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Effects of three different anti-ErbB2 antibodies on prostate tumors

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SUMMARY

Prostate cancer is the most commonly diagnosed malignancy in men in developed countries. ErbB2 contributes to prostate cancer progression by activating the androgen receptor in a steroid poor environment, thus promoting androgen-independent cell growth and survival. The consequent development of hormone refractory tumors is a major obstacle in prostate cancer therapy.

The inhibition of ErbB2 signal transduction pathways by the use of human antibodies has been considered as a valuable alternative strategy for cancer therapy.

Herein we report a comparative analysis of the antitumor effects of three different antibodies targeting different epitopes of ErbB2: Herceptin (Trastuzumab), 2C4 (Pertuzumab), and Erb-hcAb, a novel fully human compact antibody produced in our laboratory.

We demonstrate that the *in vitro* and *in vivo* growth of both androgen-dependent and –independent prostate cancer cells is efficiently inhibited by Erb-hcAb, which shows antitumor effects on some cell lines more potent than those observed for either Herceptin or 2C4.

SOMMARIO

Il cancro della prostata è il tumore maggiormente diagnosticato negli uomini nei paesi industrializzati. ErbB2 contribuisce allo sviluppo di malignità attraverso il recettore degli androgeni attivato anche in assenza di steroidi, promuovendo così la crescita delle cellule tumorali androgeno-indipendenti.

Il conseguente sviluppo di tumori resistenti alla deprivazione di androgeni è uno dei principali ostacoli nella terapia del cancro alla prostata.

L'inibizione delle vie di trasduzione del segnale di ErbB2 mediante l'impiego di anticorpi umani è stata considerata una valida strategia per la terapia del cancro.

In questo lavoro di tesi, sono stati analizzati mediante valutazione comparativa gli effetti antitumorali di tre anticorpi che legano diversi epitopi di ErbB2: Herceptin (Trastuzumab), 2C4 (Pertuzumab) e Erb-hcAb, un nuovo anticorpo compatto completamente umano prodotto nel nostro laboratorio.

Erb-hcAb inibisce efficacemente la crescita *in vitro* e *in vivo* di cellule tumorali della prostata sia androgeno-dipendenti che androgeno-indipendenti con effetti su alcune delle linee cellulari saggiate più potenti di quelli osservati per Herceptin o 2C4.

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1. INTRODUCTION

Prostate cancer is the second leading cause of cancer death among men in the US, where in 2005 it was the most frequently diagnosed cancer, accounting for approximately 33% of incident cases in men and 10% of deaths (1). Most of the patients with an advanced form of the disease at diagnosis are treated by systemic androgen ablation therapy. Initially, the cancer is responsive to this type of treatment because it exhibits an androgen-sensitive phenotype (2). Unfortunately, at a later stage the cancer typically progresses in a form that does not respond anymore to further androgen deprivation, thus becoming androgen-independent. Androgen-independent prostate cancer is resistant to therapeutic interventions and ultimately leads to the demise of the patient.

The mechanisms through which prostate cancer converts to androgen independence are still unclear. It has been reported that the onset of ErbB2 protein overexpression may coincide with the emergence of androgen independence as the androgen receptor can be activated by ErbB2 through Akt pathway, thus promoting androgen-independent prostate cancer growth and survival (3). ErbB (also known as Her2) is a transmembrane tyrosine kinase member of the epidermal growth factor receptor (EGFR) family, which is overexpressed in many human cancer types. In particular, ErbB2 is implicated in malignant transformation and tumorigenesis (4,5), and it is overexpressed in breast, ovary, urinary, bladder, prostate and non-small cell lung cancer and in several other carcinomas (6,7,8), whereas on normal tissues it is not detectable or it is expressed at very low levels. Owing to their role in cancer pathogenesis, ErbB2 and EGFR have become important targets of anticancer therapy: many drugs have been developed against one or both of these receptors.

Herceptin (Trastuzumab) is the only humanized monoclonal antibody available for the treatment of ErbB2-positive breast cancer since 1998 when it was approved by FDA, and is now used for the treatment of both metastatic and early-stage breast cancer (9). Although Herceptin significantly improves the outcome for patients with ErbB2-positive breast cancer, not all the patients benefit from the treatment. A significant fraction of the patients do not respond at all, and most of the patients who initially respond to Herceptin generally tend to develop resistance during the treatment (10).

Furthermore, also carcinomas with a high expression of ErbB2, such as non-small cell lung carcinoma, gastric and prostate tumours, have been found to be resistant or much less sensitive to Herceptin treatment (11,12,13). Finally, large-scale clinical studies with Herceptin have shown that it

engenders cardiotoxicity (14,15). It especially occurs in patients treated previously or concurrently with anthracyclines.

Thus, there is an urgent need to identify new antibodies capable of disrupting the ErbB2 signaling pathway in cancer patients.

Recently, we produced a novel fully human anti-ErbB2 immunoagent, engineered by fusing Erbicin, a human anti-ErbB2 scFv, with the Fc region of a human IgG1 (16)(Fig.1A).

The human anti-ErbB2 antibody construct has been called Erb-hcAb for its “compact” size (100kDa), compared with the full size (155kDa) of a natural IgG. It has been reported that Erb-hcAb is capable of selective binding to malignant cells that express ErbB2, and of inhibiting their growth *in vitro* and *in vivo*, with no effects on ErbB2-negative cells. Moreover Erb-hcAb is endowed with both ADCC and CDC cytotoxic effects(16,17)

More recently, it has been shown that Erb-hcAb does not display the cardiotoxic effect of Herceptin *in vitro* on rat cardiomyocytes and *in vivo* on a mouse model, whereas Herceptin was strongly toxic. This difference was found to be due to their different mechanism of action, which can explain their different effect: Herceptin, at difference with Erb-hcAb, induces apoptosis in cardiac cells (18). Finally Erb-hcAb is active *in vitro* and *in vivo* against some Herceptin-resistant, ErbB2-positive breast cancer cell lines (19).

In the present study, we investigated the antitumor effects of Erb-hcAb, towards androgen-dependent and androgen-independent ErbB2-positive prostate cancer cells in comparison with Herceptin and 2C4 the mouse antibody version of Pertuzumab, a new humanized anti-ErbB2 monoclonal antibody, currently in phase II clinical trials for metastatic breast cancer (20), ovarian, prostate and non small-cell lung cancer (21,22).

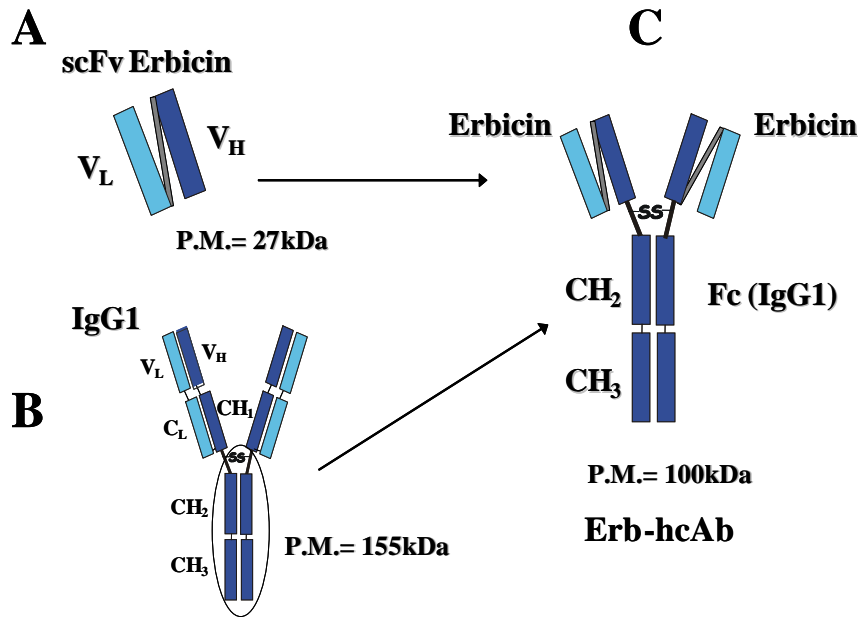


Fig. 1A Schematic representation of Erb-hcAb (C), the human compact anti-ErbB2 antibody made up of two Erbicin molecules (A) fused to the CH₂ and CH₃ regions of human IgG1 (B).

2. MATERIALS AND METHODS

2.1 Cell cultures and antibodies

The hybridoma cells producing 2C4 antibody were grown in DMEM-RPMI-1640 medium in a 1:1 ratio (Gibco Life Technologies, Paisley, UK BRL)..

The CHO (Chinese Hamster Ovary) cell line of clone K1, transfected with the recombinant plasmid pIgPlus, was cultured in RPMI-1640 medium containing neomycin at a concentration of 1 mg/ml.

The LnCap and PC3 cells lines, were cultured in RPMI-1640 medium. The Du145 cells, were cultured in EMEM (Eagle's Minimum Essential Medium) containing sodium pyruvate and non-essential amino acids at a concentration of 1 mM. The 22Rv1 cells were cultured in DMEM containing HEPES at a concentration of 15 mM. All the cell lines were supplemented with 10% heat-inactivated foetal bovine serum (FBS), 50 Units/ml penicillin, and 50 µg/ml streptomycin (all from Gibco), cultured at 37°C in 5% CO₂ atmosphere.

The antibodies used were: Herceptin (Genentech, South San Francisco, CA, USA); horseradish peroxidase-conjugated goat anti-human affinity isolated IgG1 (Fc-specific, Sigma, St Louis, MO, USA), monoclonal anti-human IgG (Fc-specific, Sigma), horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (Pierce, Rockford, IL, USA) horseradish peroxidase-conjugated anti β actin (Pierce).

2.2 Production and purification of Erb-hcAb

For recombinant protein production, transfected CHO cells were expanded to near confluence in selective medium containing neomycin, and then were grown for 3–4 days in serum-free medium. The recombinant fusion protein, henceforth termed Erb-hcAb, secreted by transfected CHO cells, was purified from culture medium by affinity chromatography on a protein A-Ceramic Hyper D®F column (BioSeptra, Cergy-Saint-Christophe, France) loaded with 300–500 ml of conditioned medium, washed with 10 volumes of 100 mM Tris-HCl, pH 8.0 containing 0.5 M NaCl, and 10 volumes of 10 mM Tris-HCl, pH 8.0. The protein eluate was obtained with 50 mM glycine pH 3.0, and immediately neutralised with 1/10 volume of 1 M Tris-HCl, pH 8.0.

2.3 Production and purification of 2C4

2C4 hybridoma cells were expanded to near confluence in complete medium, and then were grown for 3–4 days in serum-free medium. The secreted antibody was purified from culture medium by affinity chromatography on a protein G sepharose loaded with 300–500 ml of conditioned medium. Wash and elution steps were carried out as described above for Erb-hcAb.

2.4 SDS-PAGE and western blotting analyses of Erb-hcAb and 2C4 antibodies

The expression of Erb-hcAb and 2C4 was evaluated by 8% SDS-PAGE analyses of conditioned medium, under nonreducing condition, followed by electroblotting onto poly-vinylidene difluoride membranes (Millipore Corporation, Bedford, MA, USA). For Erb-hcAb detection, was used horseradish peroxidase-conjugated anti-human IgG1 (Fc-specific) mAb, while for 2C4 detection were used horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (Pierce, Rockford, IL, USA) both followed by enhanced chemiluminescence (Super signal West Pico, Pierce Rockford, IL, USA).

2.5 Cell lysis and immunoblotting analyses

The cells, washed with PBS, were collected by centrifugation at 1200 rpm for 7 minutes. The pellet was resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 0.5% Nonidet P-40, 150 mM NaCl), containing Complete™ proteases inhibitor (Boehringer Mannheim, Germany). After 20 min at 0°C, the extracts were clarified by centrifugation at 12.000 rpm at 4°C for 15 min. Aliquots of 20 µg were run on 7.5% SDS-PAGE, followed by electroblotting onto PVDF membranes (Millipore, Bedford, Ma). The ErbB2 protein was detected using Neu anti-ErbB2 mAb (Santa Cruz), followed by rabbit anti-mouse horseradish peroxidase conjugated antibody. The signal intensity of reactive bands was quantitatively measured by a phosphorimager (45-710, Bio Rad, Hercules, CA)

2.6 ELISA assays

ErbB2-positive prostate tumor cells, harvested in nonenzymatic dissociation solution (Sigma), were washed and transferred to U-bottom microtitre plates (1×10^5 cells per well). After blocking with PBS containing 6% bovine serum albumin (BSA), cells were treated with the antibodies in enzyme-linked immunosorbent assay (ELISA) buffer (PBS/BSA 3%) for 90 min. After centrifugation and removal of supernatants, the pelleted cells were washed twice in 200 μ l of ELISA buffer, resuspended in 100 μ l of ELISA buffer, and incubated with peroxidase-conjugated anti-human IgG (Fc-specific) antibody (Sigma) or peroxidase-conjugated anti-mouse IgG antibody (Pierce) for detection of human or mouse antibodies, respectively. After 1 h, the plates were centrifuged, washed with ELISA buffer, and reacted with 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma). Binding values were determined from the absorbance at 450 nm, and reported as the mean of at least three determinations (standard deviation $\leq 5\%$).

2.7 Cell growth inhibition assays

Prostate tumor cells were seeded in 96-well, flat-bottom plates at a density of 7.5×10^3 well. After addition of the protein under test, viable cells by the Trypan blue-exclusion test were counted at suitable time intervals. Cell survival was expressed as percent of viable cells in the presence of the protein under test with respect to control cultures grown in the absence of the protein. Typically, cell survival values were obtained from at least three separate experiments in which triplicate counts were determined; standard deviations were below 5%.

2.8 *In vivo antitumour activity*

In vivo experiments, were performed in collaboration with Prof. P. Laccetti, (University Federico II, Naples, Italy) with 6-week-old male Balb/c Nude mice (Charles River laboratories, Calco, Italy). LnCap or PC3 cells (2×10^6) were suspended in 0.2 ml sterile PBS and injected subcutaneously (day 0) in the right paw. At day 20, when tumours were clearly detectable, Erb-hcAb, Herceptin or 2C4 dissolved in PBS were administered to three different groups of 5 mice intraperitoneally at doses of 5 mg/kg of body wt for seven times at 72 h intervals.

Another group of control mice was treated with identical volumes of sterile PBS. During the period of treatment, tumour volumes (V) were measured with calliper and calculated by the formula of rotational ellipsoid $V = A \times B^2/2$ (A is the axial diameter and B the rotational diameter). SD values were <10%. All mice were maintained at the animal facility of the Department of Cellular and Molecular Biology and Pathology, University of Naples Federico II. The animal experimentations described herein were conducted in accordance with the Italian regulation for experimentation on animals.

2.9 *Pharmacokinetics and Biodistribution of Erb-hcAb*

For assessing the pharmacokinetic and biodistribution properties of Erb-hcAb, *in vivo* experiments were performed in a mouse model in collaboration with Prof. L. Aloj (Nuclear Medicine, IRCCS National Cancer Institute, Fondazione G. Pascale, Naples, Italy). To this aim, CD1 nude mice were subcutaneously injected with LnCap cells (1×10^6 cells) in the flank to generate xenografts. After 3 weeks tumor weights were in the range of 0.2 to 0.5 g. Animals were divided into 3 groups of 5 mice each and ^{125}I labeled Erb-hcAb was injected ip (1-2 E6 cpm/animal). At preset times ranging from 5 min to 96 h retro-orbital sinus sampling was performed alternating group of animals for each time point in order to evaluate blood clearance of the radiolabelled antibody fragment. At 96h animals were sacrificed and residual activity in organs was determined by gamma counting and weighing. Radioactivity levels are expressed as percentage of injected dose per gram (%ID/g) normalized for a 20 g mouse.

The blood clearance parameters of the labeled protein were fitted using Prism 4.0 software (Macintosh version, GraphPad Software, San Diego California

Materials and Methods

USA, www.graphpad.com) according to the equation: $X(t) = A \exp(-(\alpha t)) + B \exp(-(\beta t))$, where $X(t)$ is the %ID/g of radiolabeled antibody at time t . This biexponential blood clearance profile was calculated from the time of maximal concentration of blood radioactivity.

3. RESULTS

3.1 Purification of Erb-hcAb and 2C4

Erb-hcAb was expressed by CHO (Chinese Hamster Ovary cells) transfected with the recombinant plasmid (pIg1plus) containing the cDNA encoding Erb-hcAb. 2C4, the parental mouse antibody of Pertuzumab, was produced by hybridoma cells (LGC Promochem). Both the antibodies were secreted into the culture medium, and purified by affinity chromatography (see Materials and Methods) with a final yield of 1,5 mg L⁻¹ for Erb-hcAb and 3 mg L⁻¹ for 2C4.

The protein concentration was determined by absorbance at 280 nm and by Bradford colorimetric assays.

When the purified fractions of Erb-hcAb and 2C4 were analysed by SDS-PAGE (Fig.1), they were found to migrate with the expected molecular sizes of about 100 kDa for Erb-hcAb and 155 kDa for 2C4. Western blotting analyses of Erb-hcAb and 2C4 performed with an anti-human Fc (Sigma) and an anti-mouse mAb (Sigma) demonstrated immunoreactivity of the purified proteins (Fig.1).

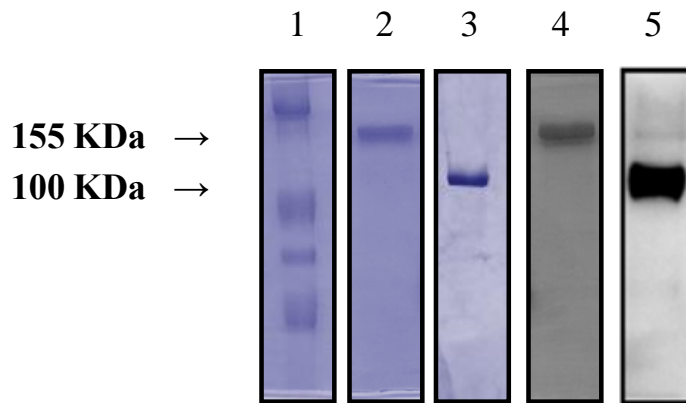


Fig. 1. SDS-PAGE and Western blotting analyses of purified 2C4 and Erb-hcAb. Coomassie Blue staining of 2C4 and Erb-hcAb fractions eluted by affinity chromatography are in *Lane 2* and *Lane 3* respectively; Western blotting analyses of purified 2C4 and Erb-hcAb are in *Lane 4* and in *Lane 5* respectively. Molecular weight standards are in *Lane 1*.

3.2 ErbB2 levels on prostate cancer cells

The level of ErbB2 receptor in prostate tumors was investigated by Western blotting analyses of cell extracts from four different prostate cancer cells lines: Lncap, Du145, Pc3 and 22rv1. Briefly, cells were lysed and analyzed by Western blotting with a commercial anti-ErbB2 antibody (Neu, Santa Cruz) and an anti-actin mAb. As a positive control, we used SKBR3 human breast cancer cells, which express high levels of ErbB2. As shown in Fig.2, ErbB2 is expressed in all the prostate cell lines tested, even though at lower levels with respect to those observed in mammary carcinoma cells.

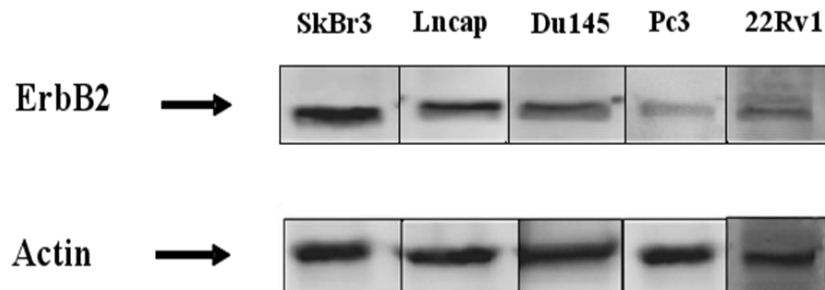


Fig. 2. Western blotting analyses with the anti-ErbB2 (Neu) mAb of the expression levels of ErbB2 in the indicated cell lines. To normalize the band signals, an anti-actin mAb was used.

Results

These results were confirmed by ELISA assays, performed by incubating the prostate cancer cells with increasing concentrations of Erb-hcAb, Herceptin or 2C4. As shown in Fig.3, the three antibodies bind to all the prostate cells tested with a comparable affinity even though Herceptin shows a lower binding ability on 22RV1 and PC3 cells.

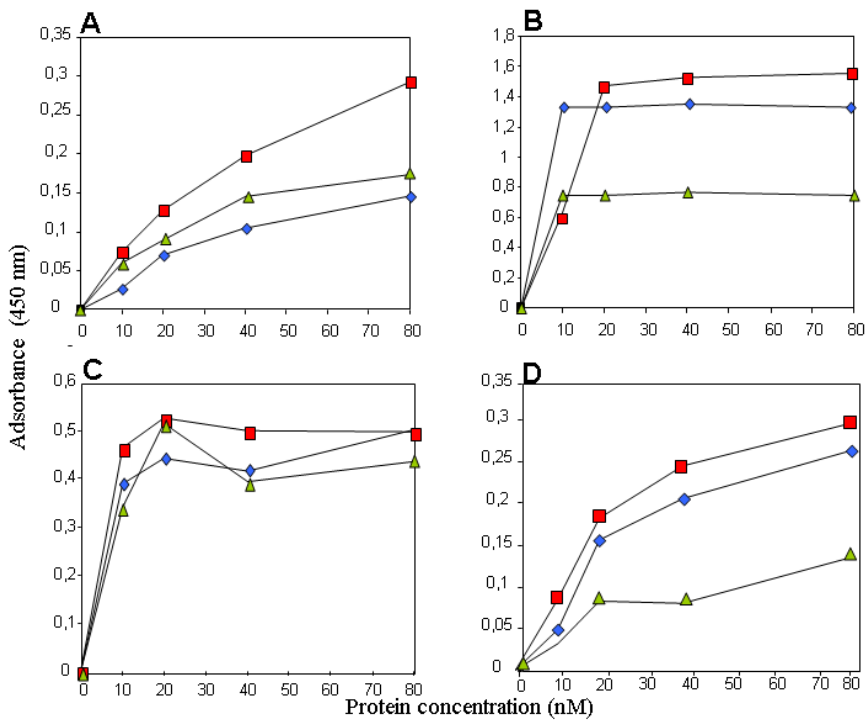


Fig. 3. Binding curves of the antibodies to prostate cancer cell lines. Herceptin (triangles), Erb-hcAb (rhomboids) and 2C4 (squares) were tested on DU145 (A), 22RV1(B), LnCap (C) and PC3 (D) cells

3.3 In vitro effects of Erb-hcAb on prostate cancer cells

The antitumor effects of the antibodies were tested on ErbB2-positive prostate cancer cells by incubating Lncap, Du145, 22RV1 and PC3 cells in the absence or in the presence of increasing concentrations (50-200nM) of Erb-hcAb, Herceptin or 2c4.

After 72 hours, cells were counted and their survival was expressed as percentage of viable cells in the presence of the protein under test, with respect to control cells grown in the absence of the protein. The SD was calculated on the basis of the results obtained from six different experiments.

As shown in Fig.4, Erb-hcAb was found to selectively inhibit the growth of all the cells tested in a dose-dependent manner, showing a stronger effect with respect to Herceptin and 2C4, when high protein concentrations were tested.

Results

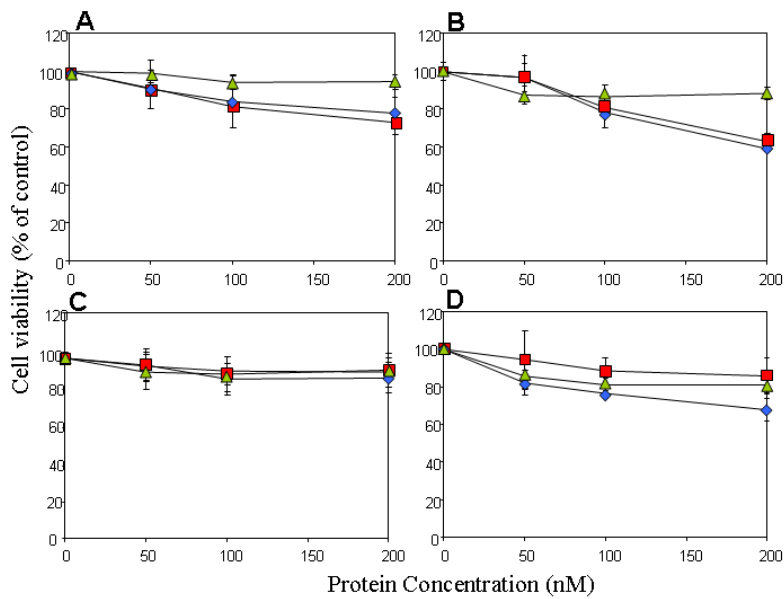


Fig. 4. Effects of the antibodies on cell survival. Dose-response curves of Herceptin (triangles), Erb-hcAb (rhomboids) and 2C4 (squares) on prostate cancer cells: 22RV1 (A), LnCap (B), DU145 (C) and PC3 (D).

3.4 *In vitro* effects of combination of Erb-hcAb, Herceptin and 2C4 prostate cancer cells

On the basis of the observation that Herceptin, Pertuzumab and Erb-hcAb bind to different epitopes, we investigated whether the combination of the three antibodies could allow for more effective and lower therapeutic doses.

To this aim, ErbB2-positive LnCap androgen-dependent and DU145 androgen-independent prostate cancer cells, were treated for 72 h at 37°C with Herceptin (100 nM) or Erb-hcAb (100 nM) or Pertuzumab (100 nM), or with a combination of each antibody with the others at the same concentrations. As shown in Figure 5, the combinatorial treatment of the three antibodies inhibited the growth of prostate tumor cells more effectively than the treatment with either each single immunoagent or combination of two antibodies (data not shown), thus showing additive effects.

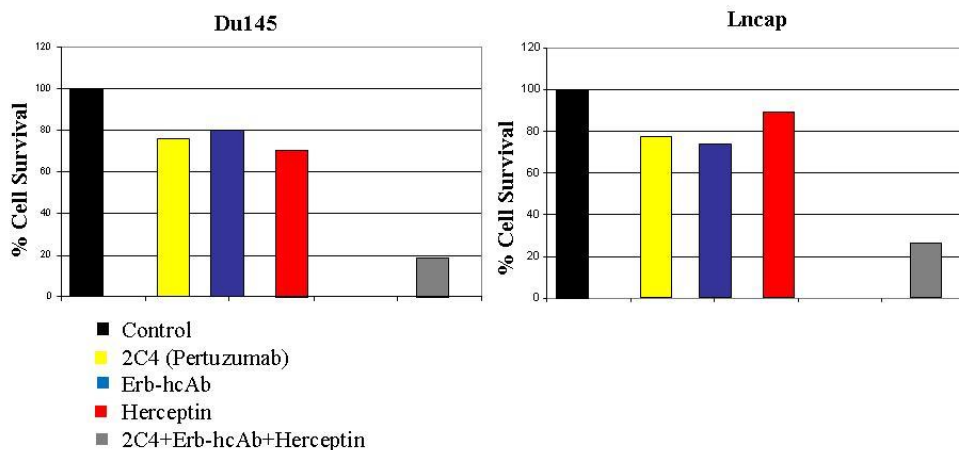


Fig. 5. Growth inhibition effects on ErbB2-positive prostate cancer cells of combinatorial treatments (grey bars) of 2C4 (yellow bars), Erb-hcAb (blue bars) and Herceptin (red bars).

3.5 In vivo Antitumor Activity of Erb-hcAb

For in vivo studies, Erb-hcAb was tested on human prostate, LnCap androgen-dependent and PC3 androgen-independent cancer cells.

As shown in Fig.6, the treatment of mice bearing LnCap and PC3 tumors with seven doses, at 72h intervals, of 5 mg /Kg⁻¹ of body wt of Erb-hcAb induced a dramatic reduction in tumour volume. In parallel experiments, we tested the effects of Herceptin and 2C4, administered as indicated above for Erb-hcAb.

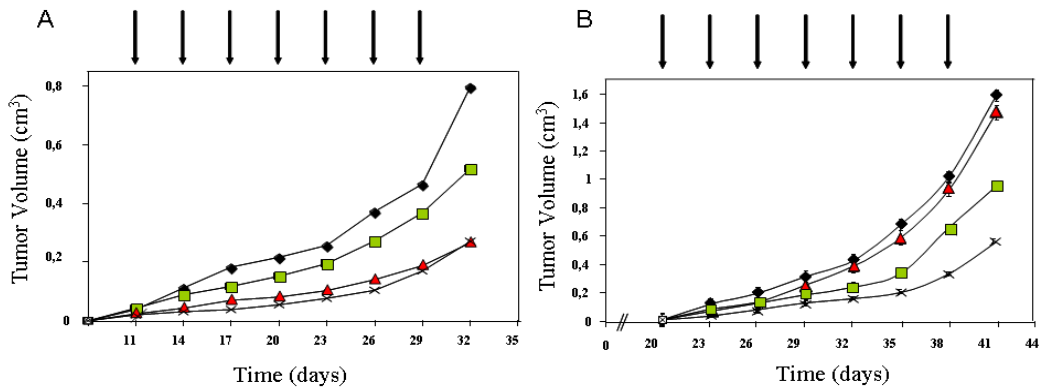


Fig. 6. In vivo effects on tumor growth of Erb-hcAb. Mice inoculated with 2x10⁵ PC3 (A) or LnCap (B) prostate cancer cells were untreated (rhomboids) or treated with 2C4 (triangles), Herceptin (squares) and Erb-hcAb (crosses).

Surprisingly, 2C4 was found to be effective for PC3 tumors but inactive on LnCap androgen-dependent tumors, whereas Herceptin, as expected (20), showed only slight effects on both androgen-dependent and androgen-independent tumors.

No growth inhibition was observed in mice treated with PBS. During the period of treatment, the animals did not show signs of wasting or other visible signs of toxicity.

3.6 Pharmacokinetics and Biodistribution of Erb-hcAb

Erb-hcAb was labeled with Na ^{125}I and injected i.p. (1-2 E6 cpm/animal) in mice bearing LnCap xenografts. At increasing time intervals, ranging from 5 min to 96 h retro-orbital sinus sampling was performed to evaluate blood clearance of the radiolabelled compact antibody. At 96h animals were sacrificed and residual radioactivity in organs was determined by gamma counting and weighing. Radioactivity levels are expressed as percentage of injected dose per gram (%ID/g) normalized for a weight of 20 g per mouse.

The blood clearance profile of ^{125}I -Erb-hcAb is well described by the biexponential function with a rapid early clearance phase and a slower late phase (Fig.7). The values found ($t_{1/2}$ of 7.92 h and 43.62h for the alpha and beta phase, respectively) are those expected for radiolabeled proteins of similar molecular weight (23) and indicate stability of the immunoconjugate.

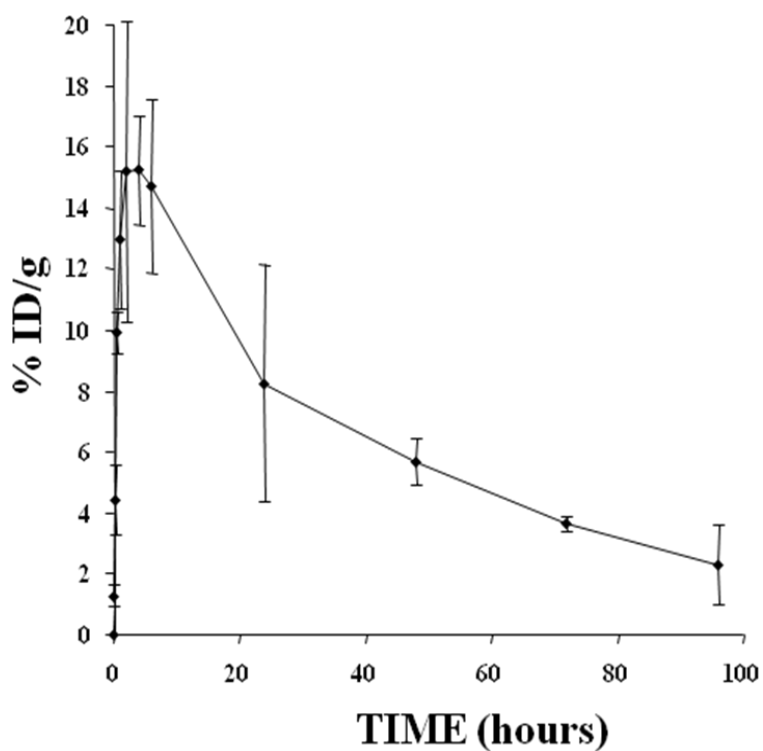


Fig. 7. Blood clearance profile of ¹²⁵I-Erb-hcAb. The estimated alpha and beta rate parameters corresponding respectively to the rapid early and the slower late clearance phases are as expected for radiolabeled proteins of similar weight.

Biodistribution at the 96 h time point showed higher accumulation of ^{125}I -Erb-hcAb in the LnCaP xenografts compared to all the other organs measured. These results taken together indicate adequate biodistribution properties of the radiolabeled compound due to the extended circulating half-life which yield high concentrations in the ErbB2 expressing xenografts necessary for biological activity.

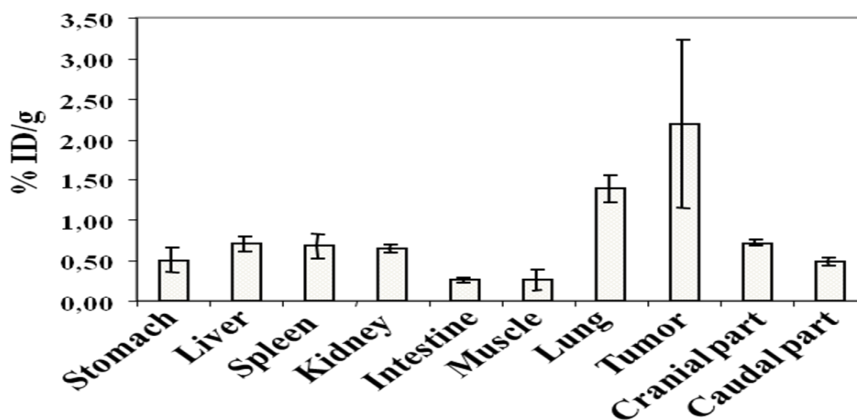


Fig. 8. Tissue and tumor biodistribution of radiolabeled Erb-hcAb at 96 h post-injection. The LnCap xenografts retain higher amounts of the radiolabeled immunoconjugate compared to all the other organs measured, with tumor to normal tissue ratios ranging from 1.5 (tumor to lung ratio) to approximately 8 times (tumor to muscle ratio).

4. DISCUSSION/CONCLUSIONS

Prostate cancer is one of the most common malignancies in men in developed countries. The developing and adult prostate is regulated by androgens acting through the androgen receptor (AR). High levels of AR expression are observed in primary prostate cancer and can be detected throughout progression in both androgen-sensitive and hormone refractory cancers. The development of androgen-independence creates a major challenge for treatment of prostate cancer patients because the hormone refractory tumor is minimally responsive to most of the current treatments, such as the androgen ablation therapy.

Prostate cancer progression is often associated with alteration of growth factor or growth factor receptor expression leading to androgen receptor activation. Unlike steroid hormones, growth factors regulate cellular responses through binding to membrane receptors. Growth factor or cytokine binding initiates a phosphorylation cascade that ultimately results in phosphorylation of transcription factors or transcription factor-interacting proteins (24,25). A number of recent reports suggest that activation of ErbB kinase axis results in AR activation.

The overexpression of the ErbB family members, particularly ErbB2, has been observed clinically in a number of cancer types and it is associated with poor prognosis in breast cancer patients (4,5). It is possible that increased expression of ErbB2 in prostate carcinomas is related to the development of hormone resistance. This is likely due to the protection by ErbB2 of prostate cancer cells from the androgen ablation therapy, as it allows AR transcription under conditions of extremely low levels of circulating hormone. ErbB2 activation of AR through Akt has been proposed as a major pathway that promotes androgen independent prostate cancer growth (26,27). The mechanisms by which ErbB2 is activated in prostate cancer are still unclear, but the mutual regulation between androgens and the ErbB2 network could contribute to shed light on the coincidence of onset of ErbB2 overexpression with the emergence of androgen independence. Indeed, androgen ablation could promote the survival of some cells by stimulating ErbB2 expression that is negatively regulated by androgens.

Thus, the use of antibodies capable of inhibiting the ErbB2 signaling could provide some clinical benefits for prostate cancer patients. Despite the advances made by the discovery and characterization of Herceptin, the only humanized antibody currently used for the treatment of breast cancer, most of the prostate cancer cells are less sensitive than breast tumor cells to Herceptin treatment (13) and many patients eventually relapse. Consequently, there is a need for additional therapeutics directed to the ErbB2 signaling pathway.

In previous studies it has been shown that Erb-hcAb, a fully human compact anti-ErbB2 antibody developed in our laboratory, is able to inhibit both *in vitro* and *in vivo* the growth of breast cancer cells either sensitive (16,17) or resistant to the treatment with Herceptin(19). Thus, we tested whether Erb-hcAb could be useful for the treatment of prostate cancer by determining its effects on prostate cancer cells *in vitro* and *in vivo* in comparison with those of Herceptin and Pertuzumab (2C4), another anti-ErbB2 antibody currently in clinical trials for prostate cancer (21). To this aim, we firstly examined the levels of ErbB2 expression on a panel of prostate cancer cells by western blotting and measured the binding abilities of the antibodies to the receptor by ELISA assays, then we tested their effects on the cells *in vitro* and *in vivo* in a mouse model.

Here, we show that Erb-hcAb is able to efficiently inhibit the growth of both androgen-dependent and androgen-independent tumor cells, with antitumor effects more potent than those observed with either 2C4 (Pertuzumab) or Herceptin.

In particular, the antibody 2C4 (Pertuzumab) significantly inhibited the growth of androgen-independent tumor cells but strikingly it had no effects on the androgen-dependent tumors, whereas Herceptin, as reported in the literature (13), showed only slight antitumor effects on both types of tumor cells.

We then tested whether the combination of these antibodies targeting different epitopes of ErbB2 led to more potent antitumor effects as it has been previously reported in literature that the combination of Herceptin and Pertuzumab as well as the combination of Herceptin and Erb-hcAb had synergistic or additive effects on breast tumor cells (28,29). Indeed, we found that the combination of the three different antibodies was the most effective in inhibiting the *in vitro* growth of prostate cancer cells.

These results therefore suggest a potential use of Erb-hcAb in the immunotherapy of prostate cancer in addition to that of breast cancer resistant to the treatment with Herceptin, the antibody currently used in the clinic.

5. REFERENCES

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