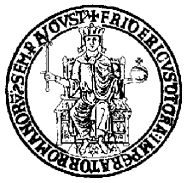

Università degli studi di Napoli
"Federico II"

Dottorato di ricerca in Biochimica e Biologia Cellulare e Molecolare
XX ciclo

Novel human immunoagents anti-ErbB2: interactions with the ErbB2 receptor

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Summary

Overexpression of the tyrosine kinase receptor ErbB2 is a typical feature of many carcinomas, in particular breast cancer where it is associated with progression of malignancy, and is a sign of a poor prognosis. Thus, ErbB2 represents a validated target of therapeutic intervention.

Herceptin, a humanized anti-ErbB2 antibody, has proved to be very effective in the immunotherapy of breast carcinoma. However, some ErbB2-positive carcinomas are resistant to Herceptin and it is cardiotoxic in a high percent of patients. We have engineered novel fully human immunoagents directed to ErbB2. Erbicin, an anti-ErbB2 scFv ; Erb-hRNase, a human immunoRNase made up of Erbicin fused to a human RNase; Erb-hcAb, a human, “compact” antibody, in which two Erbicin molecules are fused to the CH2 and CH3 regions of a human IgG1. Both, Erb-hRNase and Erb-hcAb severely inhibit the growth of ErbB2-positive cells in vivo.

To define and implement the antitumor potential of Erbicin, ERB-hcAb, and ERB-hRNase, I have deemed essential to gather further and hard data on their interactions with ErbB2. The purified antigen was used, i.e. the extracellular domain of ErbB2 (ErbB2-ECD), and three different methods of investigation. The results provided for the first time a systematic analyses of the binding parameters of Erbicin, the Erbicin-Derived-ImmunoAgents (EDIA), in comparison with Herceptin. They indicated that the EDIA bind soluble ECD with a lower affinity than that of Herceptin, and ErbB2 exposed on the cell surface with a higher affinity than that of Herceptin.

These results suggest that the fraction of immunoagent neutralized by free ECD shed into the bloodstream is much higher for Herceptin than for the novel immunoagents.

Finally I show here for the first time that these novel immunoagents recognize on the ErbB2 receptor an epitope different from that targeted by Herceptin.

Sommario

L'iperespressione del recettore transmembrana tirosinachinasico ErbB2 (Her2/neu) è caratteristica di diversi tipi di carcinomi, in particolare del cancro della mammella, ed è associata a maggiore aggressività e prognosi infausta. ErbB2 rappresenta dunque un bersaglio ideale per l'immunoterapia.

Herceptin, un anticorpo umanizzato anti-ErbB2 largamente impiegato nella terapia del carcinoma mammario, risulta essere cardiotossico per molte pazienti. Inoltre molti carcinomi ErbB2-positivi sono resistenti ad Herceptin.

Basandoci su queste considerazioni nel nostro laboratorio sono stati costruiti nuovi immunoagenti (IA) completamente umani anti-ErbB2: Erbicina un scFv (*single chain variable fragment*) anti-ErbB2 costituito dalle regioni variabili della catena pesante e leggera dell'anticorpo unite tramite un oligopeptide flessibile; Erb-hRNasi, una immunoRNasi costituita dalla fusione di Erbicina con la ribonucleasi pancreatica umana (HP-RNasi); hERB-hcAb, un anticorpo umano compatto in cui due molecole di Erbicina sono legate alle regioni CH2 e CH3 di una IgG1 umana. Entrambi Erb-hRNasi ed Erb-hcAb, risultano essere citotossici per cellule ErbB2-positive, inibiscono efficacemente la crescita di tumori ErbB2 positivi in vivo e studi preliminari in vitro hanno dimostrato che tali IA non presentano effetti cardiotossici. Allo scopo di definire e sfruttare il potenziale antitumorale del frammento anticorpale Erbicina e degli immunoagenti da essa derivati è stato necessario procedere alla caratterizzazione degli stessi mediante studi sulle interazioni con il dominio extracellulare del recettore ErbB2 (ErbB2-ECD) utilizzando per la prima volta l'antigene in forma solubile.

L'attività si è articolata in varie fasi:

a) clonaggio, espressione e purificazione del dominio extracellulare di ErbB2.

Per tale fine, la regione comprendente i residui 1-624 di ErbB2-ECD è stata stabilmente espressa in cellule eucariotiche 293 e secreta nel mezzo di coltura, ErbB2-ECD è stato poi purificato mediante cromatografia di immunoaffinità con una resa di circa 10mg/L;

b) ottenuto l'antigene puro, determinazioni dell'affinità di legame di ErbB2-ECD con gli IA sono state effettuate in parallelo con Herceptin mediantesaggi ELISA, saggi calorimetrici e saggi di risonanza plasmonica di superficie (*SPR*);

c) mappa dell'epitopo, riconosciuto dagli immunoagenti su ErbB2, al fine di determinare se tali IA, riconoscono epitopi differenti rispetto ad anticorpi attualmente utilizzati nella cura dei tumori ErbB2 positivi.

I risultati ottenuti con i diversi approcci metodologici hanno dimostrato che Erbicina e gli IA da essa derivati contrariamente ad Herceptin legano ErbB2-ECD solubile con minore affinità rispetto a quella determinata con ECD inserito in cellule ErbB2-positive. Inoltre si è trovato che mentre il legame di ERB-hcAb a cellule ErbB2-positive non è influenzato dalla presenza di ECD solubile, quello di Herceptin è fortemente inibito. Ciò rappresenta un vantaggio ai fini terapeutici poiché i nuovi IA tendono a legarsi con maggiore affinità al dominio extracellulare del recettore ErbB2 nella sua conformazione nativa associata alla superficie cellulare, rispetto all'ECD libero nel circolo ematico. Questa frazione di ECD, prodotta dal taglio proteolitico di metalloproteasi, aumenta quando ErbB2 è iperespresso. L'impiego di IA anti-tumorali con minore affinità per ECD solubile permetterebbe l'uso di dosi terapeutiche inferiori rispetto a quelle usate per Herceptin. La possibilità di eseguire saggi ELISA *sandwich* e saggi *SPR* in cui ErbB2-ECD è riconosciuto sia da Herceptin, che da Erbicina o ERB-hRNasi, ha fornito inoltre per la prima volta una prova indiretta del fatto che Erbicina e gli IA da essa derivati riconoscono un epitopo di ErbB2 differente da quello riconosciuto da Herceptin. Per l'identificazione dell' epitopo, il complesso ERB-hcAb-ECD immobilizzato su matrice di agarosio, è stato trattato con proteasi specifiche. La regione dell' antigene in contatto con l'anticorpo protetta dal taglio proteolitico è stata identificata mediante analisi di spettroscopia di massa MALDI.

L'epitopo identificato è compreso nella regione N-terminale del primo dominio dell'ECD, una regione differente rispetto a quella identificata per gli anticorpi anti-ErbB2 noti. Tale risultato offre la prospettiva che gli immunoagenti derivati da Erbicina possano agire con meccanismo differente e pertanto possano essere impiegati in alternativa o in associazione con Herceptin con un conseguente incremento della loro azione antitumorale.

Abbreviations

BSA	Albumin from bovine serum
DMEM	Dulbecco modified Eagles medium
ECD	Extracellular domain
EDIA	Erbicin-Derived-ImmunoAgents
ELISA	Enzyme Liked Immuno Sorbant Assay
ErbB2-ECD	Extracellular domain of ErbB2 receptor
ERB-hcAb	Human anti-ErbB2-compact Antibody
FBS	Foetal bovine serum
Fc	CH2 and CH3 regions of a human IgG1
HP-RNasi	Human pancreatic RNase
HRP	Horseradish peroxidase
IA	Immunoagents
ITC	Isothermal titration calorimetry
PBS	Phosphate buffer saline
PVDF	Poly-vinylidene difluoride
ScFv	Single-chain antibody fragment
SDS	Sodium dodecyl sulphate
SPR	Surface plasmon resonance
TRIS	Trishydroxymethylaminomethane
$\mathbf{V_H}$	The variable domains of heavy chains
$\mathbf{V_L}$	The variable domains of light chains

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INTRODUCTION

*Erb*B2 (HER2/*neu*) is a proto-oncogene of the *erb*B family of tyrosine kinase receptors (1). It encodes the ErbB2 185 kDa transmembrane glycoprotein receptor, which comprises an extracellular domain (ECD) and an intracellular tyrosine kinase activity (2) (Figure 1). While no natural ligand has been identified for this receptor, it has been ascertained that its overexpression is associated with various carcinomas, in particular with human breast cancer. As ErbB2 overexpression is involved in progression of the malignancy, and is a sign of a poor prognosis, (3) recent evidence indicates that ErbB2 amplifies the signal provided by other receptors of the ErbB family by heterodimerizing with them (4). The important biological role of ErbB2 in the signaling network that drives epithelial cell proliferation and transformation together with its extracellular accessibility and its overexpression has led to consider ErbB2 as a validated target of therapeutic intervention. However, when ErbB2 is overexpressed not all the ErbB2 protein is embedded in the membrane of malignant cells, as it has been reported that a fraction of the extracellular domain of ErbB2 (ErbB2-ECD) is proteolytically removed from the receptor and is shed as a soluble protein in the sera of breast cancer patients (5).

Herceptin (6), a humanized anti-ErbB2 antibody, has proved to be an essential tool in the immunotherapy of breast carcinoma. However, some ErbB2-positive carcinomas are resistant to the growth inhibitory effect of Herceptin (7), while in other patients resistance of malignant cells is induced at a later stage in the treatment (8). Furthermore, it has been found that Herceptin can engender cardiotoxicity in a significant fraction of treated patients (9, 10).

An alternative approach to the use of Herceptin in immunotherapy has been promoted, based on

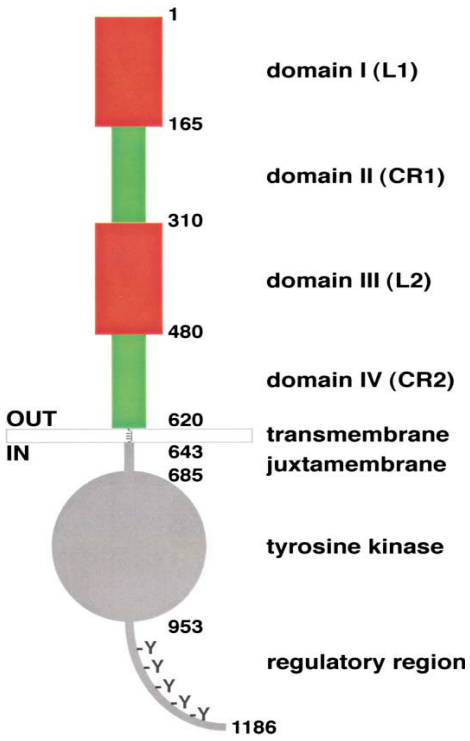

Figure 1

Domains organization of ErbB2 receptor: domain I and III (red); domain II e IV (green) of the extracellular region; the transmembrane domain and the intracellular tyrosine kinase domain are in gray.

the administration of Herceptin combined with other anti-ErbB2 antibodies (11, 12). A prerequisite for this strategy is that the latter antibodies are directed to epitopes on ErbB2-ECD different from that recognized by Herceptin.

Based on these considerations, we have been searching for novel immunoagents directed to epitopes different from that recognized by Herceptin, with no cardiotoxic side effects, and able to fulfil the therapeutic need of Herceptin unresponsive patients.

This led us to the production of a novel, fully human anti-ErbB2 single-chain antibody fragment (scFv), isolated from a large phage display library through a double selection strategy performed on live cells. This scFv (Figure 2), named Erbicin (13), specifically binds to ErbB2-positive cells, inhibits the receptor autophosphorylation, and is internalized in target cells. Furthermore, Erbicin has been used to construct more effective human anti-ErbB2 immunoagents by two different strategies.

The first was based on immunoconjugates made up of Erbicin fused with an RNase, i.e. a pro-toxin, as the RNase becomes toxic only when Erbicin promotes its internalization by target cells. One of these fully human immunoconjugates, an immunoRNase (IR), denominated ERB-hRNase (Erbicin-human-RNase), was produced by the fusion of Erbicin with human pancreatic RNase (14) as shown in Figure 3. The alternative strategy was aimed at producing a therapeutic reagent with an increased half-life, a prolonged tumor retention, and the ability to recruit host effector functions. Erbicin was thus fused to the CH2, CH3, and hinge regions from a human IgG1 to obtain an immunoglobulin-like antibody (15, 16).

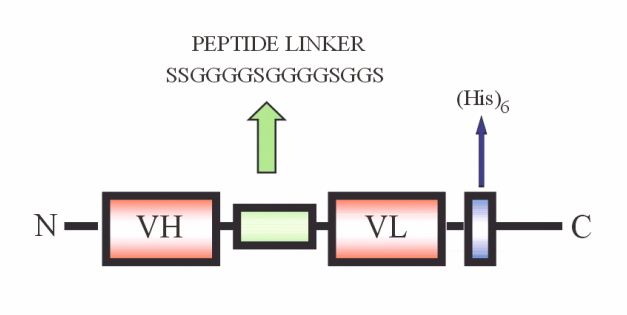

Figure 2

Schematic representation of Erbicin an anti-ErbB2 single chain variable fragment (scFv). VH and VL, the variable domains of heavy and light chains respectively; LINKER, the 15-residue junction peptide, $(His)_6$, a 6-residue His tag.

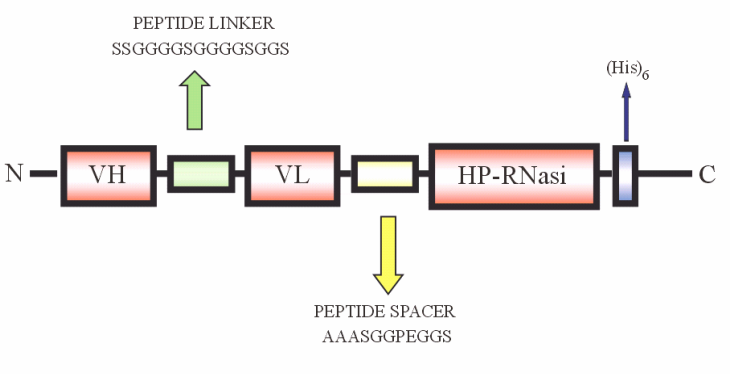

Figure 3

Schematic representation of the human immunoRNase ERB-hRNase. VH and VL, the variable domains of heavy and light chains respectively, of Erbicin the anti-ErbB2 single chain variable fragment (scFv); LINKER, the 15-residue junction peptide; SPACER, the peptide connecting Erbicin and the HP-RNase moieties and $(His)_6$, a 6-residue His tag.

The engineered antibody was called ERB-hcAb (human anti-ErbB2-compact Antibody) for its "compact" size (100 kDa), compared with the full size (155 kDa) of a natural IgG (Figure 4).

Both Erbicin-Derived-Immunoagents were found to selectively and strongly kill ErbB2-positive cells, both *in vitro* and *in vivo* (13-16). However, to define and implement the antitumor potential of Erbicin, ERB-hcAb, and ERB-hRNase, I have deemed essential to gather further and hard data on their interaction with ErbB2 by using the purified antigen, i.e. the extracellular domain of ErbB2.

The majority of antibodies so far isolated from combinatorial libraries expressed on phages have been selected using purified antigens, or peptides immobilized on artificial surfaces. The disadvantage of this approach is that it may lead to the selection of antibodies that do not recognize the antigen in its native state within the cell membrane, i.e. in its physiological context (17). On the other hand, direct panning of an scFv library on live cells, as it was performed for the isolation of Erbicin (13), has the potential of isolating phage antibodies that recognize cell surface antigens in their native configuration.

To determine and quantitatively evaluate the affinity for ErbB2 of the Erbicin and Erbicin-Derived-ImmunoAgents (EDIA), I have used for the first time the recombinant extracellular domain of ErbB2 as a homogenous, soluble antigen.

Furthermore, three different analytical methods were employed, and the results obtained with Erbicin and the Erbicin-Derived-ImmunoAgents were compared with the results obtained with Herceptin.

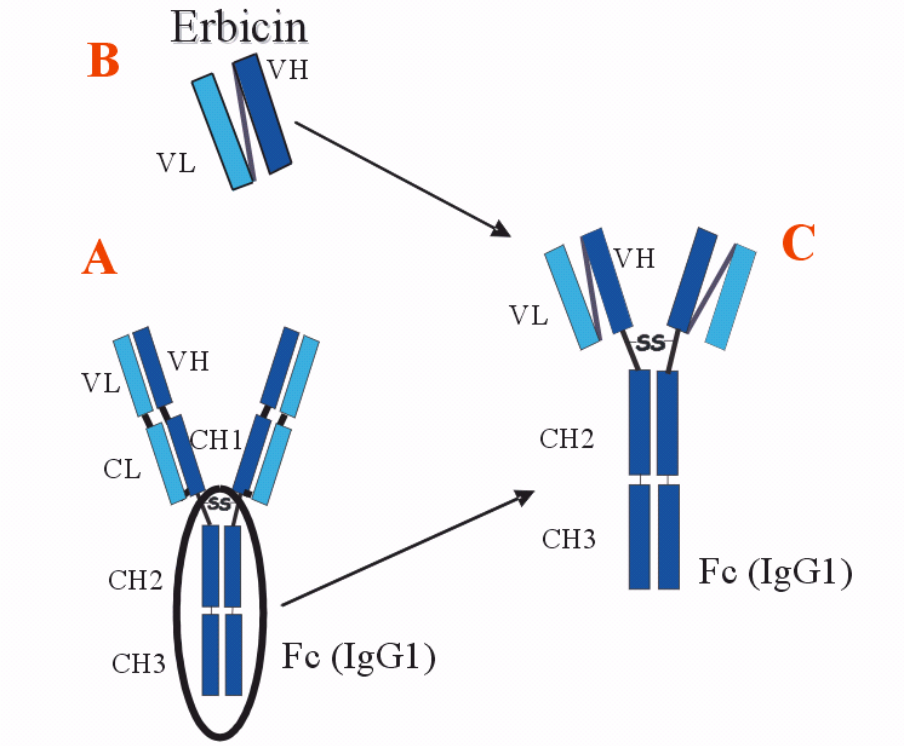

Figure 4

Schematic representation of ERB-hcAb (C), the human compact anti-ErbB2 antibody made up of two Erbicin molecules (B) fused to the CH2 and CH3 regions of a human IgG1 (A).

All three methodological approaches led to convergent affinity values, which *de facto* validates the results as obtained. Furthermore, we determined and compared the affinity values of Herceptin and the Erbicin-Derived-ImmunoAgents for the ECD structured within the whole receptor molecule, natively inserted into the cell membrane, with the values measured using the isolated ECD. I found that the Erbicin-Derived-ImmunoAgents bind soluble ECD with a lower affinity than Herceptin. On the other hand, the EDIA bind ErbB2 exposed on the cell surface with a higher affinity than that of Herceptin (14, 15). These results suggest that the fraction of immunoagent neutralized by the free ECD shed into the bloodstream is much higher for Herceptin than for the novel immunoagents.

Finally, I report that these novel immunoagents recognize on the ErbB2 receptor an epitope different from that targeted by Herceptin (18).

Materials and Methods

Cell lines and Antibodies

The 293 cell line (human embryonic kidney), was cultured in DMEM (Gibco Life Technologies, Paisley, UK BRL). The SKBR3 cell line (human breast cancer), was cultured in RPMI 1640 (Gibco BRL). The media were supplemented with 10% heat-inactivated foetal bovine serum (FBS), 50 Units/ml penicillin, and 50 µg/ml streptomycin (all from Gibco). All the cell lines were obtained from ATCC (Rockville, MD) and cultured at 37°C in 5% CO_2 atmosphere.

The antibodies used were: Herceptin (Genentech**,** South San Francisco, CA, USA); horseradish peroxidase-conjugated anti-His antibody (Qiagen**,** Valencia, CA, USA); horseradish peroxidase-conjugated goat anti-human affinity isolated IgG1 (Fc- specific, Sigma**,** St Louis, MO, USA). Erbicin, ERB-hcAb and ERB-hRNase were prepared as previously described (13-15).

Production of the extracellular domain of ErbB2 receptor (ErbB2-ECD)

ErbB2-ECD, the extracellular domain of ErbB2 (residues 1–624) was stably expressed and secreted by 293 cells. In brief, cells grown in DMEM containing 10% FBS at 70-80% confluency were co-transfected with 5µg of expression vector pVij-hECDopt, coding for the cDNA of ErbB2-ECD, and 100ng of selectable plasmid *Signal pIg1plus* (R & D System, Minneapolis, USA) using the SuperFect reagent (Qiagen).

Stable transfectants were selected in the presence of G418 (Sigma) at concentration of 1mg/mL and subcloned. The expression of ErbB2-ECD was evaluated by 8% SDS-PAGE analyses of conditioned medium, under non-reducing condition, followed by electroblotting onto poly-vinylidene difluoride membranes (Millipore Corporation, Bedford, MA, USA). For detection, Herceptin was used as primary anti-ErBb2 antibody, followed by horseradish peroxidase-conjugated anti-human IgG1 (Fc-specific) mAb for visualization by enhanced chemioluminescence (Super signal West Pico, Pierce Rockford, IL, USA). The culture medium of 293 cells before transfection was used as negative control. For recombinant protein production, co-transfected 293 cells were expanded to near confluence in selective medium containing neomycin (SIGMA) at concentration of 0,4mg/mL, and then grown for 3-4 days in serum-free medium. The conditioned medium (1 liter), with an estimated ErbB2-ECD concentration about 20 mg/L, was concentrated by ultrafiltration on AMICON membranes to 100mL and frozen at -80°C for further processing.

Immunoprecipitation assays

The ErbB2-ECD immunoprecipitation assays were carried out by incubation of 10 mL aliquots of 293 cells conditioned medium with 10 µg/ml (final concentration) of Herceptin or ERB-hcAb in PBS for 3 h at 4°C. The immune complexes were then collected by adsorption to protein A-Agarose (Sigma) for 1 h at 4°C. After washes with PBS, the proteins were released by boiling in loading buffer (19), and run on 8% SDS-PAGE, followed by Coomassie staining and immunoblotting assays as described above.

Protein purification

ErbB2-ECD secreted by transfected 293 cells was purified from culture medium by immunoaffinity chromatography with the AKTA Purifier system (GE Healthcare, Amersham Bioscience AB, Uppsla, Sweden). The affinity column was prepared by coupling 8 mg of Herceptin to 1.5 g of CNBr-activated Sepharose 4B Fast Flow (GE Healthcare). The antibody was immobilised to the agarose via secondary amine according to the manufacturer's instructions. The resulting 4-ml column was loaded with 10 mL of the concentrated conditioned medium, washed with 3 volumes of 10 mM Tris-HCl, pH 7.4 and eluted with 50 mM Glycine pH 3.0 containing 1M NaCl. The collected fractions were immediately neutralized with a 1/10 volume of 1 M Tris-HCl pH 8.0.

The purity of the preparation was evaluted by 8% SDS-PAGE, followed by Coomassie staining and Western Blotting analyses with either Herceptin or ERB-hcAb as anti-ErbB2 antibodies, followed by horseradish peroxidase-conjugated anti-human IgG1 (Fc-specific) mAb. The purified protein was stored until use at –20°C after addition of glycerol at a concentration of 10%.

ELISA assays for the complex of ErbB2-ECD and Erbicin or ERB-hRNase

The affinity of Erbicin and ERB-hRNase for soluble ErbB2-ECD was measured by an ELISA sandwich assay. A 96-well plate was coated with 5 µg/ml of Herceptin in PBS (Sigma), kept overnight at 4°C and blocked for 1 h at 37 °C with 5% bovine serum albumin (BSA) (Sigma) in PBS. To the plate rinsed with PBS, a solution of purified ErbB2-ECD in PBS (5 µg/ml) was added.

After 1 h at room temperature, the plate was washed and increasing concentrations of purified ERB-hRNase or Erbicin (50-500 nM) were added in ELISA buffer (PBS/BSA 1%) in triplicate wells, and incubated for 2 h at room temperature with a blank control of PBS. After rinsing with PBS, an anti-His HRP-conjugated antibody was added at 1/1000 dilution in ELISA buffer. After 1 h at room temperature, the plate was rinsed with PBS, and bound immunoagents were detected by using 3,3',5,5-tetramethylbenzidine (TMB) as a substrate (Sigma). The product was measured at 450 nm using a microplate reader (Multilabel Counter Victor 3, Perkin Elmer, Cologno Monzese, Italy). The reported affinity values are the mean of at least three determinations (standard deviation ≤ 5%).

ELISA assays for the complex of ErbB2-ECD and ERB-hcAb or Herceptin

The affinity of ERB-hcAb and Herceptin for ErbB2-ECD was measured as follows. A 96-well plate was coated with 5 µg/ml of purified ECD in PBS and left overnight at 4°C. After blocking with 5% BSA as above, increasing concentrations of ERB-hcAb (10-60 nM) or Herceptin (0.1-10 nM) were added in ELISA buffer and allowed to bind for 2 h at room temperature. The plate was rinsed with PBS and an anti-human IgG1 (Fc-specific) HRP-conjugated antibody was added at 1/1000 dilution in ELISA buffer for antibody detection. After 1 h, the plate was rinsed with PBS and bound ERB-hcAb or Herceptin was detected as described above.

ELISA assays with ErbB2-positive cells

ELISA assays with ErbB2-positive cells were carried out on SKBR3 cells harvested in non-enzymatic dissociation solution (Sigma), washed and transferred to U-bottom 96-well microtiter plates (2 x 10^5 cells per well). After blocking with PBS containing 6% BSA, cells were treated with ERB-hcAb or Herceptin at increasing concentrations (1-8 nM) in ELISA buffer (PBS/BSA 3%) in the presence or absence of soluble ErbB2-ECD. ErbB2-ECD was added either in equimolar amounts or in a 10-fold molar excess to the ErbB2 receptor number on SKBR3 cells (20). After an incubation of 90 minutes at room temperature, centrifugation and removal of supernatants, pelleted cells were washed twice with ELISA buffer and incubated with peroxidase-conjugated anti-human IgG1 (Fc-specific) mAb for antibodies detection. After 1 h, the plates were centrifuged, washed with ELISA buffer, and reacted with TMB as above. Binding values were determined from the absorbance at 450 nm, and reported as the mean of at least three determinations (standard deviation ≤ 5%).

Isothermal titration calorimetry (ITC)

ITC measurements were carried out in collaboration with Prof. C. Giancola, Department of Chemistry, Federico II University, Napoli, Italy, as described in previous reports (20). In brief, tritrations were performed using a CSC 4200 calorimeter from Calorimetry Science Corporation (CSC, Utah) with a cell volume of 2 mL (Figure 5).

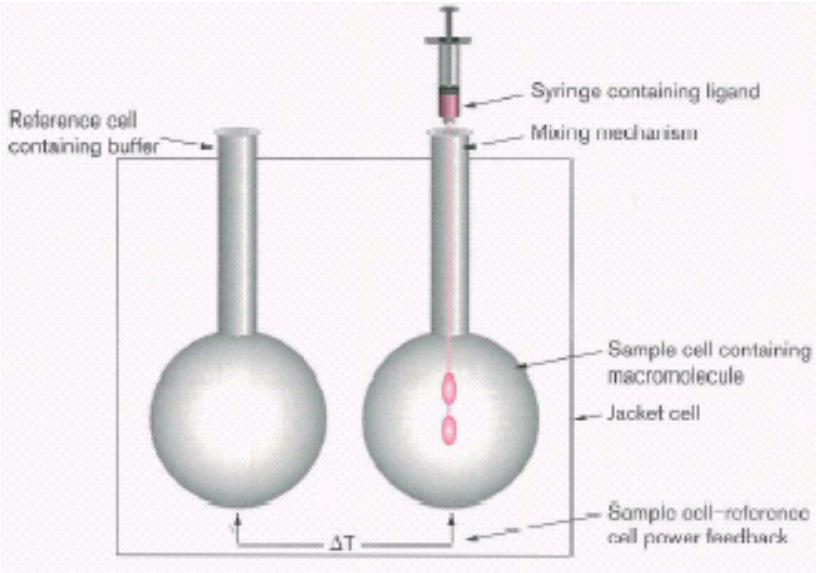

Figure 5

Schematic representation of the ITC instrument.

The concentration of the immunoagents (Erbicin, ERB-hRNase, ERB-hcAb), in the instrument cell, were 30 µM, and ErbB2-ECD ligand concentration in the syringe was 3 µM. For each titration, 10 µL aliquots of ErbB2-ECD in PBS solution were injected into the immunoagents solution in PBS at 400 s intervals allowing for complete equilibration. Binding curves involved the addition of about 25-injections. The heat of dilution of the immunoagents into the solvent was measured in a separate experiment and appropriate corrections were made. The data were integrated, corrected for dilution heats, normalized for concentration, and analyzed, assuming a model based on a single set of identical independent binding sites, with the Bindwork software supplied with the instrument, which provided a stoichiometry of binding (*n* ligand:protein), change in enthalpy (ΔH), and binding constant (K_b).

For the experiments with immunoagents binding directly to ErbB2-positive cells, SKBR3 cells were grown to half-confluency, left overnight in the absence of serum, and collected from the plate by cell dissociation solution. After equilibration in a binding buffer made up of PBS containing 3% BSA, $2.5x10^5$ cells, corresponding to 0.83 pMol of ErbB2 receptor (21) were diluted to 2 mL in binding buffer and introduced in the sample cell of the instrument. The immunoagents solutions in PBS were added by successive injections as above for a total of 1.66 pMol. Data were treated as described above for the experiments with soluble proteins. In the experiments in which cell endocytosis was blocked, cells were treated, before their introduction in the apparatus sample chamber, with 2-deoxy-glucose (50 mM) and Na azide (10 mM) for 2 h at 37°C.

Surface plasmon resonance (SPR) analyses

Surface plasmon resonance (SPR) analyses were performed at 25°C on a BIAcore X instrument (BiacoreAB, Uppsala, Sweden), equipped with research-grade CM5 sensor chips (Biacore). The running buffer was HBS-EP buffer (10 mM Hepes, 0.15 M NaCl, 3.4 mM EDTA and 0.005% surfactant P20 at pH 7.4). Coupling reagents, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethyl aminopropyl) carbodimide hydrochloride (EDC), ethanolamine hydrochloride and HBS-EP running buffer were purchased from Biacore (Uppsala, Sweden). Soluble carboxymethyl-dextran was from Fluka (Buchs SG, Switzerland) and Protein A from *Staphylococcus aureus* was purchased from GE Healthcare (Bio-Sciences).

Kinetic analyses by SPR of soluble ErbB2-ECD binding to captured ERB-hcAb or Herceptin

SPR analyses were carried out in collaboration with Valeria Cafaro Doctor, Department of Structural and Functional Biology, Federico II University, Napoli, Italy. To investigate the binding properties of Herceptin and ERB-hcAb to the soluble extracellular domain of the ErbB2 receptor (ErbB2-ECD), a capturing method was chosen. Herceptin and ERB-hcAb were captured by Protein A from *Staphylococcus aureus* immobilized onto the surface of a CM5 sensor chip using standard amine coupling chemistry. The surface of the chip was activated with 70 µL of a mixture 1:1 of 0.4 M 1-ethyl-3-(3-dimethyl aminopropyl) carbodimide hydrochloride (EDC) and 0.1 M N-hydroxysuccinimide (NHS), followed by 100 µL of Protein A (70 µg/ml) dissolved in 10 mM sodium acetate buffer, pH 4.5, at a constant flow-rate of 10 µL/min. Typically, 5000 response units (RU) of Protein A were captured. To block unreacted activated esters, 70 µL of 1 M ethanolamine hydrochloride, pH 8.5, were injected. Non-covalently associated Protein A was washed from the surface using four 30-s injections of 10 mM glycine–HCl, pH 2.2. The reference flow cell was inactivated by amine coupling chemistry as already described, but omitting the Protein A injection. Herceptin (2 µg/mL) or ERB-hcAb (1 µg/mL) diluted in HBS-EP buffer was injected onto the chip at a flow rate of 30 µL/min, giving typically a response of 800-1200 RU for Herceptin and 100-200 RU for ERB-hcAb, depending on the contact time. ErbB2-ECD diluted in HBS-EP buffer at concentrations of 10-950 nM was passed over the immobilized antibodies at a constant flow rate of 30 µL/min, and association

and dissociation phases were recorded for 200 s and 300-600 s, respectively. The sensor surface was regenerated by 30-s injections of 10 mM Glycine–HCl, pH 2.2, at the end of each binding cycle. A blank curve in which only running buffer was passed over the captured antibodies was subtracted from each sensorgram.

Kinetic analyses by SPR of Erbicin and ERB-hRNase binding to immobilized ErbB2-ECD

To measure the binding properties of Erbicin and ERB-hRNase to ErbB2-ECD, the ECD was immobilized onto the surface of sensor chip CM5 using standard amine coupling chemistry (see above). Carboxymethylated dextran on the sensor surface was activated with a 1:1 mixture of EDC and NHS. ErbB2-ECD was diluted in 10 mM sodium acetate, pH 4.5, at a final concentration of 1 μg/mL, and 60 μL were then applied to the activated carboxymethylated dextran surface to capture typically 500 RU of ErbB2-ECD. After immobilization, the remaining esters were deactivated with ethanolamine. The sensor surface was washed with four 30-s injections of 10 mM NaOH to remove unbound ErbB2-ECD. The reference flow cell was activated and inactivated by amine coupling chemistry as described above.

Binding curves were recorded injecting Erbicin (5-700 nM) or ERB-hRNase (3–700 nM) over the immobilized ErbB2-ECD at a constant flow rate of 30μL/min. Association and dissociation phases were recorded for 200 s and 300 s, respectively. At the end of each detection, the sensor surface was regenerated by injecting 10 μL of 10 mM NaOH.

For the analysis, each sensorgram was subtracted from the response of the surface on which only running buffer was passed over. A second set of binding curves was recorded for ERB-hRNase in the presence of soluble carboxymethyl-dextran at a final concentration of 5 mg/mL.

Evaluation of kinetic and affinity constants from sensorgram data

The rate constants of the interactions as described above were calculated by a non-linear analysis of the association and dissociation curves using the SPR kinetic evaluation software package BIAevaluation 3.2 (BIAcore), fitting to the 1:1 Langmuir binding model. Values of χ^2 for fittings were ≤ 0.8, indicating good fits. The equilibrium dissociation constants (K_D) were calculated from the values of association rate constant, k_a, and dissociation rate constant, k_d, according to the thermodynamic relationship: $K_D = k_d/k_a$. Standard deviations were obtained from three independent analyses using different biosensors, sample preparations, and ligand densities on the flow cell surfaces.

Determination by SPR of apparent affinity constants for Herceptin and ERB-hcAb binding to the immobilized ErbB2-ECD

Herceptin or ERB-hcAb were passed over ErbB2-ECD immobilized to a CM5 sensor chip made up as described above. Herceptin (0.16-300 nM) or ERB-hcAb (0.7-350 nM), diluted in HBS-EP buffer, were injected at 5 µL/min.
The equilibrium responses for each concentration were determined by subsequent injections of the analytes over the chip.

The equilibrium responses were plotted versus analyte concentrations and the apparent equilibrium constants (K_D) were calculated from the concentration corresponding to half-maximal saturation.

Determination by SPR of competition in binding to ErbB2-ECD between Herceptin and ERB-hcAb or ERB-hRNase

ErbB2-ECD was immobilized to the surface of sensor chip as described above. The binding of ERB-hcAb and ERB-hRNase to ErbB2-ECD immobilized on the surface were performed by two subsequent injections of 213 nM ERB-hcAb (corresponding to 426 nM binding site concentration) or 500 nM Erb-hRNase using a 2 min contact time for each injection at a constant flow rate of 5 µl/min. The concentrations of ERB-hcAb and ERB-hRNase were chosen after several experiments at different concentrations. The sensor chip surface was regenerated by a 30 s injection of 25 mM NaOH/1M NaCl.

Competition in binding to ECD between Herceptin and ERB-hcAb or ERB-hRNase was investigated by saturating the sensor chip with Herceptin and injecting over the surface ERB-hcAb or ERB-hRNase. To this purpose two subsequent injections of 440 nM Herceptin at a constant flow rate of 5 µl/min were performed using 2 min contact time for each injection, followed by two subsequent injections of 213 nM ERB-hcAb or 500 nM ERB-hRNase using a 2 min contact time for each injection. No regeneration of sensor surface was made between injections.

Epitope Mapping

Aliquots of ERB-hcAb (800µg) were immobilized on to 400µL of CNBr-activated Sepharose (GE Healtcare), as described above. Following the blocking of the unreacted groups with 1 M ethanolamine hydrochloride, the resin was washed with PBS and ErbB2-ECD (400µg) in PBS was added to the resin containing the immobilized ERB-hcAb. Binding of the antigen was performed at 4°C while gently rotating overnight.

The cleavage of antigen-antibody complex with trypsin or endoproteinase Glu-C (SIGMA) was performed with a E/S ratio of 1/10 for 1 h. Following proteolysis, a 10uL aliquot of the beads was removed for direct analyses by mass spectrometry (MALDI). Mass spectrometry analyses were carried out in collaboration with Prof. P. Pucci, CEINGE Advanced Biotechnology and Department of Organic Chemistry and Biochemistry, Federico II University, Napoli, Italy, as described in previous reports (22).

Results

1.1 Production and characterization of ErbB2-ECD

The cDNA coding for the extracellular domain of ErbB2 receptor (ErbB2-ECD) was stably transfected in 293 cells. The encoded protein was expressed as a secretion product into the culture medium, as revealed by western blotting (Figure 6) and immunoprecipitation analyses performed (see Methods) with ERB-hcAb or Herceptin as anti-ErbB2 antibodies (see Figure 7A). The final yield of ErbB2-ECD purified by affinity chromatography (see Methods) was 12 mg/L of medium. The protein was analyzed by SDS-PAGE followed by Coomassie staining and Western blotting with Herceptin or ERB-hcAb (Figure 7B). Its molecular size was about 80 kDa, as expected.

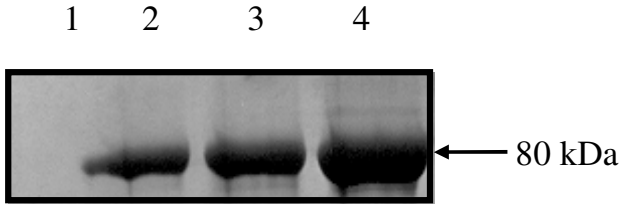

Figure 6

Western Blot analyses of conditioned 293 transfected cells medium with Herceptin as an anti-ErbB2-ECD antibody

Lane 1 negative control (non-transfected 293 cells conditioned medium)

Lane 2, 3, 4 conditioned medium of different selected clones.

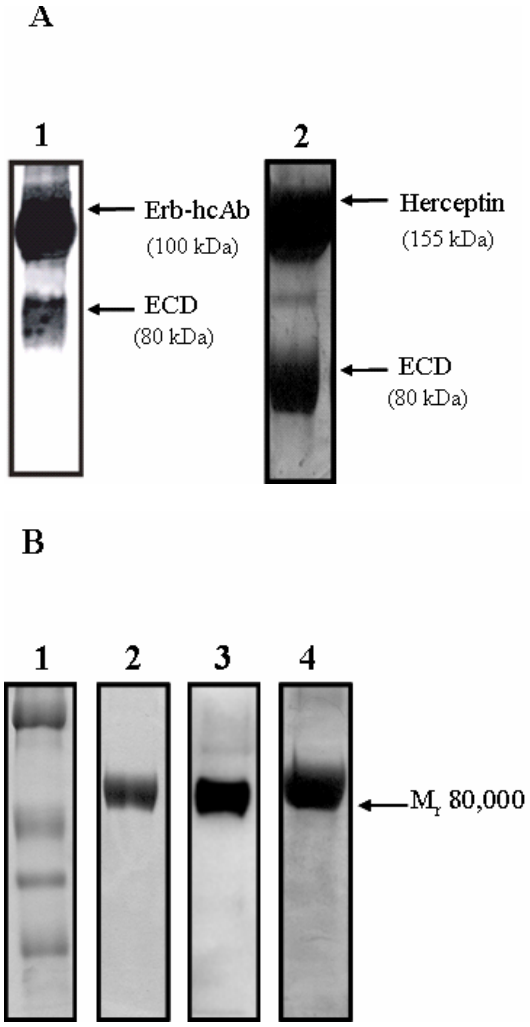

Figure 7

(A) Immunoprecipitation analyses of ErbB2 ECD from conditioned 293 cells medium with ERB-hcAb (*lane 1*) or Herceptin (*lane 2*). Immune complexes were evaluated by Western blotting with anti-ErbB2 antibody (Herceptin); (B) SDS-PAGE analyses of purified ErbB2-ECD, *Lane 1*, standards; *Lane 2*, ECD eluted from the immunoaffinity chromatography stained with Coomassie Blue; *Lanes 3-4*, Western blot analyses of the sample as in *line 2* using ERB-hcAb or Herceptin, respectively, as anti-ErbB2-ECD antibody.

1.2 Analyses by ELISA of the interactions of Erbicin, Erbicin-Derived-ImmunoAgents and Herceptin with ErbB2-ECD

ELISA sandwich assays were performed to determine the ability of Erbicin and ERB-hRNase to recognize ErbB2-ECD. Herceptin fixed on the microplate was used to capture ErbB2-ECD, which in turn could interact with the anti-ErbB2 immunoagents. A peroxidase-conjugated anti-His mAb was used to reveal the bound immunoagents.

The affinity of ERB-hcAb or Herceptin for ErbB2-ECD was instead measured by ELISA directly on ECD coated wells. Antibody binding was detected with a peroxidase-conjugated anti-human, Fc-specific IgG1 mAb.

The results are tabulated in Table I as apparent binding constants, measured from the binding curves, shown in Figure 8, as the concentrations corresponding to half-maximal saturation.. The obtained values (see Table I) were found to be higher than previously reported values (14, 15), obtained directly with ErbB2-positive cells, hence with ErbB2 inserted in cell membrane. This is an indication of lower affinities of the novel immunoagents for ECD.

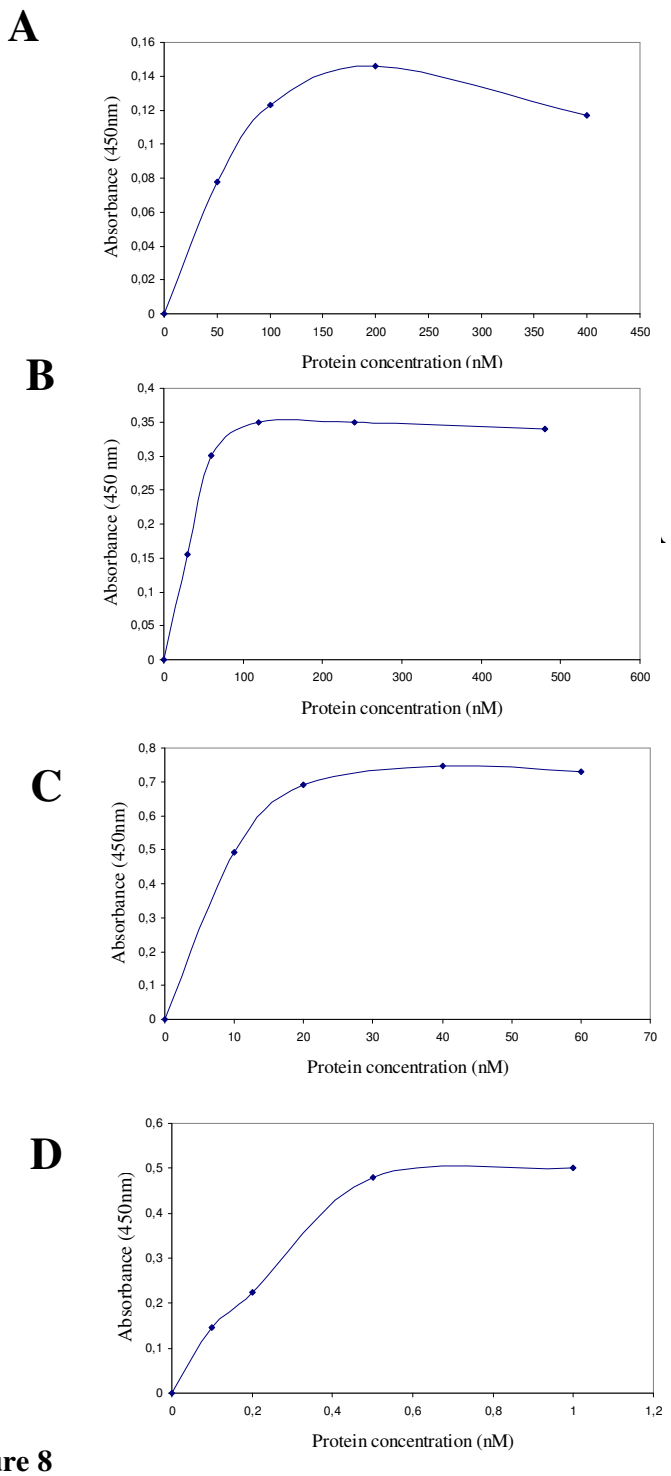

Figure 8

Binding curves of : (A) ERB-hRNase, (B) Erbicin, (C) ERB-hcAb, (D) Herceptin to ErbB2-ECD obtained by ELISA assays.

TABLE I	ErbB2-ECD	ErbB2-positive cells (14, 15)
	K_D apparent (nM)	**K_D apparent (nM)**
Erbicin	50	5
ERB-hRNase	30	4.5
ERB-hcAb	7	1
Herceptin	0.1	5

These results appear to be of special interest, as the lower binding affinity of Erbicin-Derived-ImmunoAgents for soluble ErbB2-ECD is not shared by Herceptin, which displays a high affinity for soluble ErBb2-ECD (0.1 nM), about 50-fold higher than that determined when Herceptin was tested with ErbB2-ECD embedded in live cells (see Table I). These findings can be explained by considering that parental Erbicin was selected from a phage library by using ErbB2-ECD inserted into ErbB2-positive cells, whereas for the isolation of Herceptin free, soluble ECD was used (23).

Another interesting indication stems from the results of the ELISA sandwich type of assays with Herceptin as a capturing agent. They indicate that Erbicin and the novel immunoconjugates (ERB-hRNase, ERB-hcAb) recognize on ErbB2-ECD epitopes different from that selected by Herceptin.

This conclusion is in line with recent data from our laboratory (18). Thus, we tested whether soluble ErbB2-ECD could interfere with the binding to ErbB2-positive cells of anti-ErbB2 antibodies by performing ELISA with

ERB-hcAb or Herceptin in the presence or absence of free ECD. Each antibody was tested at increasing concentrations with soluble ECD added either in equimolar amounts, or in a 10-fold molar excess to the number of receptor molecules on the cell membrane (21). In a parallel experiment, binding was assayed in the absence of added ErbB2-ECD. The binding of antibodies was detected with a peroxidase-conjugated anti-human, Fc-specific IgG1 mAb.

As shown in Figure 9A, when ERB-hcAb was tested the binding curves obtained in the presence or absence of soluble ECD were found to be superimposable. This suggested that the binding ability of ERB-hcAb to ErbB2-positive cells was unaffected by the presence of soluble ECD. The binding instead of Herceptin to ErbB2-positive cells (Figure 9B) was strongly reduced by ECD used at a 1:1 ratio with the receptor number, and fully inhibited with a 10-fold molar excess of ECD.

These results, in line with those described above on the high affinity of Herceptin for soluble ECD, indicate that for Herceptin there is a favourable competition of soluble ErbB2-ECD over the ECD on the cell membrane, whereas there is no detectable competition by free ECD in the case of ERB-hcAb.

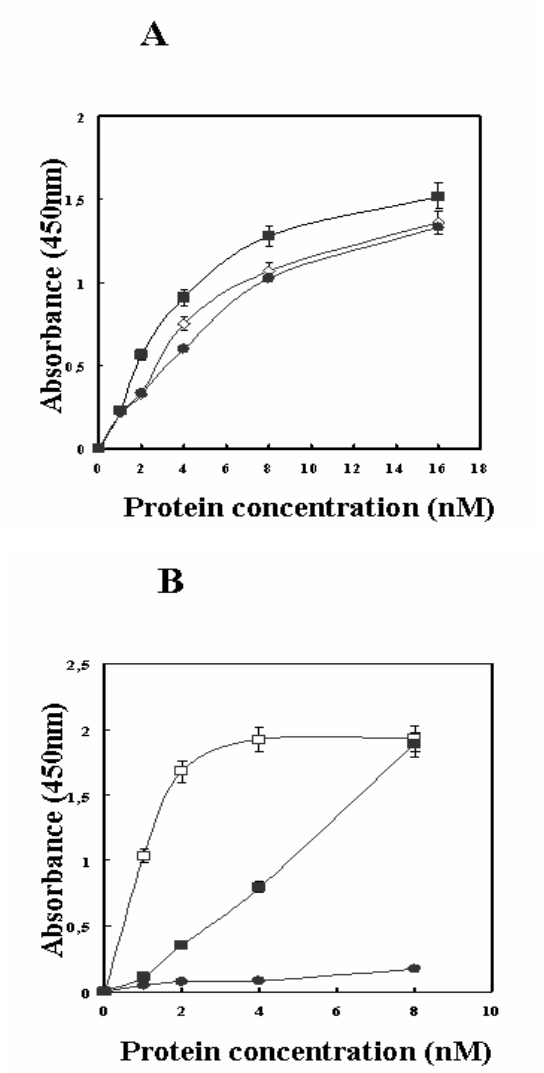

Figure 9

Binding curves of ERB-hcAb (A) or Herceptin (B) to SKBR3 cells obtained through ELISA assays performed in the absence (empty symbols) or in the presence of soluble ECD. Soluble ECD was added at a ratio of 1:1 with the number of receptor molecules on cell membrane (black squares) or at a ratio of 10:1 (black circles).

1.3 Analyses by Isothermal Titration Calorimetry (ITC) of the interactions of Erbicin, Erbicin-Derived-ImmunoAgents and Herceptin with soluble ErbB2-ECD

Figure 10 shows the results of a binding study carried out by calorimetric titrations of soluble ErbB2-ECD with Erbicin, EDIA and Herceptin. Exothermic heat pulses were observed after each injection of the immunoagent into the ErbB2-ECD solution (see insets of Fig 10). Integration of the heat produced upon injections as a function of time, and conversion to per-mole of ErbB2-ECD gives binding isotherms. The data were plotted as a function of molar ratio. The stoichiometry of binding (n), binding constants (K_b) and enthalpy changes ($\Delta_b H°$) were obtained from the fitted curves. The Gibbs free energy change ($\Delta_b G°$) and the entropy change ($\Delta_b S°$) were calculated from the equation,

$$\Delta_b G° = -RT \ln K_b = \Delta_b H° - T\Delta_b S°$$

The obtained thermodynamic parameters are summarized in Table II.

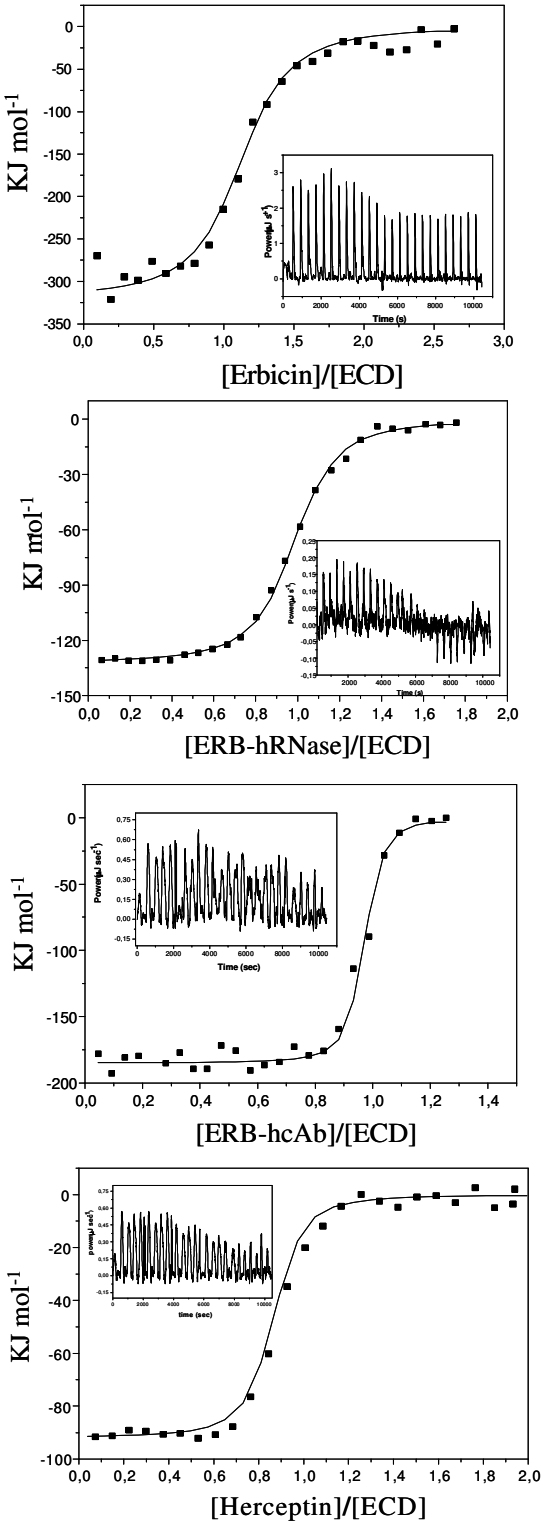

Figure 10

Calorimetric titrations for the interaction of the immunoagents with ErbB2-ECD

Table II	n	K_b (M^{-1})	K_D (nM)	Δ_bH (kJ mol^{-1})	$T\Delta_bS$ (kJ mol^{-1})	Δ_bG (kJ mol^{-1})
ECD/Erbicin	1.0	$1.4x10^7$±0.2	77±10	-300±18	-260±4	-40±6
ECD/ERB-hRNase	1.0	$4.8x10^7$±0.6	21±3	-139±8	-95±3	-44±6
ECD/ERB-hcAb	1.0	$2.4x10^8$±3	4±0.5	-183±11	-135±2	-48±6
ECD/Herceptin	1.0	$8.4x10^7$	12±1	-94±6	-49±5	-45±5

Their inspection reveals that the binding stoichiometry is 1:1 also for the complexes with the bivalent antibodies ERB-hcAb and Herceptin.

As shown by the $\Delta_bH°$ values, binding is driven by a favourable binding enthalpy, opposed by unfavourable binding entropy change, $\Delta_bS°$.

It is of interest that Erbicin shows the lowest affinity for ErbB2-ECD among the studied systems, in agreement with ELISA and SPR results (see Tables I and IV). This indicates a greater number of non-covalent interactions (H-bonds, van der Waals contacts and salt bridges) upon binding of ECD to immunoagents with more stable complexes likely due to cooperation by the non-immune moieties.

When we attempted to study the interactions of the immunoagents with live cells, i.e. with ErbB2 inserted on cell membrane, surprising results were obtained. The $\Delta_bH°$ values were about 100-fold higher than those measured with soluble receptor, and the binding constants were about 1000-fold higher. The possibility that these findings could be attributed not to the binding events, but to the internalization process was excluded, as when we tested an anti-ErbB2 antibody which is not internalised (N28) identical result were obtained.

This conclusion was confirmed when we tested cells poisoned to inhibit endocytosis (see Table III).

Table III	**n**	**$K_b/10^{10}$ (M^{-1})**	**K_D (pM)**	**$\Delta_bH/10^5$ (kJ mol^{-1})**	**Δ_bG (kJ mol^{-1})**
ErbB2-cells/ Erbicin	1.6	6.2±0.7	16±2	-1.15±0.07	-62±0.3
ErbB2-cells/ ERB-hRNase	1.1	5.9±0.9	17±2	-3.20±0.05	-61.±9
ErbB2-cells/ ERB-hcAb	1.2	27.7±1.9	3.6±0.2	-1.20±0.03	-65.±4
ErbB2-cells/ Herceptin	1.0	7.5±0.8	13±1	-1.46±0.07	-62±7
ErbB2-cells/N28	1.0	11.0±5.2	9±4	-1.55±0.09	-63.0±30
ErbB2-cells poisoned/ERB-hRNase	1.0	4.7±0.9	21±4	-1.85±0.04	-61±12

Apparently, the interactions of anti-ErbB2 immunoagents with ErbB2 on live cells cannot be interpreted as simple ligand-to-receptor interactions. It has been anticipated in recent reports that ligand binding to cell receptors may trigger higher order events in the membrane of targeted cells. In these events directly stimulated receptors and other seemingly unrelated receptors and effectors are engaged in the formation of complex networks and receptor mosaics (24), and may induce membrane bending and remodelling (25).

An ErbB signalling network has been proposed (26) after an analysis at the systems level, with ErbB2 as an amplifier of the network (27). Thus antibody binding, which mimics ligand binding, may set off events beyond binding, which are evidenced by an incredibly higher binding affinity and a high heat production.

1.4 Analyses by Surface Plasmon Resonance (SPR) of the interactions of ERB-hcAb and Herceptin with soluble ErbB2-ECD

To compare the binding properties of ERB-hcAb and Herceptin to soluble ErbB2-ECD with a different methodology based on physico-chemical principles, SPR analyses were carried out. Herceptin and ERB-hcAb are bivalent molecules, each endowed with two identical antigen binding sites. Hence, a kinetic binding study by SPR was carried out by capturing the antibodies with Protein A covalently immobilized onto the surface of a sensor chip, and by passing soluble ErbB2-ECD (a monovalent analyte) over the chip surface (see Methods).

Figure 11 shows the binding curves recorded for determining the association and dissociation rate constants for ERB-hcAb (A) and Herceptin (B) complexes with ErbB2-ECD. These analyses highlighted completely different kinetic behaviours for the two antibodies.

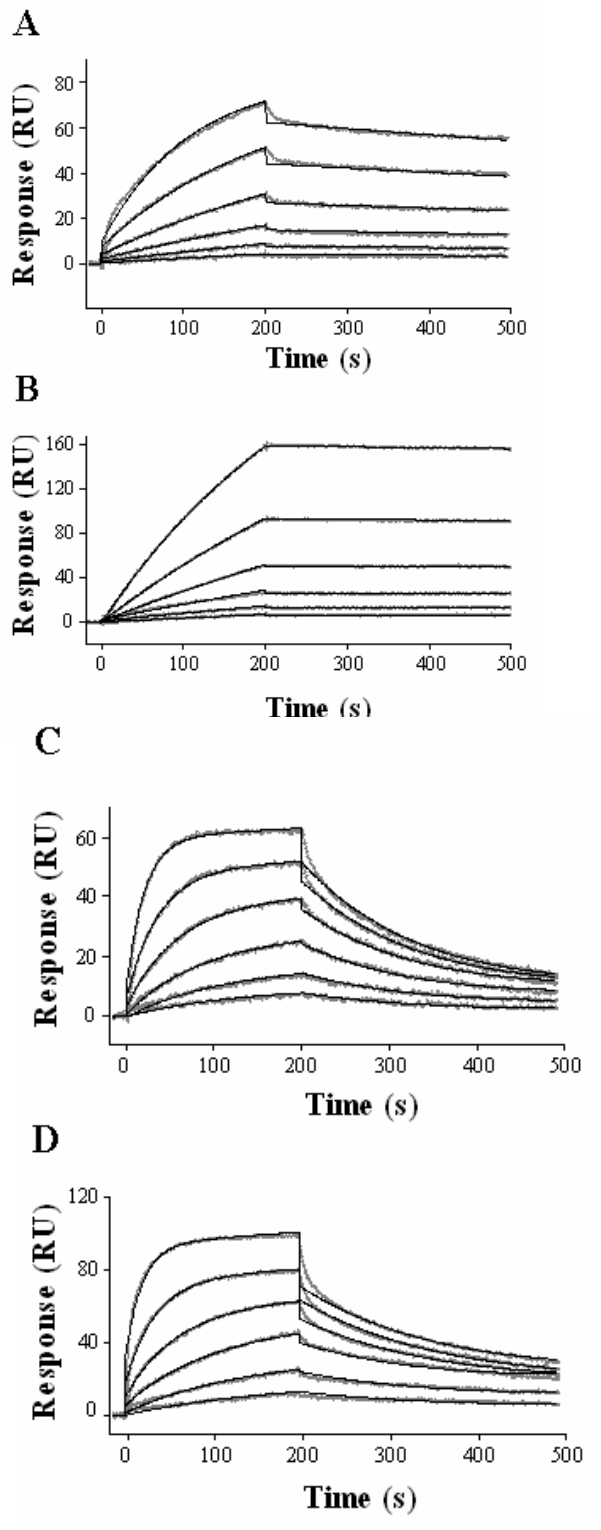

Figure 11

Sensorgrams recorded of ErbB2-ECD binding to ERB-hcAb (A), Herceptin (B),Erbicin (C), ERB-hRNase (D).

Herceptin bound ErbB2-ECD with a relatively low k_a value of 7.25 x 10^3 M^{-1} sec^{-1}, about 3-fold lower than the value determined for the ERB-hcAb complex (1.77 x 10^4 M^{-1} sec^{-1}). As for the dissociation step, the Herceptin/ErbB2-ECD complex was found to be much more stable, with a k_d of 6.5 x 10^{-5} sec^{-1}, about one order of magnitude lower than the k_d value of the ERB-hcAb/ErbB2-ECD complex (4.35 x $10^{-4}sec^{-1}$).

The calculated equilibrium dissociation constant (K_D) for Herceptin/ErbB2-ECD and ERB-hcAb/ErbB2-ECD 1:1 complexes were 9.4 and 24.7 nM per binding site, respectively. These data indicated that Herceptin binds to soluble ErbB2-ECD with a higher affinity than ERB-hcAb, so that Herceptin is strongly sequestered into the immuno-complex. They are in line with those reported above and obtained with ELISA (see Table I), but the K_D values 9.4 nM and 24.7 were higher than the apparent constants (half-maximal saturation concentrations) measured with ELISA for the same complexes (0.1 and 7 nM, respectively).

These large differences could however be ascribed to the different experimental system used for the ELISA and the SPR binding assays.

The system was thus investigated by SPR with a different experimental set up, similar to that used for ELISA (see above), based on ErbB2-ECD covalently immobilized on the chip surface, with Herceptin or ERB-hcAb injected over the chip. Half-maximal saturation values were measured and found to be 11 nM for ERB-hcAb/ErbB2-ECD and 0.13 nM for Herceptin/ErbB2-ECD.

These values (see Table IV) are in good agreement with the data obtained with the ELISA assays reported above (7 and 0.1 nM, respectively).

The conclusion can hence be drawn that the differences in affinity values obtained with the kinetic study are essentially due to the different modes of interaction of bivalent antibodies. When the antibodies are immobilized through Protein A on the SPR chip, each antigen binding site can interact with a free ECD molecule in a 1:1 complex. When instead it is ECD to be immobilized onto the SPR chip, both antibody binding sites can interact with ECD. Binding of antibody to the first site will increase the chances of collision and binding at the second site, but dissociation from the second site does not release easily the antibody from the adsorbed antigen. This is in line with the findings of low k_d values for the dissociation of the antibodies complexes as determined by kinetic SPR analyses.

TABLE IV	k_a **(M^{-1} sec^{-1})** [a]	k_d **(sec^{-1})** [a]	K_D **(nM)** [a]	K_D **apparent (nM)**
Erbicin	1.33 ±0.13 x10^5	6.16 ±0.42 x 10^{-3}	46.7 ±0.55	
Erb-hRNase	1.5 ±0.18 x 10^5	4.12 ±0.84 x 10^{-3}	27.2 ±0.25	
Erb-hRNase [b]	1.61 x 10^5	4.47 x 10^{-3}	27.8	
ERB-hcAb	1.77 ±0.13 x1 0^4	4.35 ±0.09 x 10^{-4}	24.7 ±0.24	9.24
Herceptin	7.25 ±2.4 x 10^3	6.5 ±1.12 x 10^{-5}	9.4 ±0.15	0.13

1.5 SPR analyses of Erbicin and ERB-hRNase binding to ErbB2-ECD

We investigated by SPR the interactions with soluble ErbB2-ECD of the single-chain fragment (Erbicin) and its RNase conjugate (ERB-hRNase). Binding was measured with ErbB2-ECD covalently immobilized onto a sensor chip, while Erbicin or ERB-hRNase were flowed across the chip. The kinetic constants were obtained for these monovalent analytes through fitting the curves with a 1:1 interaction model. Similar binding curves were obtained with Erbicin and ERB-hRNase (see Figure 11 C and D), showing very similar association rate constants, but slightly different dissociation rate constants (see Table IV). Erbicin, with a k_d value of 6.16 x 10^{-3} sec^{-1}, dissociated from ErbB2-ECD 1.5 times faster than ERB-hRNase (k_d = 4.12 x 10^{-3} sec^{-1}).

This indicated a higher stability for the ERB-hRNase/ErbB2-ECD complex. Equilibrium K_D values were 46.7± 5 and 27.2 ± 3.3 nM, respectively, for Erbicin/ErbB2-ECD and Erb-hRNase/ErbB2-ECD, as tabulated in Table IV. The significant difference in K_D values could be clearly ascribed to the different, lower dissociation rate constant measured for the ERB-hRNase/ErbB2-ECD complex. These data, in good agreement with those obtained from the ELISA experiments reported above (50 and 30 nM, respectively), can be ascribed, as suggested above, to interactions with the non immune moieties, in particular to aspecific electrostatic interactions between the positively charged RNase linked in the immunoconjugate and the negatively charged carboxymethyl-dextran matrix of the SPR chip. Thus, the kinetic analyses of the ERB-hRNase/ErbB2-ECD complex were repeated in the presence of soluble carboxymethyl-dextran.

However, identical constants were measured for the ERB-hRNase/ErbB2-ECD complex in the presence or absence of soluble carboxymethyl-dextran (Table IV). This indicates that the higher stability of ERB-hRNase/ErbB2-ECD complex was not due to aspecific ionic interaction, but to specific structural features of immunoagent-ErbB2-ECD complex.

1.6 ErbB2-ECD epitope recognized by Erbicin-Derived-ImmunoAgents (EDIA) is different from that Herceptin

To determine if the novel immunoagents recognize an epitope different from that targeted by Herceptin, competition experiments were carried out by plasmon resonance assays of EDIA binding in the presence of Herceptin. In these experiments, ERB-hcAb and ERB-hRNase were each injected over ErbB2-ECD immobilized on the chip, before and after saturation with Herceptin (Figure 12). After the ErbB2-ECD surface was saturated by repeated injections of Herceptin until no significant additional response was observed (Figure 12), equivalent molar amounts of each immunoagent were injected.

The binding curves obtained for ERB-hcAb and ERB-hRNase were found to be identical to those obtained in the absence of Herceptin (Figure 12, insets). This indicates that each immunoagent could bind to the ECD on the chip surface irrespective of the previous saturation with Herceptin.

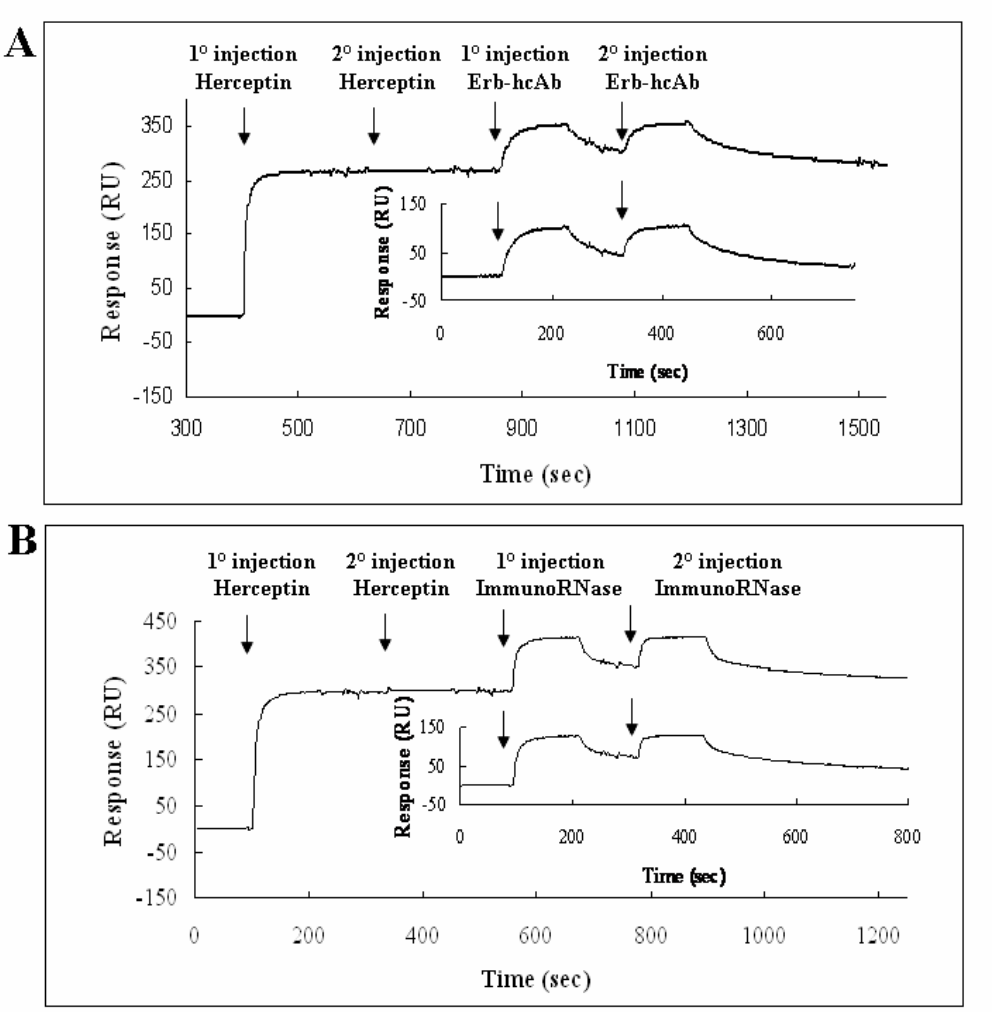

Figure 12

Binding of ERB-hcAb (**A**) and ERB-hRNase (**B**) to the ErbB2-ECD immobilized on plasmon resonance chips, before or after their saturation with Herceptin. From left to right: ECD saturated with Herceptin by two subsequent injections (marked by arrows) before the addition of either ERB-hcAb (**A**) or ERB-hRNase (**B**) by two subsequent injections (marked by arrows) over the chip. The insets show the binding of ERB-hcAb (**A**) or ERB-hRNase (**B**) to the ECD in the absence of Herceptin.

In conclusion, the novel immunoagents do not compete with Herceptin for binding to ErbB2, hence they bind an epitope different from that of Herceptin.

To identify the epitope recognized by Erbicin and EDIA, ErbB2-ECD was coupled to the ERB-hcAb antibody previously immobilized on Sepharose beads. The immobilized antigen-antibody complex was subjected to proteolysis with endoproteinase Glu-C and trypsin. The peptide segments protected within the antibody/ECD complex indicate that the epitope is contained within the first domain of ErbB2-ECD (Figure 13). These results confirm that the novel immunoagents (Erbicin, ERB-hRNase, ERB-hcAb) recognize a different epitope on ErbB2-ECD from that of Herceptin and other available anti-ErbB2 monoclonal antibodies such as Pertuzmab, 7c2 and MAB74 (28, 18).

TQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDIQEVQG 60

61
YVLIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLNNTTPVTGASPGGLRELQL
120

121
RSLTEILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPMCKGS
180

181
RCWGESSEDCQSLTRTVCAGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSG2
40

241
ICELHCPALVTYNTDTFESMPNPEGRYTFGASCVTACPYNYLSTDVGSCTLVCPLHNQEV
300

301
TAEDGTQRCEKCSKPCARVCYGLGMEHLREVRAVTSANIQEFAGCKKIFGSLAFLPESFD
360

361
GDPASNTAPLQPEQLQVFETLEEITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNGAYS
420

421
LTLQGLGISWLGLRSLRELGSGLALIHHNTHLCFVHTVPWDQLFRNPHQALLHTANRPED
480

481
ECVGEGLACHQLCARGHCWGPGPTQCVNCSQFLRGQECVEECRVLQGLPREYVNARHCLP
540

541
CHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGA
600

601
CQPCPINCTHSCVDLDDKGCPAEQRASPLT

Figure 13

In red, the peptide segments of ErbB2 recognized by Erbicin and EDIA.

Conclusions

The novel antitumor immunoagents Erbicin, ERB-hcAb and ERB-hRNase have most, potentially all, the features which can make an immunoagent a valid, precious tool for anti-cancer immunotherapy: 1. they are all of human origin, which would seriously decrease, if not eliminate, the risks of immune response; 2. they are directed to a cell receptor, such as ErbB2, minimally present in non-malignant cells, but over-expressed in many carcinomas, especially in breast cancer cells; 3. they selectively kill ErbB2-positive cells, both *in vitro* and *in vivo*; 4. their size, smaller than that of immunoglobulins, should favour penetration in solid tumors; however, in the case of ERB-hcAb and ERB-hRNase, it should also allow for a prolonged half-life in the bloodstream.

Binding to a cell-embedded tumor-associated-antigen is the first key step in the mechanism of antitumor immunoagents. Thus, I directed my attention to studying the binding properties not only of the novel Erbicin-derived anti-ErbB2 immunoagents, but also of Herceptin, an immunoagent successfully employed in the therapy of breast cancer. Furthermore, the availability of soluble ErbB2-ECD has led us to describe for the first time the binding of these immunoagents to the isolated, free extracellular domain of ErbB2. Also for the first time the binding study was conducted not only with a semi-quantitative methodology, such as that based on ELISA, the only methodology previously used for measuring Herceptin binding (15), but also with quantitative methods based on physico-chemical principles, such as surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC).

The main results of this study can be summarized as follows.

1. For the first time extensive and conclusive information are reported on relative affinity and binding kinetics of the Erbicin-Derived-ImmunoAgents and Herceptin for soluble, or cell-linked ErbB2.

2. The results are validated through the use of three independent methodologies: ELISA, SPR, and ITC, which gave coherent results.

3. The binding of Erbicin to ErbB2-ECD was found to be enhanced and stabilized through the linking of the Erbicin scFv to either an RNase or the Fc antibody fragment, as in ERB-hRNase and ERB-hcAb, respectively. This was revealed by the higher binding affinity of the Erbicin immunoconjugates with respect to that of the free Erbicin scFv.

4. The novel, Erbicin-Derived-ImmunoAgents display a binding affinity towards soluble ErbB2-ECD lower than that measured for ECD embedded in the membrane of ErbB2-positive cells. Herceptin instead shows a higher affinity for soluble ErbB2-ECD. Furthermore, binding of ERB-hcAb to cancer cells is not affected by soluble ECD, whereas that of Herceptin is strongly inhibited. As soluble ECD is proteolytically released from the surface of ErbB2-overexpressing cancer cells, and is detected in the serum of patients with advanced breast cancer (5), a fraction of Herceptin is neutralized in these patients by serum ECD, hence it is subtracted to cell-directed antitumor action (29).

Thus, the data inserted in this thesis indicate that EDIA, the Erbicin-Derived-ImmunoAgents have a superior therapeutic potential with respect to Herceptin, as their use.

5. I reported here for the first time that the epitope recognised by the Erbicin based immunoagents is different from that targeted by Herceptin (2).

This observation is supported by results from two different types of tests: plasmon resonance analyses (18) and mass spectroscopic analyses.

6. A binding study carried out by ITC on anti-ErbB2 immunoagents tested directly on live cells has revealed that the association of the immunoagents to the receptor inserted into live cells cannot be interpreted as a simple ligand-to-receptor interaction. Apparently, antibody binding, just like ligand binding, triggers higher order events which engage other membrane receptors and effectors in the formation of complex networks and receptor mosaics.

Bibliography

1 Yamamoto T, Ikawa S, Akiyama T, Semba K, Nomura N, Miyajima N, Saito T, Toyoshima K. (1986) Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor. *Nature* **319**: 230-4

2 Cho HS, Mason K, Ramyar KX, Stanley AM, Gabelli SB, Denney DW Jr, Leahy DJ. (2003) Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nature* **924**:756-60

3 Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**: 177-182

4 Klapper LN, Vaisman N, Hurwitz E, Pinkas-Kramarski R, Yarden Y, Sela M. (1997) A subclass of tumor-inhibitory monoclonal antibodies to ErbB-2/HER2 blocks crosstalk with growth factor receptors. *Oncogene* **14**: 2099-109

5 Wu JT, Astill ME, Zhang P. (1993) Detection of the extracellular domain of c-erbB-2 oncoprotein in sera from patients with various carcinomas: correlation with tumor markers. *J clin Lab Anal* **7**: 31-40

6 Stebbing J, Copson E, O'Reilly S. (2000) Herceptin (trastuzamab) in advanced breast cancer. *Cancer Treat Rev* **26:** 287-90

7 Cardoso F, Piccart MJ, Durbecq V, Di Leo A. (2002) Resistance to trastuzumab: a necessary evil or a temporary challenge? *Clin Breast Cancer* **3**: 247-57; discussion 258-9

8 Natha R, Esteva FJ. (2006) Herceptin mechanisms of action and resistence. *Cancer Letter* **232**: 123-138

9 Chien KR. (2000) Myocyte survival pathways and cardiomyopathy: implications for trastuzumab cardiotoxicity. *Semin Oncol* **27**: 9-14; discussion 92-100

10 Sparano JA. (2001) Cardiac toxicity of trastuzumab (Herceptin): implications for the design of adjuvant trials. *Semin Oncol* **28**: 20-7

11 Spiridon CI, Ghetie MA, Uhr J, Marches R, Li JL, Shen GL, Vitetta ES. (2002) Targeting multiple Her-2 epitopes with monoclonal antibodies results in improved antigrowth activity of a human breast cancer cell line in vitro and in vivo. *Clin Cancer Res* **8**: 1720-30

12 Willems A, Gauger K, Henrichs C, Harbeck N. (2005) Antibody therapy for breast cancer. *Anticancer Res* **25**: 1483-9

13 De Lorenzo C, Palmer DB, Piccoli R, Ritter MA, D'Alessio G. (2002) A new human antitumor immunoreagent specific for ErbB2. *Clin Cancer Res* **8:** 1710-9

14 De Lorenzo C, Arciello A, Cozzolino R, Palmer DB, Laccetti P, Piccoli R, D'Alessio G. (2004) A fully human antitumor immunoRNase selective for ErbB-2-positive carcinomas. *Cancer Res* **64**: 4870-4

15 De Lorenzo C, Tedesco A, Terrazzano G, Cozzolino R, Laccetti P, Piccoli R, D'Alessio G. (2004) A human, compact, fully functional anti-ErbB2 antibody as a novel antitumour agent. *Br J Cancer* **91:** 1200-4

16 De Lorenzo C, Cozzolino R, Carpentieri A, Pucci P, Laccetti P, D'Alessio G. (2005) Biological properties of a human compact anti-ErbB2 antibody. *Carcinogenesis* **26**: 1890-5

17 De Kruif J, Van der Vuurst de Vries AR, Cilenti L, Boel E, Van Ewijk W, Logtenberg T. (1996) New perspectives on recombinant human antibodies. *Immunol Today* **10**: 453-5

18 De Lorenzo C, Troise F, Cafaro V, D'Alessio G. (2007) Combinatorial experimental protocols for Erbicin-derived immunoagents and Herceptin. *Br J Cancer* (in press)

19 Laemmli U. Cleavage of structural proteins durino the assembly of the head of bacteriophage T4. (1970) *Nature* **227**: 680-5

20 Erra E, Petraccone L, Esposito V, Randazzo A, Mayol L, Ladbury J, Barone G, Giancola C. (2005) Interaction of porphyrin with G-quadruplex structures. *Nucleosides Nucleotides Nucleic Acids*. **24**: 753-6

21 Wikman M, Steffen AC, Gunneriusson E, Tolmachev V, Adams GP, Carlsson J, Stahl S. (2004) Selection and characterization of HER2/neu-binding affibody ligands. *Protein Eng Des Sel* **5**: 455-62

22 Papac DI, Hoyes J, Tomer KB. (1994) Epitope mapping of the gastrin-releasing peptide/anti-bombesin monoclonal antibody complex by proteolysis followed by matrix-assisted laser desorption ionization mass spectrometry. *Protein Sci* **9**: 1485-92

23 Fendly BM, Winget M, Hudziak RM, Lipari MT, Napier MA, Ullrich A. (1990) Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. *Cancer Res* **5**: 1550-8.

24 Agnati LF, Genedani S, Leo G, Forni A, Woods AS, Filaferro M, Franco R, Fuxe K. (2007) A beta peptides as one of the crucial volume transmission signals in the trophic units and their interactions with homocysteine.

Physiological implications and relevance for Alzheimer's disease. *J Neural Transm*. **114**: 21-31

25 Reynwar BJ, Illya G, Harmandaris VA, Müller MM, Kremer K, Deserno M. (2007) Aggregation and vesiculation of membrane proteins by curvature-mediated interactions. *Nature* **447**: 461-4

26 Citri A, Yarden Y. (2006) EGF-ERBB signalling: towards the systems level. *Nat Rev Mol Cell Biol* **7**: 505-16

27 Clayton AH, Walker F, Orchard SG, Henderson C, Fuchs D, Rothacker J, Nice EC, Burgess AW. (2005) Ligand-induced dimer-tetramer transition during the activation of the cell surface epidermal growth factor receptor-A multidimensional microscopy analysis. *J Biol Chem.* **34**:30392-9

28 Franklin CM,. Carey CD, Vajdos FF,. Leahy DJ, de Vos AM Sliwkowski XM. (2004) Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell* **5:**317-28

29 Brodowicz T, Wiltschke C, Budinsky AC, Krainer M, Steger GG, Zielinski CC. (1997) Soluble HER-2/neu neutralizes biologic effects of anti-HER-2/neu antibody on breast cancer cells in vitro. *Int J Cancer* **6**:875-9

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