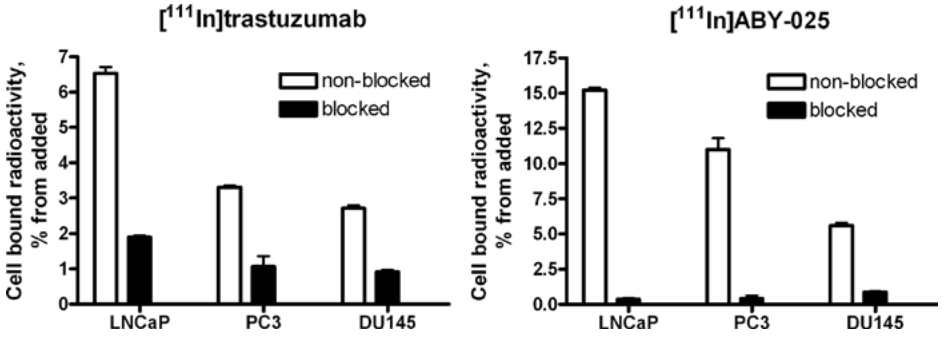

Figure 1. Specificity of the binding of the radiolabeled tracers [^{111}In]CHX-A"DTPA-trastuzumab and [^{111}In]ABY-025 to prostate cancer cells in an *in vitro* specificity test for HER2. For each of the tracers, one group of cell culture dishes was pre-treated with saturating amounts of non-labeled counterpart before incubation with the [^{111}In]-labeled tracer. Cell-associated radioactivity was calculated as the percentage of total added radioactivity. Reduction of cell-bound radioactivity due to receptor saturation confirmed specific binding. Data are presented as an average of three samples with standard deviations; error bars may not be evident as they are smaller than the point symbols.

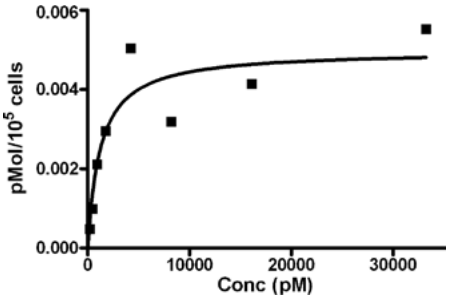

Figure 2. Non-linear regression analysis of data from a typical binding saturation experiment. Increasing concentrations of [^{111}In]CHX-A"DTPA-trastuzumab (x-axis) were added to dishes with LNCaP cells. The cells were incubated with [^{111}In]CHX-A"DTPA-trastuzumab at 4˚C to prevent internalization. After 4 h of incubation, the cells were harvested, and bound radioactivity (y-axis) was measured.

Table I. Expression of HER2 receptors by prostate cell lines *in vitro*.

Cell line	Receptors per cell
LNCaP	30,000±8,000
PC3	23,600±8,500
DU145	51,000±14,000

Data were obtained by non-linear regression analysis of saturation experiments with [^{111}In]CHX-A"DTPA-trastuzumab, and presented as an average of three samples with standard deviations.

Cellular binding and processing of radiolabeled conjugates. The data concerning the binding and cellular processing of [^{111}In]CHX-A"DTPA-trastuzumab and [^{111}In]ABY-025 are presented in Fig. 3. The binding pattern was somewhat different for the two conjugates. For Affibody molecules, an initial rapid binding was followed by slow but continuous growth of the uptake up to 24 h of incubation. Such a pattern may be due to continuous cell proliferation and an absence of HER2 down-regulation. A similar pattern was also observed with [^{111}In]CHX-A"DTPA-trastuzumab in the LNCaP cells. However, in the DU-145 and PC3 cells, a rapid initial binding of [^{111}In]CHX-A"DTPA-trastuzumab was followed by a plateau after 8 h of incubation.

The internalization of both conjugates demonstrated continued growth during the entire incubation period. The internalization rate was approximately equal for both the monoclonal antibody and the Affibody molecule. In both cases, internalization was moderately rapid. In the case of [^{111}In]ABY-025, ~10% of cell-associated radioactivity was internalized at 4 h after the initiation of incubation. At 24 h, the percentage of internalized radioactivity was 38-45%. The internalization rate of [^{111}In]CHX-A"DTPA-trastuzumab was slightly higher than that of [^{111}In]ABY-025, with a greater difference between the cell lines. For example, 50.1±2.3% of [^{111}In]CHX-A"DTPA-trastuzumab-delivered radioactivity was internalized by LNCaP cells at 24 h, while the corresponding value for DU145 cells was only 35.5±3.9%.

Discussion

Involvement of HER2 in the transition to androgen independence renders it a possible target for the therapy of disseminated prostate cancer. A common choice of therapy for breast cancer with documented overexpression of HER2 is the use of trastuzumab (Herceptin), a humanized monoclonal antibody that binds specifically to the HER2 receptor. Since a well-established drug is already in use and has been proven effective for HER2-overexpressing breast cancer tumors, the investigation of the possible use of this drug in other HER2-overexpressing tumors is warranted.

Agus *et al* demonstrated that trastuzumab is effective in androgen-independent tumor xenografts when used in combination with paclitaxel (23). The EGFR/HER2 tyrosine kinase inhibitor lapatinib has also demonstrated a promising effect in a pre-clinical *in vitro* model (24). Nevertheless, clinical trials concerning the treatment of prostate cancer with trastuzumab or lapatinib have failed to demonstrate their efficacy (25-27). It must be emphasized that unselected patients were enrolled in these trials and, at best, only biopsies from primary tumors were analyzed. An absence of patient stratification according to HER2 expression level was suggested as one possible reason for failure (25). However, the detection of HER2 expression in prostate cancer metastases remains a challenge. There have

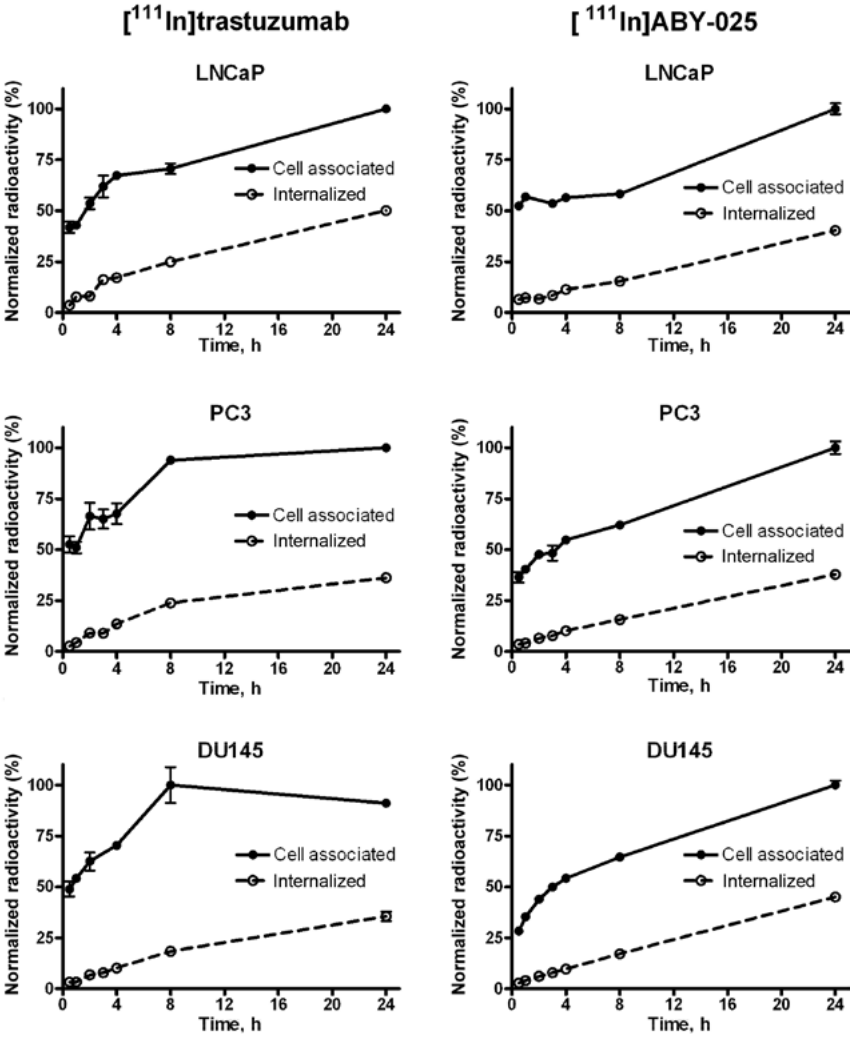

Figure 3. Binding and cellular processing of [^{111}In]-CHX-A"DTPA-trastuzumab and [^{111}In]ABY 025 by prostate cancer cells during continuous incubation at 37˚C. Data are presented as an average of three samples with standard deviations; error bars may not be evident as they are smaller than the point symbols.

been numerous attempts to establish HER2 expression levels in prostate cancer using immunohistochemistry (IHC). The results have varied from no (28) to detectable (29) overexpression in all tested samples. IHC has the downside of being a technique that requires high sample quality, well-established procedures and experienced staff to produce trustworthy data. It is difficult to compare data obtained from different studies, as some studies employ the antibody of the established HerceptTest kit (Dako Inc.), but use their own protocols, while others use the HerceptTest kit, even though the test is known to perform with less reliability in prostate tissue. Moreover, IHC requires that a biopsy be performed, and it is difficult to obtain a biopsy from bone metastases, which are the most common form in disseminated prostate cancer. Obtaining biopsies only from amenable sites is associated with the risk of false negative or positive findings, since information concerning all metastases is required for the determination of personalized treatment. The use of radionuclide molecular imaging may be a solution in this case. The present study is an initial step in the characterization of the potential imaging probes [^{111}In]ABY-025 and [^{111}In]CHX-A"DTPA-trastuzumab for the detection of HER2 overexpression *in vivo*.

The need to establish pre-clinical models for the *in vivo* detection of HER2 expression in prostate cancer cells is clear. One step towards this is the proper characterization of prostate cancer cell lines to establish the number of HER2 receptors on the cells. During this study, such a characterization was carried out for the prostate cancer cell lines DU-145, PC3 and LNCaP. Expression of HER2 was demonstrated and quantified in all three of the prostate cancer cell lines tested (Table I). As these cell lines have different degrees of androgen dependence, together they constitute a panel that can be used for the measurement of changes in HER2 expression in response to different treatments. Although the expression is moderate (20,000-50,000 receptors per cell), our previous experience with LS174T cells having similar expression level shows that such xenografts can be clearly visualized *in vivo* (30,31).

Another significant factor involved in tracer development is the internalization rate. Internalization is followed by transfer to the lysosomal compartment, where targeting proteins undergo proteolytic degradation. Charged radiocatabolites of radiometal labels remain trapped intracellularly, while lipophilic catabolites of radiohalogen labels leak from cells, decreasing tumor-associated radioactivity. Previous studies

using breast and ovarian carcinoma cell lines suggested relatively slow internalization of Affibody molecules (20,22,30), but could not exclude *a priori* that internalization of Affibody molecules proceeds at a different rate in prostate cancer cells.

Previously reported data concerning the internalization of radiolabeled trastuzumab are conflicting. Certain authors have claimed that internalization is low (32), while others have suggested a much higher internalization rate (33). Our previous data on the cellular processing of radioiodinated tracers by the gastric adenocarcinoma NCI-N87 cell line suggested a somewhat more rapid internalization of trastuzumab in comparison to Affibody molecules (34). This study demonstrated that the internalization of Affibody molecules by a prostate cancer cell line was somewhat more rapid than that of ovarian and breast cancer cell lines (22). The internalization rates of Affibody molecules and trastuzumab were rather similar, with a small variation between cell lines. Such features would definitely favor the use of radiometal labels for trastuzumab and, most likely, for Affibody molecules.

Acknowledgements

This study was supported by the Swedish Cancer Society (Cancerfonden) and the Swedish Research Council (Vetenskapsrådet). The authors thank Affibody AB, Stockholm, for providingABY-025, and Apoteket Farmaci AB (Cytostatikaberedniongen, Sjukhusapoteket, Uppsala) for assistance in obtaining the Herceptin.

References

1. Taichman RS, Loberg RD, Mehra R and Pienta KJ: The evolving biology and treatment of prostate cancer. J Clin Invest 117: 2351-2361, 2007.
2. Pienta KJ and Bradley D: Mechanisms underlying the development of androgen-independent prostate cancer. Clin Cancer Res 12: 1665-1671, 2006.
3. Tannock IF, de Wit R, Berry WR, *et al*: Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 351: 1502-1512, 2004.
4. Petrylak DP, Tangen CM, Hussain MH, *et al*: Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. N Engl J Med 351: 1513-1520, 2004.
5. Pienta KJ and Smith DC: Advances in prostate cancer chemotherapy: a new era begins. CA Cancer J Clin 55: 300-318, 2005.
6. Signoretti S, Montironi R, Manola J, *et al*: Her-2-neu expression and progression toward androgen independence in human prostate cancer. J Natl Cancer Inst 92: 1918-1925, 2000.
7. Scher HI and Sawyers CL: Biology of castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. J Clin Oncol 23: 8253-8261, 2005.
8. So A, Gleave M, Hurtado-Col A and Nelson C: Mechanisms of the development of androgen independence in prostate cancer. World J Urol 23: 1-9, 2005.
9. Culig Z, Hobisch A, Cronauer MV, *et al*: Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor and epidermal growth factor. Cancer Res 54: 5474-5478, 1994.
10. Craft N, Shostak Y, Carey M and Sawyers CL: A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nat Med 5: 280-285, 1999.
11. Gioeli D, Ficarro SB, Kwiek JJ, *et al*: Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. J Biol Chem 277: 29304-29314, 2002.
12. Slamon DJ, Godolphin W, Jones LA, *et al*: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244: 707-712, 1989.
13. Tolmachev V: Imaging of HER-2 overexpression in tumors for guiding therapy. Curr Pharm Des 14: 2999-3011, 2008.
14. Behr TM, Behe M and Wormann B: Trastuzumab and breast cancer. N Engl J Med 345: 995-996, 2001.
15. Tolmachev V and Orlova A: Update on affibody molecules for in vivo imaging of targets for cancer therapy. Minerva Biotechnologica 21: 21-30, 2009.
16. Nygren PA: Alternative binding proteins: affibody binding proteins developed from a small three-helix bundle scaffold. FEBS J 275: 2668-2676, 2008.
17. Orlova A, Magnusson M, Eriksson TL, *et al*: Tumor imaging using a picomolar affinity HER2 binding affibody molecule. Cancer Res 66: 4339-4348, 2006.
18. Baum RP, Prasad V, Müller D, *et al*: Molecular imaging of HER2-expressing malignant tumors in breast cancer patients using synthetic ^{111}In- or ^{68}Ga-labeled Affibody molecules. J Nucl Med 51: 892-897, 2010.
19. Feldwisch J, Tolmachev V, Lendel C, *et al*: Design of an optimized scaffold for Affibody molecules. J Mol Biol 398: 232-247, 2010.
20. Ahlgren S, Orlova A, Wållberg H, *et al*: Targeting of HER2-expressing tumors using ^{111}In-ABY-025, a second generation Affibody molecule with a fundamentally re-engineered scaffold. J Nucl Med 51: 1131-1138, 2010.
21. Almqvist Y, Steffen AC, Tolmachev V, Divgi CR and Sundin A: In vitro and in vivo characterization of 177Lu-huA33: a radioimmunoconjugate against colorectal cancer. Nucl Med Biol 33: 991-998, 2006.
22. Wållberg H and Orlova A: Slow internalization of anti-HER2 synthetic affibody monomer ^{111}In-DOTA-ZHER2:342-pep2: implications for development of labeled tracers. Cancer Biother Radiopharm 23: 435-442, 2008.
23. Agus DB, Scher HI, Higgins B, *et al*: Response of prostate cancer to anti-Her-2/neu antibody in androgen-dependent and -independent human xenograft models. Cancer Res 59: 4761-4764, 1999.
24. Liu Y, Majumder S, McCall W, *et al*: Inhibition of HER-2/neu kinase impairs androgen receptor recruitment to the androgen responsive enhancer. Cancer Res 65: 3404-3409, 2005.
25. Ziada A, Barqawi A, Glode LM, *et al*: The use of trastuzumab in the treatment of hormone refractory prostate cancer; phase II trial. Prostate 60: 332-337, 2004.
26. Lara PN Jr, Chee KG, Longmate J, *et al*: Trastuzumab plus docetaxel in HER-2/neu-positive prostate carcinoma: final results from the California Cancer Consortium Screening and Phase II Trial. Cancer 100: 2125-2131, 2004.
27. Sridhar SS, Hotte SJ, Chin JL, *et al*: A multicenter phase II clinical trial of lapatinib (GW572016) in hormonally untreated advanced prostate cancer. Am J Clin Oncol 33: 609-613, 2010.
28. Visakorpi T, Kallioniemi OP, Koivula T, Harvey J and Isola J: Expression of epidermal growth factor receptor and ERBB2 (HER-2/Neu) oncoprotein in prostatic carcinomas. Mod Pathol 5: 643-648, 1992.
29. Gu K, Mes-Masson AM, Gauthier J and Saad F: Overexpression of HER-2/neu in human prostate cancer and benign hyperplasia. Cancer Lett 99: 185-189, 1996.
30. Ahlgren S, Wållberg H, Tran TA, *et al*: Targeting of HER2-expressing tumors with a site-specifically 99mTc-labeled recombinant affibody molecule, ZHER2:2395, with C-terminally engineered cysteine. J Nucl Med 50: 781-789, 2009.
31. Tran TA, Rosik D, Abrahmsén L, *et al*: Design, synthesis and biological evaluation of a multifunctional HER2-specific Affibody molecule for molecular imaging. Eur J Nucl Med Mol Imaging 36: 1864-1873, 2009.
32. Austin CD, de Maziere AM, Pisacane PI, *et al*: Endocytosis and sorting of ErbB2 and the site of action of cancer therapeutics trastuzumab and geldanamycin. Mol Biol Cell 15: 5268-5282, 2004.
33. Lub-de Hooge MN, Kosterink JG, Perik PJ, *et al*: Preclinical characterisation of 111In-DTPA-trastuzumab. Br J Pharmacol 143: 99-106, 2004.
34. Orlova A, Wållberg H, Stone-Elander S and Tolmachev V: On the selection of a tracer for PET imaging of HER2-expressing tumors: direct comparison of a 124I-labeled affibody molecule and trastuzumab in a murine xenograft model. J Nucl Med 50: 417-425, 2009.