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Technical note

Development and validation of cell-based ELISA for the quantification of trastuzumab in human plasma

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ABSTRACT

Trastuzumab is a therapeutic monoclonal antibody against the Her2 oncoprotein, which is over-expressed in approximately 30% of breast cancers, and is now used routinely in the management of early and metastatic Her2+ disease. However, not all Her2+ breast cancer patients respond to trastuzumab and the pharmacodynamic and pharmacokinetic parameters behind this variation in response are unknown. Pharmacological investigations into variable response to trastuzumab have been hampered by the lack of a published feasible method to determine trastuzumab concentration in plasma. Here we describe the development and validation of a cell-based ELISA to measure trastuzumab in human plasma. The assay specifically measures the interaction between trastuzumab and Her2 and has a dynamic range of between 10 and 120 μg/ml. The mean intra-assay and inter-assay variability of the ELISA was 9%. Trastuzumab in plasma was stable for at least 10 weeks at −20 °C and 72 h at 4 °C, and was unaffected by 5 freeze/thaw cycles. Having validated the assay, the trough plasma trastuzumab concentrations of 30 patients being treated for metastatic or early disease were measured. The median trough concentration was 62 (range 21 to 441) μg/ml.
This cell-based ELISA method has undergone appropriate validation and is suitable for quantification of trastuzumab in the plasma of patients treated with Herceptin.

1. Introduction

Breast cancer is the most commonly diagnosed cancer and, despite improvements in treatment, remains the leading cause of cancer-related mortality in women in Europe (Ferlay et al., 2007). Incidence of breast cancer has been rising over the last 30 years, however improvements in early detection and therapeutic management of the disease have led to a concomitant decrease in mortality over the last 20 years in many countries (Botha et al., 2003). The improvements in therapy have been largely due to an increased understanding of the molecular aetiology of the disease. Breast cancer is a heterogeneous disease, and bio-molecular elements responsible for this heterogeneity have been exploited in attempts to specifically target cancer cells. For example ~70% of breast cancers are estrogen and/or progesterone receptor positive. Many of these tumours are initially dependent on estradiol for proliferation and estrogen antagonism is central to the management of hormone receptor positive tumours (EBCTCG, 1998). More recently the over-expression, due to gene amplification, of the receptor tyrosine kinase human epidermal growth factor receptor 2 (cErb2, Her2) has been exploited in cancer chemotherapy (Cobleigh et al., 1999).

Her2 gene amplification occurs in upto 30% of breast cancers (Slamon et al., 1987) and is an oncogenic event, driving ligand-independent proliferation (Worthylake et al., 1999) and survival via aberrant PI3K/AKT signalling (Yakes et al., 2002). The oncogenic overexpression of Her2 has provided a tumour specific target for the therapeutic management of breast cancer.

Trastuzumab (Herceptin) is a humanised monoclonal antibody targeting an epitope resulting from the tertiary structure of Her2 (Cho et al., 2003). It is currently used

Abbreviations: ELISA, Enzyme-Linked Immunosorbent Assay; TBS, Tris Buffered Saline; HRP, Horseradish peroxidise.

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clinically as an adjuvant therapy in early breast cancer and in the treatment of metastatic disease (Baselga et al., 2006). The exact mechanism of action of trastuzumab is uncertain, but appears to be multifactorial and includes the direct inhibition of proliferative signalling and induction of antibody-dependent cell-mediated cytotoxicity (ADCC) (Cooley et al., 1999). Four major trials have shown that trastuzumab reduces the risk of recurrence of early Her2 positive breast cancer by approximately 50% (Piccart-Gebhart et al., 2005; Romond et al., 2005; Slamon et al., 2005). When coadministered with docetaxel in the treatment of metastatic disease, trastuzumab improves overall survival from 23 to 31 months (Marty et al., 2005). However only 38% of patients with Her2-positive metastases respond to single agent trastuzumab therapy (Vogel et al., 2002), and cancer recurs in the majority of patients with metastatic disease (Slamon et al., 2001).

There are few published clinical investigations of biomarkers that may be predictive of response or susceptibility to resistance to trastuzumab. Several studies have reported pharmacokinetic data on trastuzumab and interindividual variability in trough concentrations of between 10-fold and 1000-fold have been observed, with low concentrations being associated with high circulating Her2 extracellular domain (ECD) in plasma (Baselga et al., 2005). It has also been observed that response is associated with a 1.6-fold higher mean trough trastuzumab concentration than that seen in non-responders (Cobleigh et al., 1999).

A barrier to investigating potential predictive pharmacokinetic parameters and pharmacodynamic biomarkers is the lack of a published assay to measure trastuzumab concentration in plasma. Pharmacokinetic studies published so far have failed to provide sufficient detail to allow the assay to be carried out independently (Baselga et al., 1996), (Cobleigh et al., 1999), (Tokuda et al., 1999), or have used purified Her2 protein that is not commercially available (Pegram et al., 1998). Although a very detailed method for quantification of trastuzumab has been published (Maple et al., 2004) the method described requires a capture antigen that is supplied only as a standard in a commercially-available Her2 ELISA kit. The quantities required mean that only a limited number of samples could be analysed for each kit purchased, in addition to the cost of capture antibodies and routine ELISA reagents.

We are currently undertaking clinical trials in breast cancer where measurement of trastuzumab pharmacokinetics would be advantageous. Given the lack of commercially-available purified trastuzumab antigens, we have developed and validated a cell-based ELISA to quantify trastuzumab in diluted human plasma. The assay uses a high density of formaldehyde-fixed SKBR3 Her2-positive breast cancer cells as the capture antigen in what is otherwise a conventional direct ELISA.

2. Materials and methods

Trastuzumab (Herceptin) was purchased from Roche (Welwyn Garden City, UK). TMB (3,3′,5,5′-tetramethylbenzidine) substrate kit was purchased from Pierce (Cramlington, UK). Fetal calf serum (FCS), HRP conjugated goat anti-human antibody and Alexa fluor488 conjugated goat anti human antibody were purchased from Invitrogen (Paisley, UK). Goat serum was purchased from Millipore (Herts, UK). Human plasma from nine individuals was purchased from the Blood Transfusion Service (Newcastle upon Tyne, UK). Phosphate buffered saline, Tris base, sodium chloride, concentrated hydrochloric acid, Tween 20, sodium azide, poly-D-lysine, Hoescht 33342, RPMI 1640, Formalin and Costar 96 well tissue culture plates were all purchased from Sigma (Poole, Dorset, UK). Sterile μ-clear black-walled optical 96 well plates were purchased from Greiner (Gloustershire, UK). SKBR3 breast adenocarcinoma cells and MDA MB 231 breast adenocarcinoma cells were gifts from Dr. Felicity May at the NICR, UK.

2.1. Patient samples

Plasma samples from 30 patients with Her2-positive breast cancer were collected during 2008. All the patients received trastuzumab every three weeks (8 mg/kg loading dose followed by 6 mg/kg) as part of their treatment of metastatic or non metastatic disease at the Newcastle General Hospital, Newcastle, UK. Samples were withdrawn immediately before the administration of the next dose of trastuzumab, after a minimum of 6 weeks of treatment. Patient samples were collected under appropriate ethical approval and with full informed patient consent.

2.2. Immunocytochemistry

SKBR3 and MDA MB 231 cells were plated at 5000 cells per well in optical 96 well plates and allowed to adhere for 24 h at 37 °C and 5% CO_2. After washing with PBS cells were fixed with 10% formalin for 20 min. The cells were blocked for 1 h with 20% goat serum in Tris buffered saline (TBS) with 0.1% Tween 20 for 1 h at room temperature and subsequently incubated with human plasma spiked with trastuzumab and diluted 1/4000 in wash buffer (TBS with 0.1% Tween 20 and 1% goat serum). Each well was washed 3 times with wash buffer, then incubated for 1 h at room temperature with an Alexafluor 488 tagged goat anti-human antibody diluted 1/500 in wash buffer with 10 μg/ml Hoescht 33342. Wells were washed 3 times with PBS and imaged with a ×20 objective on a Pathway HT Inverted fluorescent microscope (BD, Oxford, UK).

2.3. Trastuzumab ELISA

SKBR3 cells were propagated in RPMI 1640 with 10% FCS at 37 °C and 5% CO_2. To prepare single-use aliquots, SKBR3 cells were maintained and trypsinised at approximately 90% confluence, and split 1 in 2 until thirty two 125 cm^2 tissue culture flasks with cells at approximately 90% confluence were attained. The cells were trypsinised, pooled and counted on a Coulter Z1 cell counter (BD, Oxford, UK). The resuspended cells were centrifuged at 1000 rpm in a Centaur 2 bench top centrifuge (MSE, London, UK) for 5 min, the medium was aspirated and the pellet resuspended in RPMI 1640 with 10% FCS and 10% DMSO at a concentration of 2.6×10^7 ml^{-1}. This suspension was stored at −80 °C in 0.5 ml aliquots until needed for the assay.

On day one of the assay the inner 60 wells of two 96 well tissue culture plates were incubated with 100 μl per well of 100 μg/ml 70,000–150,000 kDa poly-D-lysine for 5 min at room temperature under sterile conditions. The poly-D-lysine was aspirated and each well was washed with sterile deionised