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COMPARISON OF CARDIOTOXIC EFFECTS OF DIFFERENT ERB-B2 INHIBITORS AND THEIR ASSOCIATED MECHANISMS

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RINGRAZIAMENTI E DEDICHE

SUMMARY

The ErbB2 receptor is a proto-oncogene associated with a poor prognosis in breast cancer. Herceptin, the only humanized anti-ErbB2 antibody currently in clinical use, has proved to be an essential tool in the immunotherapy of breast carcinoma, but induces cardiotoxicity.

ErbB2 is involved in the growth and survival pathway of adult cardiomyocytes; however its levels in the adult heart are much lower than those found in breast cancer cells, the intended targets of anti-ErbB2 antibodies. Furthermore, Lapatinib, a dual kinase inhibitor of EGFR and ErbB2, and Pertuzumab, a new anti-ErbB2 monoclonal antibody currently in clinical trials, which recognizes an epitope distant from that of Herceptin, have shown relatively low cardiotoxicity in clinical trials.

Two novel human antitumor immunoconjugates, made up of a human anti-ErbB2 scFv, Erbicin, fused with either a human RNase or the Fc region of a human IgG1, are selectively cytotoxic for ErbB2-positive cancer cells in vitro and vivo, and target an epitope of ErbB2 different from that of Herceptin.

As Herceptin has shown cardiotoxic effects, we evaluated if any side effects were exerted also by Erbicin-derived immunoagents (EDIA), used as single agents or in combination with anthracyclines. Furthermore, we compared the in vitro and in vivo cardiotoxic effects of EDIA with those of the other available anti-ErbB2 drugs: Herceptin, 2C4 (Pertuzumab) and Lapatinib.

Here we show that EDIA, in contrast with Herceptin, 2C4 and Lapatinib, have no toxic effects on human fetal cardiomyocytes in vitro, and do not induce additive toxicity when combined with doxorubicin.

Moreover, EDIA do not impair cardiac function in vivo in mice, as evaluated by Color Doppler echocardiography, whereas Herceptin significantly reduces radial strain at day 2 and fractional shortening at day 7 of treatment in a fashion similar to doxorubicin. Also 2C4 and Lapatinib significantly reduce radial strain after only two days of treatment, even though they showed cardiotoxic effects less pronounced than those of Herceptin.

We investigated also the molecular basis of the different cardiotoxic effects among the ErbB2 inhibitors by testing their effects on the formation of the Neuregulin 1β (HRG)/ErbB2/ErbB4 complex and on the activation of its downstream signaling. We report herein that Erb-hcAb at difference with Herceptin, 2C4 (Pertuzumab) and Lapatinib,

does not affect the ErbB2-ErbB4 signaling pathway activated by HRG in cardiac cells.

These findings may have important implications for the mechanism and treatment of anti-ErbB2-induced cardiotoxicity, and suggest that EDIA could fulfil the therapeutic need of patients ineligible to Herceptin treatment due to cardiac dysfunction. Furthermore, these results strongly indicate that radial strain could become a reliable marker to detect early cardiac dysfunction.

RIASSUNTO

Il recettore transmembrana tirosina–chinasico (RTK) ErbB2 è iper-espresso su diversi tipi di carcinomi, in particolare in quello mammario.

Attualmente l'unico anticorpo monoclonale umanizzato impiegato nella terapia del carcinoma mammario è Herceptin. Nonostante la sua efficacia, l'impiego di Herceptin presenta alcuni svantaggi, quali l'insorgenza di un fenomeno di resistenza nelle pazienti trattate per lungo tempo con il farmaco, e l'induzione di effetti cardiotossici, che aumentano in modo considerevole quando Herceptin è somministrato in combinazione con le antracicline.

Allo scopo di ottenere anticorpi più efficienti e privi di tali effetti collaterali, nel nostro laboratorio sono stati ingegnerizzati due nuovi immunoagenti. A partire dalla fusione di un frammento anticorpale umano specifico per ErbB2, chiamato Erbicin, o con una RNasi umana o con la regione cristallizzabile di una IgG umana sono stati ottenuti rispettivamente Erb-hRNasi ed Erb-hcAb, un anticorpo umano compatto così chiamato per le sue ridotte dimensioni (100 KDa) rispetto a quelle delle IgG naturali (155 KDa). Entrambi gli immunoagenti derivati da Erbicin (EDIA, Erbicin derived immunoagents) legano selettivamente ed inibiscono la crescita di cellule ErbB2-positive, sia in vitro che in vivo. Risultati recenti hanno inoltre dimostrato che gli EDIA riconoscono un epitopo di ErbB2 diverso da quello di Herceptin e che non presentano effetti tossici su cellule cardiache di ratto in vitro, nè su modelli animali di topo in vivo. Nell'ambito di tale progetto, la mia attività di ricerca ha avuto lo scopo di completare la valutazione della cardiotossicità di Erb-hcAb su cardiomiociti umani, in assenza o in presenza di antracicline e di comprendere quali fossero le basi molecolari della differente cardiotossicità tra Erb-hcAb, Herceptin e altri inibitori di recettori tirosina chinasici, come Lapatinib o 2C4 (Pertuzumab), l'altro anticorpo anti-ErbB2, attualmente in fase di sperimentazione clinica.

I risultati ottenuti hanno dimostrato che gli EDIA, differentemente da Herceptin, 2C4 e Lapatinib, non mostrano effetti tossici in vitro su cardiomiociti fetali umani quando utilizzati in monoterapia, e non potenziano gli effetti tossici della doxorubicina quando impiegati in combinazione con l'antraciclina. Inoltre i due EDIA non alterano la funzionalità cardiaca in vivo in modelli sperimentali di topo, come dimostrato da studi di ecocardiografia Color doppler con analisi *speckle tracking*. Al contrario, il trattamento con Herceptin riduceva in modo significativo lo strain radiale dopo 2 giorni e la

frazione di accorciamento del ventricolo sinistro dopo 7 giorni. Anche 2C4 e Lapatinib riducevano lo strain radiale dopo soli due giorni dal trattamento, anche se era stato precedentemente riportato che i loro effetti cardiotossici sono meno pronunciati rispetto a quelli di Herceptin.

Tali risultati sono stati interpretati alla luce dei diversi meccanismi molecolari dei diversi inibitori, chiariti nel corso della mia attività sperimentale. Erb-hcAb, a differenza di Herceptin, 2C4 e Lapatinib non interferisce con il *pathway* di sopravvivenza cellulare promosso dall'eterodimerizzazione di ErbB2 con ErbB4 in presenza del ligando Neuregulina 1β.

Questi risultati potrebbero avere importanti implicazioni per la comprensione del meccanismo e del trattamento della cardiotossicità indotta da inibitori di ErbB2, ed indicano un possibile impiego dello strain radiale come marcatore per l'identificazione precoce di disfunzioni cardiache. Alla luce di questi risultati gli EDIA potrebbero essere utilizzati a scopo terapeutico in pazienti che non possono essere trattate con Herceptin a causa delle complicanze cardiache.

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1. INTRODUCTION

Immunotherapy is a precious strategy to overcome the limits of the conventional anti-cancer treatments. Indeed, targeting cancer cells via antibodies specific for tumor associated surface proteins could fulfil the lack of selectivity of radiotherapy and chemotherapy, and is a new interesting biomedical approach as it combines the rational drug design with the progress in understanding cancer biology.

ErbB2 (Figure 1) is an attractive target for immunotherapy, as it is a transmembrane tyrosine kinase receptor highly expressed on many breast, ovary, lung and other carcinoma cells [1-5], whereas in normal tissues it is expressed at low levels only in certain epithelial cell types [6]. Overexpression of this receptor is associated with progression of malignancy of breast cancer, and is a sign of a poor prognosis [7]. Herceptin, the only anti-ErbB2 humanized monoclonal antibody approved by FDA for the therapy of mammary carcinoma, has proved to be effective in the immunotherapy of breast carcinoma [8]. However, large-scale clinical studies with Herceptin, have shown that up to 7 % or 28 % of patients suffer from cardiac dysfunction when Herceptin is used either in monotherapy, or in combination with anthracyclines, respectively [9-11].

The mechanism of cardiotoxicity induced by Herceptin has not been yet elucidated but it has been supposed to be associated with inhibition of ErbB2 signaling, as the receptor is thought to participate in an important pathway for growth, repair, and survival of adult cardiomyocytes [12-14]. However, ErbB2 levels in the adult heart are low when compared with the levels found in ErbB2-overexpressing breast cancer cells, the intended targets of a therapy based on anti-ErbB2 antibodies. Furthermore, Lapatinib, a dual kinase inhibitor of the

Epidermal Growth Factor Receptor (EGFR/ErbB1) and ErbB2, and Pertuzumab, a new anti-ErbB2 monoclonal antibody at present tested in clinical trials which recognizes an epitope distant from that of Herceptin, have shown relatively low cardiotoxicity [15-17]. If inhibition of ErbB2 was responsible for cardiomyocyte dysfunction, then cardiotoxicity would have been expected also with the quinazoline compound and all the other anti-ErbB2 antibodies. Thus, Herceptin-associated cardiotoxicity must be explained by some alternative mechanisms [18]. The hypothesis has been made that Herceptin cardiotoxicity is related to the inhibition of the neuregulin 1-activated pathway which directly promotes cardiac myocyte survival via ErbB2/ErbB4 heterodimerization [12].

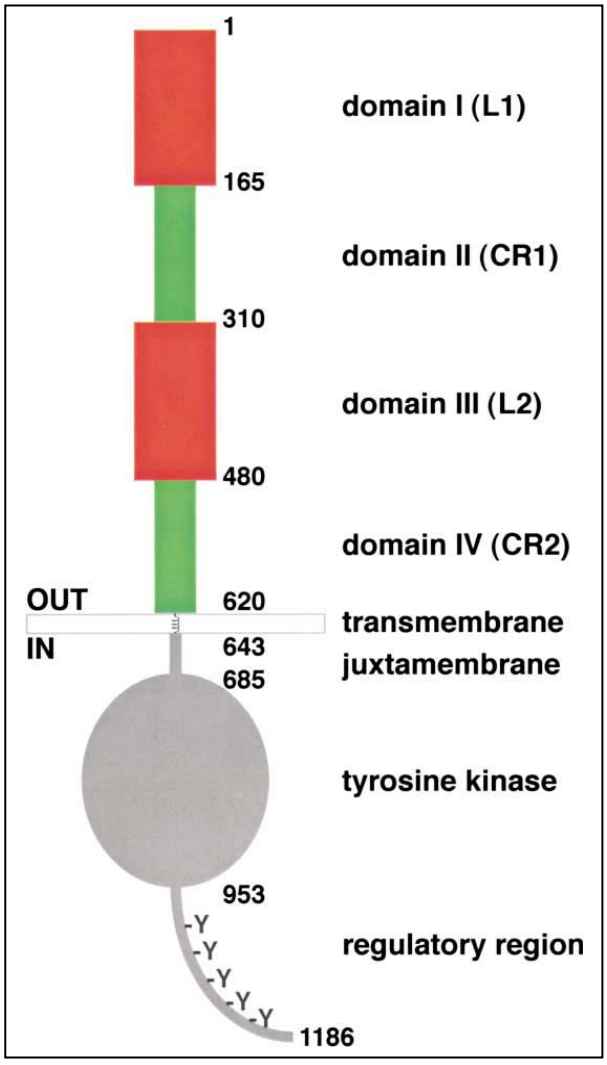

Figure 1. Schematic representation of ErbB2 receptor

ErbB2 and ErbB4 receptors are expressed in differentiated cardiomyocytes [19]. Binding of Neuregulin-1β (HRG) to ErbB4 increases its kinase activity and leads to heterodimerization with ErbB2 or homodimerization with ErbB4, though heterodimer with ErbB2 appears to be a more potent signalling complex than homodimer [20], stimulating stronger and prolonged intracellular signal transduction [21].

In vitro studies have suggested that the HRG/ErbB2/ErbB4 complex controls cardiomyocytes survival and myofibril disarray [22, 23]. It has also been shown that neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation thus promoting myocardial regeneration following myocardial injury [24]. Despite these findings, the basis of cardiac dysfunction after inhibition of this system by Herceptin has remained unclear.

Two novel human antitumor immunoconjugates were engineered in our laboratory by fusion of a human anti-ErbB2 single chain variable antibody fragment (scFv) [25], termed Erbicin, with either a human RNase [26], denominated Erb-hRNase (Erbicin-human-RNase) (Figure 2), or the Fc region of a human IgG1 [27]. The latter has been called Erb-hcAb (Erbicin-human-compact Antibody) (figure 3) for its "compact" reduced size (100 kDa), compared with the full size (155 kDa) of a natural IgG, which should promote an increased extravascular diffusion and tumor penetration. Both these Erbicin-Derived Immunoagents (EDIA) are selectively cytotoxic for ErbB2-positive cancer cells *in vitro* and *vivo* [26-28]. The antitumor action of Erb-hRNase is dependent on the ability of this molecule to be internalized by receptor mediated endocytosis and to reach the cytosol where the non toxic and non immunogenic human RNase can degrade RNA and exert a selective cytotoxic action [29]. Erb-hcAb is also capable of selective binding to malignant ErbB2-

positive cells and of inhibiting the receptor signaling thus inducing cell cycle arrest and apoptosis. Finally, due to the presence of Fc region Erb-hcAb is endowed with both ADCC and CDC cytotoxic effects [27].

More interestingly, EDIA are also active on some Herceptin-resistant breast cancer cells both in vitro and in vivo [30]. The sensitivity of these cells to treatment with EDIA is likely due to their different epitope [31, 32], since EDIA, differently from Herceptin, are capable of inhibiting the signaling pathway downstream ErbB2 [30] also in these cells.

The findings that EDIA recognize an epitope different from that of Herceptin [31, 32] led us to ascertain whether they might not present the most negative property of Herceptin: cardiotoxicity.

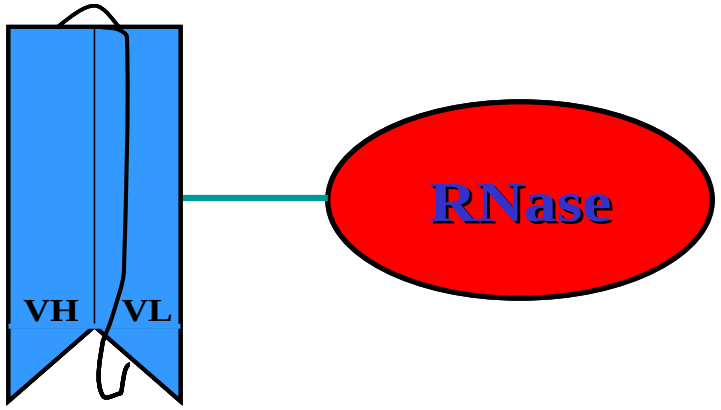

Figure 2 Schematic representation of the human immunoRNase **Erb-hRNase** (Erbicin-human-RNase).

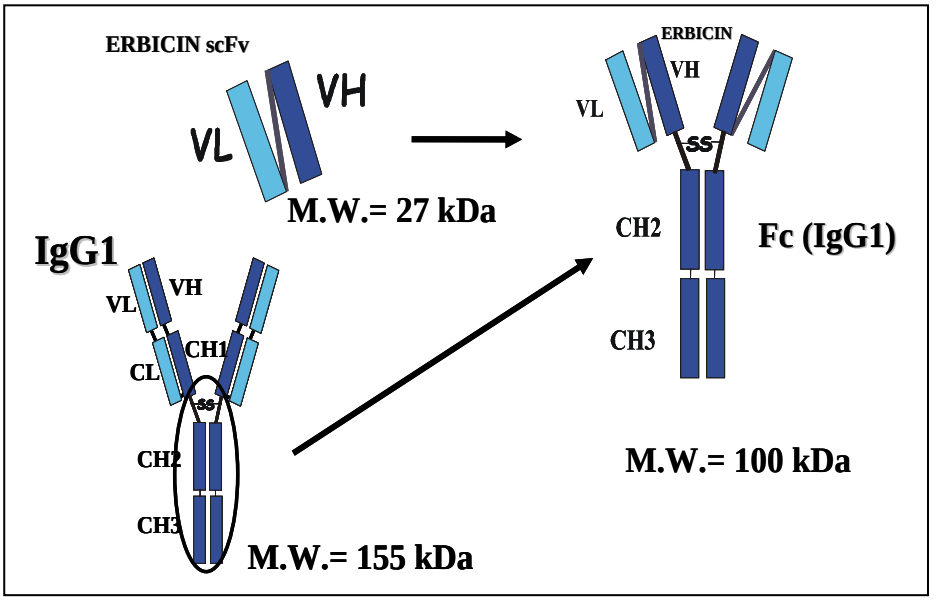

Figure. 3-The construction of Erb-hcAb, a human compact anti-ErbB2 antibody. *Left*: Erbicin, the parental anti-ErbB2 scFv. *Right*: Erb-hcAb. VH and VL are the heavy and light chain variable domains, respectively, as derived from Erbicin. H, the hinge region with disulfide bridges. CH2 and CH3 are the heavy chain constant domains of a human IgG1. *Bottom*: a full size IgG1

EDIA did not show *in vitro* adverse effects on rat cardiomyocytes and cardiomyoblasts for which Herceptin is severely toxic [33]. These differences have been found to be due to their different mechanism of action: in fact Herceptin, differently from Erb-hcAb, induces apoptosis in cardiac cells. In vivo studies on a mouse model have shown that EDIA do not alter the cardiac function, whereas Herceptin significantly reduces fractional shortening (FS) in a fashion similar to that of doxorubicin [33].

Furthermore, we demonstrated that the treatment with either Erb-hcAb or Erb-hRNase induced in treated mice cardiac fibrosis and apoptosis at a much lower extent than treatment with Herceptin or doxorubicin [33].

Thus, to test whether EDIA could fulfil the therapeutic need of cancer patients ineligible to Herceptin treatment due to cardiac dysfunction and primary or acquired Herceptin-resistance, we implemented new pre-clinical protocols to assess their cardiotoxic effects in combination with anthracyclines on human fetal cardiomyocytes *in vitro*, and in animal models *in vivo*.

Moreover, we investigated the potential therapeutic use of EDIA by comparing their cardiotoxic effects with those of all the other available anti-ErbB2 drugs, such as Herceptin, 2C4 (Pertuzumab) and Lapatinib. Finally, to investigate the molecular basis of the different cardiotoxic effects among the different ErbB2 inhibitors, we analyzed in the present study the effects of Erb-hcAb on the formation of the HRG/ErbB2/ErbB4 complex and on the activation of its downstream signaling in comparison with those of Herceptin, 2C4 (Pertuzumab) and Lapatinib. We report herein that the ErbB2-ErbB4 signaling pathway, activated by HRG in cardiac cells, is not affected by Erb-hcAb. These data may have important implications for the mechanism and treatment of anti-ErbB2-induced cardiotoxicity.

2. MATERIALS AND METHODS

2.1 Antibodies and cell lines

The hybridoma cells producing 2C4 antibody (LGC Promochem, Sesto San Giovanni, Italy) were grown in DMEM-RPMI-1640 medium in a 1:1 ratio (Sigma, St Louis, MO, USA).

The H9C2 cardiomyoblasts were cultured in DMEM containing sodium pyruvate (1.0 mM). The media were supplemented with 10% heat-inactivated fetal bovine serum, 2.0 mM L-Glutammine, 50 Units/ml penicillin, and 50 μg/ml streptomycin (all from Sigma).

The Human Fetal Cardiac Myocytes (HFC) (Innoprot, Derio, Spain), were cultured according to the manufacturer's recommendations.

The antibodies used were: Herceptin (Genentech, South San Francisco, CA, USA); Erb-hcAb, produced from PER.C6® cells (Crucell N.V., Leiden, Netherlands) transfected with the recombinant vector and purified as previously described [27]. Erb-hRNase was produced and purified, as previously reported [26].

The antibody 2C4 was produced and purified as follows. 2C4 hybridoma cells were expanded to near confluence in complete medium, and then 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 for Erb-hcAb [27].

2.2 ELISA Assays

Cells, harvested in non-enzymatic dissociation solution (Sigma), were washed and tranferred to U-bottom microtiter plates (2 x 10^5 cells per well).

After blocking with PBS containing 6% bovine serum albumin (BSA), cells were treated with the antibodies in ELISA buffer (PBS/BSA 3%) for 90 minutes. After centrifugation and removal of supernatants, the pelleted cells were washed twice in 200 µl of ELISA buffer, resuspended in 100 µl of ELISA buffer, and incubated with peroxidase-conjugated anti-human IgG (Fc-specific) or anti-mouse antibody (Sigma) for detection of Herceptin and Erb-hcAb or 2C4, 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 < 5%). Standard deviations were calculated on the basis of the results obtained by three different experiments.

2.3 Intracellular levels of p-Akt/Akt and p-Erk/Erk

H9C2 or HFC cells were seeded in 6-well plates at a density of $6x10^5$ cells/wells, incubated at 37°C and allowed to attach overnight. The following day cells were starved for 2 h in growth medium without serum, then briefly washed with PBS, and finally treated with 100 nM of each antibody diluted in RPMI medium, containing 10 mM Hepes (pH 7.2), 0,2% w/v BSA (binding buffer). After 1h at room temperature, the cells were incubated in the absence or in the presence of HRG (5 nM) for 10min at 25 C°. Supernatant was removed by aspiration and the cells were lysed in RPMI medium, containing 10mM HEPES (pH 7.2), 1,0% Triton x-100, 1% CHAPS, 2% sodium ortovanadate and protease inhibitor (Roche). Protein concentration was determined by the Bradford colorimetric assay (Sigma). Aliquots of 100 µg were run on 12 % SDS-PAGE, electroblotted onto PVDF membranes (Millipore Corporation, Bedford, MA, USA), which were treated with anti-pErk, anti-Erk, anti-pAkt or

anti-Akt antibodies (all from SantaCruz Biotecn. CA. USA), followed by anti-mouse or anti-rabbit, HRP-conjugated IgGs, respectively. The signal from secondary antibodies was visualized by enhanced chemiluminescence detection (ECL western blotting detection kit, Amersham Biosciences). The signal intensity of reactive bands was quantitatively measured with a phosphorimager (GS-710, Biorad, Hercules, CA, USA).

2.4 Immunoprecipitation and Western blotting Analyses

ErbB2/ErbB4 co-immunoprecipitation was carried out by incubating cell lysates (prepared as described above) with 10 μg/ml of Herceptin, 2C4 or ERB-hcAb in PBS for 16 h at 4°C. The immune complexes were then collected by adsorption to protein A-Sepharose (Sigma) for 1 h at 4°C. After four washes with PBS, the proteins were released by boiling in loading buffer, run on 8% SDS-PAGE, and electroblotted onto PVDF membranes (Millipore Corporation, Bedford, MA, USA). The ErbB4 protein was detected by using the anti-ErbB4 mAb (Santa Cruz Biotecn.).

2.5 In Vitro cardiotoxicity tests

To test the cardiotoxicity of Erb-hcAb, Herceptin, Lapatinib (Tykerb®, GlaxoSmithKline, Brentford, UK) and 2C4, HFC or H9C2 cells were seeded in 96-well microtiter plates at a density of $1x10^4$ cells/wells, and allowed to adhere overnight. Following the initial incubation for 16 h at 37°C, the medium was replaced with medium containing Herceptin, Erb-hcAb, 2C4 or Lapatinib in the absence or in the presence of HRG (5nM). After the treatment carried out for 72 h at 37 °C, cells were washed with PBS and stained with Trypan Blue or tested by (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) for the determination of cell survival, as previously described [33].

Cell survival was expressed as percent of viable cells in the presence of drugs under test with respect to control cultures grown in the absence of the drugs. Typically, cell survival values were obtained from at least three independent experiments in which five determinations were performed for each sample. Standard Deviations were calculated on the basis of the results obtained from all the experiments.

2.6 Cell morphology analysis

HFC cells were plated at a density of 6×10^5/well in six-well plates and incubated at 37°C with Herceptin (2 μM), Erb-hcAb (2 μM), doxorubicin (0.5 μM), or with the combination of each antibody with doxorubicin. After 24 h, cultured cells were observed by light microscopy (Nikon ECLIPSE E1000, Melville, NY, USA), and photographed (Nikon digital camera DXM 1200F), to analyze cell morphology.

2.7 Transthoracic echocardiography

In vivo cardiac function was assessed by transthoracic echocardiography in sedated 7 wk old WT C57Bl/6 mice (Harlan Italy, San Piero al Natisone, UD, Italy) using a Vevo 2100 high-resolution imaging system (VisualSonics, Toronto, ON, Canada). Mice were anesthetized with Tilotamine (0.09 mg/g), Zolazepam (0.09 mg/g), and 0.01% atropine (0.04 ml/g). Cardiac function was evaluated by noninvasive echocardiography in basal condition and after intraperitoneal treatment of equimolar doses (2 nmol/mouse) of Herceptin, Erb-hcAb, immunoRNase, 2C4; or doxorubicin (15 mg/kg), used as a positive control. Oral treatment was carried out for the Tyrosine Kinase Inhibitor Lapatinib (100 mg/kg/day). The left ventricular (LV) echocardiogram was assessed in both parasternal long-axis and short-axis views at a frame rate of

233 Hz. End-systole and end-diastole dimensions were defined as the phases corresponding to the ECG T wave, and to the R wave, respectively. M-mode LV end-systolic dimensions (LVESDs) and LV end-diastolic dimensions (LVEDDs) were averaged from 3–5 beats. Left ventricular end-diastolic dimension (LVEDD) and left ventricular end-systolic dimension (LVESD) were measured from the LV M-mode at the midpapillary muscle level. Fractional shortening percentage (%FS) was calculated as [(LVEDD-LVESD)/LVEDD] x100 [34]. Radial strain (SR) was evaluated by speckle tracking echocardiography, a novel technique that enables the assessment of myocardial strain (an index of myocardial deformation) through the analysis of speckle motion inherently present in a standard, 2-D echocardiographic image [35].

Studies and analyses were performed blinded to heart condition. Data are presented as mean±SD unless otherwise noted. Between group differences were assessed by Student's t-test or one-way ANOVA as appropriate. Statistical significance was defined as $p<0.05$.

The animal experimentations described herein were conducted in accordance with the Italian regulation for experimentation on animals. All in vivo experiments were carried out with ethical committee approval and met the standards required by the Directive 2010/63/EU of the European Parliament.

2.8 Cardiac Fibrosis Analysis

Interstitial fibrosis was evaluated by staining of 5 μm-tick tissue sections with 1% Sirius red in picric acid (Carlo Erba Laboratories, Milan, Italy) as previously described [36]. The positively stained (red) fibrotic area was measured using a computer-assisted image analysis system (Nikon NIS ELEMENTS BRV, Melville, NY, USA) and expressed as a percentage of total

area. The percentage of red staining was calculated from 3 samples/group, with 2 sections for each sample and 5 images for each section.

3. RESULTS

3.1 Binding of anti-ErbB2 antibodies to rat and human cardiac cells

As previously reported [33], Erb-hcAb and Herceptin were both capable of binding to ErbB2 receptor on rat and mouse cardiomyocytes. Here we tested if they were also capable of recognizing ErbB2 receptor on Human Fetal Cardiomyocytes (HFC). Furthermore, for a comparison with Pertuzumab, the other humanized anti-ErbB2 antibody currently tested in clinical trials, we used also its cognate mouse antibody 2C4, and tested if it was capable of binding ErbB2 both on rat H9C2 cardiomyoblasts and HFC. These experiments were carried out by immunoprecipitation assays performed by incubating the anti-ErbB2 antibodies with protein cell extracts of HFC, and for 2C4, also with extracts of H9C2 cardiomyoblasts. The immune complexes were collected by protein A-Sepharose and analyzed by Western blotting with Neu, a commercial anti-ErbB2 mAb.

As shown in figure 4 A, Erb-hcAb and Herceptin were capable of immunoprecipitating ErbB2 from HFC, and 2C4 recognized ErbB2 from both human fetal cardiomyocytes and H9C2 cardiomyoblasts (Figure 4 A,B).

These results were confirmed by ELISA assays, performed by testing Erb-hcAb, Herceptin and 2C4 for their binding to HFC at increasing concentrations.

The binding curves obtained for Erb-hcAb, Herceptin or 2C4 on HFC were found to be superimposable (figure 4 C), thus suggesting that the antibodies recognize human ErbB2 with comparable affinity.

The antibody 2C4 was tested by ELISA assays also on H9C2 cardiomyoblasts and showed the ability to bind the receptor also on these cells (figure 4 D).

3.2 *In vitro* effects of the anti-ErbB2 immunoagents on human cardiac cells

To test the cardiotoxicity of Erb-hcAb and Erb-hRNase on HFC, the cells were incubated at 37°C in the absence or in the presence of increasing concentrations of immunoagents for 24 hours and then either tested by MTT assays or counted to measure cell viability. Control tests, carried out in parallel assays with Herceptin, 2C4 (the mouse antibody version of Pertuzumab), Lapatinib or doxorubicin are presented for comparison.

As shown in Fig. 5, Erb-hcAb and Erb-hRNase did not show cardiotoxic effects, whereas Herceptin, 2C4, Lapatinib and doxorubicin were all found to be significantly toxic for cardiac cells.

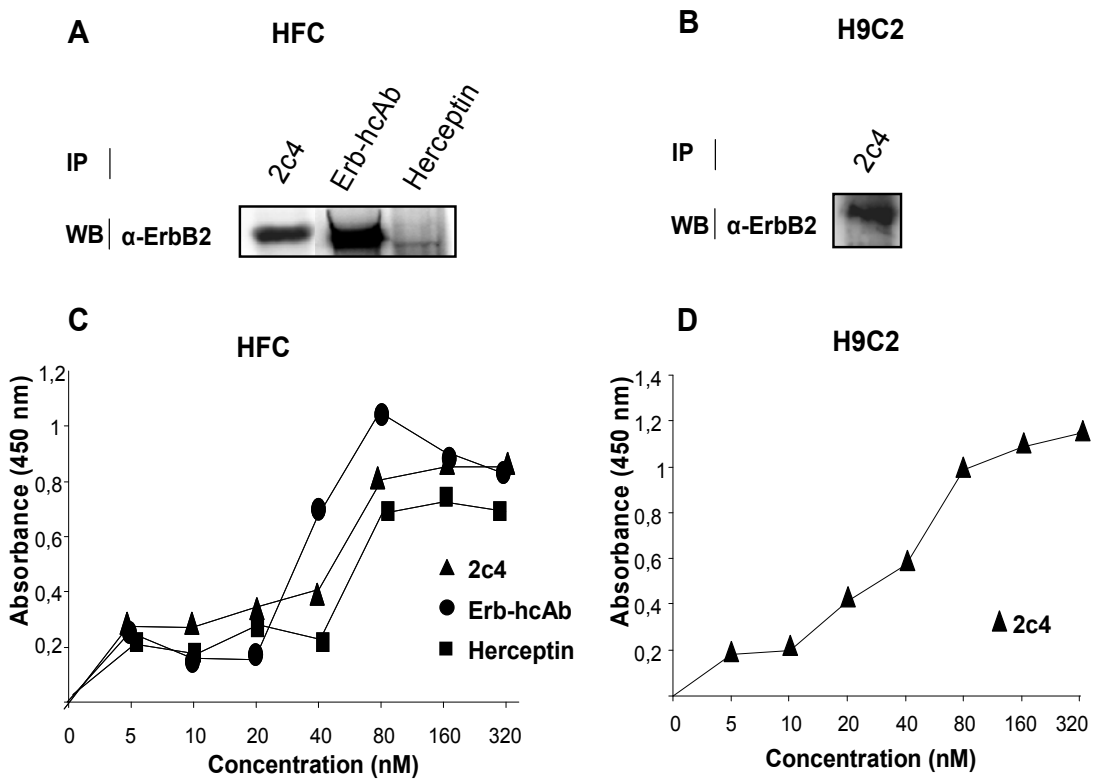

Figure 4: Binding of the antibodies to human and rat ErbB2. Western blotting analyses with Neu, an anti-ErbB2 mAb of cell extracts from HFC (A), previously immunoprecipitated with Erb-hcAb, Herceptin or 2C4, and from H9C2 (B) immunoprecipitated with 2C4. Binding curves of Herceptin (squares), Erb-hcAb (circles) or 2C4 (triangles) to HFC (C) and H9C2 cardiomyoblasts (D).

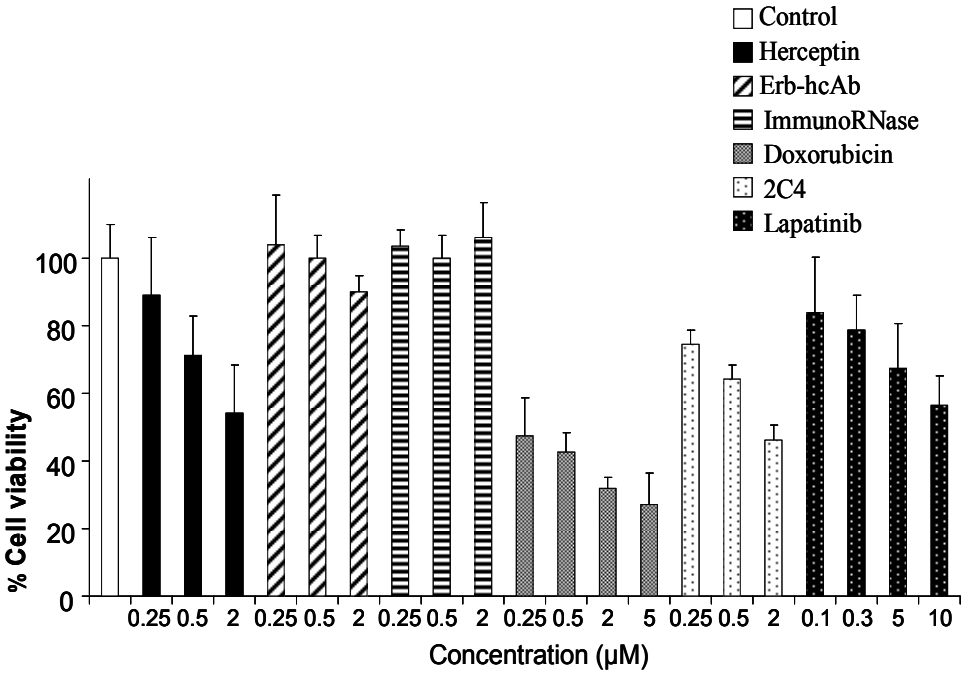

Figure 5: Effects of the anti-ErbB2 drugs on human fetal cardiomyocytes (HFC). Dose-response tests of the cells treated for 24h with Herceptin, Erb-hcAb, ImmunoRNase, 2C4, Lapatinib or Doxorubicin used as a positive control.

3.3 Effects of the anti-ErbB2 drugs on ligand-induced growth of cardiac cells

When we treated H9C2 cardiomyoblasts or HFC with the ErbB4 ligand HRG, a significant growth stimulation (between 120% and 150% of control growth) was observed. We then tested the inhibitory effects of all the investigated anti-ErbB2 drugs by adding the antibodies or Lapatinib at the concentrations reported in Figure 6, in the absence or in the presence of HRG.

As shown in Figure 6, the antibodies 2C4 and Herceptin, and the kinase inhibitor Lapatinib inhibited the growth of HFC and H9C2 in a dose dependent manner either in the absence or in the presence of HRG. On the contrary, Erb-hcAb did not show toxic effects in the absence of HRG and did not inhibit the ligand induced growth stimulation.

3.4 Effects of the anti-ErbB2 antibodies on the association of ErbB2 with ErbB4 in cardiac cells

We studied whether ErbB4-ErbB2 complexes exist on the plasma membrane in the absence of the ErbB4 ligand, HRG. To answer this question, HFC and H9C2 cells were treated with HRG or control buffer, and lysed. Immunoprecipitation was performed by adding an anti-ErbB2 antibody that recognizes the intracellular domain of ErbB2. The immunoprecipitated proteins were then analyzed by Western blotting with an anti-ErbB4 monoclonal antibody. As shown in figure 7, ErbB4 was detected in ErbB2 immunoprecipitates only when the cardiac cell cultures were treated with the ErbB4 ligand HRG. These data suggest that the recruitment of ErbB2 to ErbB4 occurs in a HRG-dependent manner.

We then studied whether antibodies to the extracellular domain of ErbB2 were capable of disrupting the formation of ErbB4-HRG-ErbB2 complexes. The antibodies to ErbB2 used in these studies, Herceptin, Erb-hcAb and 2C4, are known to bind distinct epitopes on the extracellular domain of the ErbB2 receptor [31, 32, 37].

As shown in figure 7, Herceptin and 2C4 were capable of disrupting the formation of ligand-mediated ErbB4-ErbB2 complex, whereas Erb-hcAb did not show any effects on the ErbB2-ErbB4 heterodimerization.

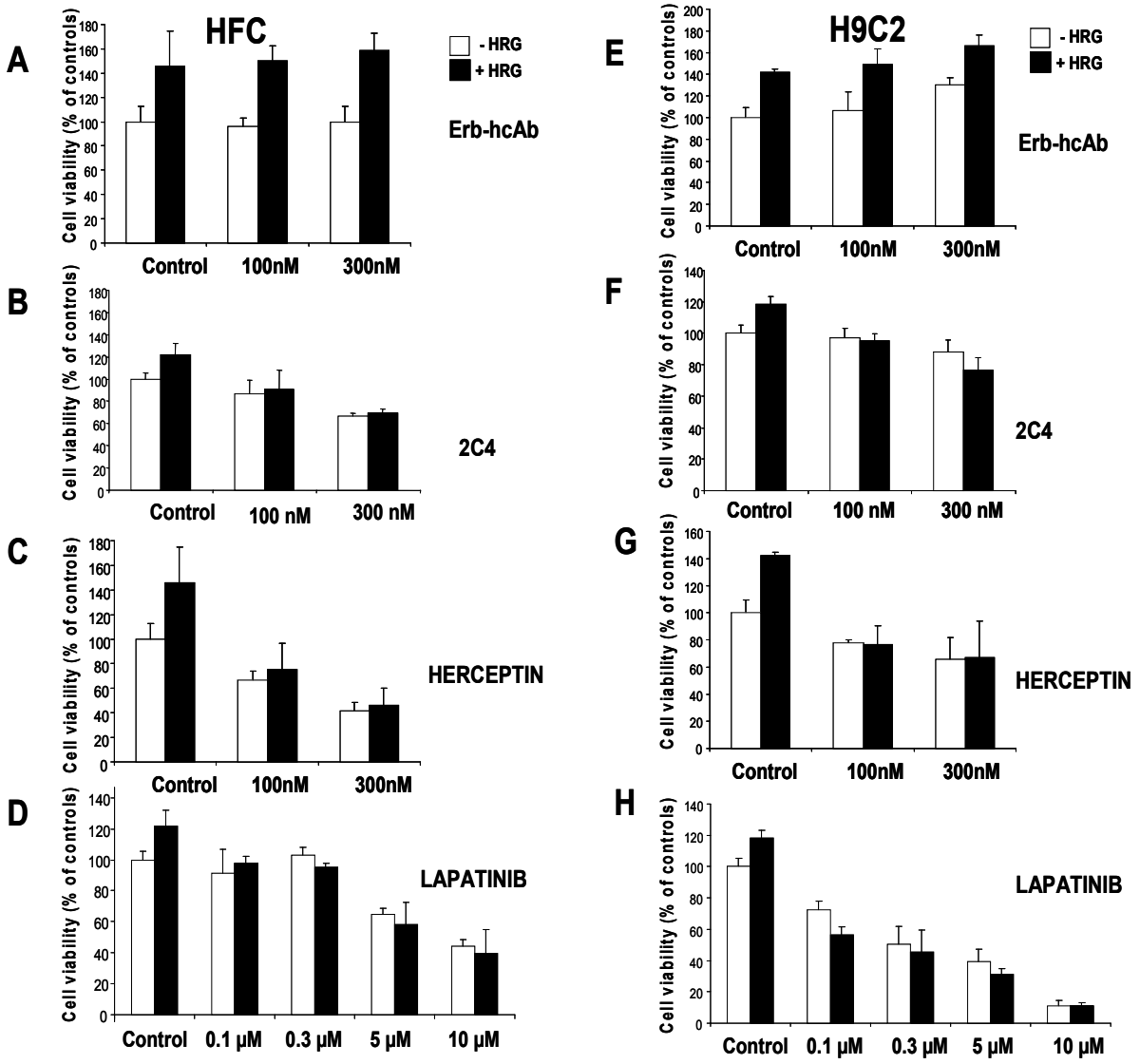

Figure 6: Effects of the anti-ErbB2 drugs on the proliferation of HFC (A-D) and H9C2 (E-H) in the absence (white bars) or in the presence (black bars) of HRG.

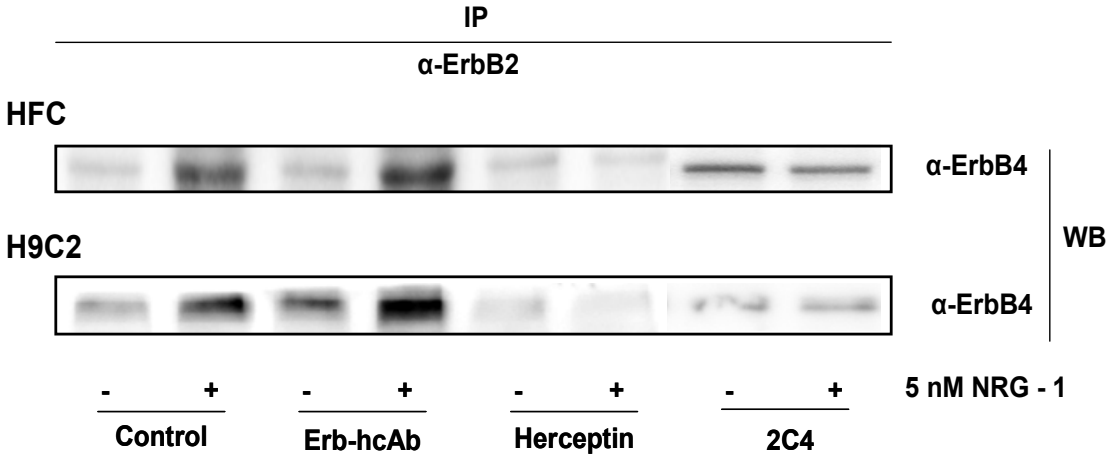

Figure 7: Association of ErbB2 with ErbB4 in the absence or in the presence of HRG in cardiac cells treated with the indicated antibodies.

3.5 Effects of the anti-ErbB2 drugs on ligand-activated ErbB2 signaling in cardiac cell lines

Since ErbB4 requires the association with ErbB2 to initiate signal transduction in cardiac cells, we determined the effects of anti-ErbB2 antibodies and Lapatinib on receptor activation. With this aim we assessed the effects of anti-ErbB2 antibodies on MAPK activation, a downstream target of ErbB2. Briefly, HFC and H9C2 cells were treated with HRG in the absence or in the presence of the antibodies or Lapatinib, and then lysed. MAPK activation was measured by western blotting of lysates with a phospho-specific MAPK antibody. As shown in Figure 8, cells treated with HRG showed a strong activation of MAPK (Erk1, Erk2) which was strongly reduced by 2C4 , Herceptin and Lapatinib treatment, whereas it was unaffected by Erb-hcAb.

To test the effects of anti-ErbB2 monoclonal antibodies or Lapatinib on PI3 kinase activation, another pathway downstream ErbB2 in cardiac cells [23], HFC and H9C2 cells were treated as described above, and the activity of the downstream Akt kinase was measured by Western blotting with a phospho-specific Akt antibody. As shown in Figure 8, the treatment of cardiac cells with the ErbB4 ligand, HRG, results in the activation of Akt that is inhibited by the pretreatment with 2C4, Herceptin or Lapatinib, but it is not affected by Erb-hcAb.

These results suggest that Herceptin, 2C4 and Lapatinib exert their cardiotoxic effects by inhibiting the pathways downstream ErbB2-ErbB4 complexes upon activation by HRG, whereas Erb-hcAb does not show cardiotoxic effects because it does not inhibit the ErbB2-ErbB4 heterodimerization and its downstream signaling activation induced by HRG.

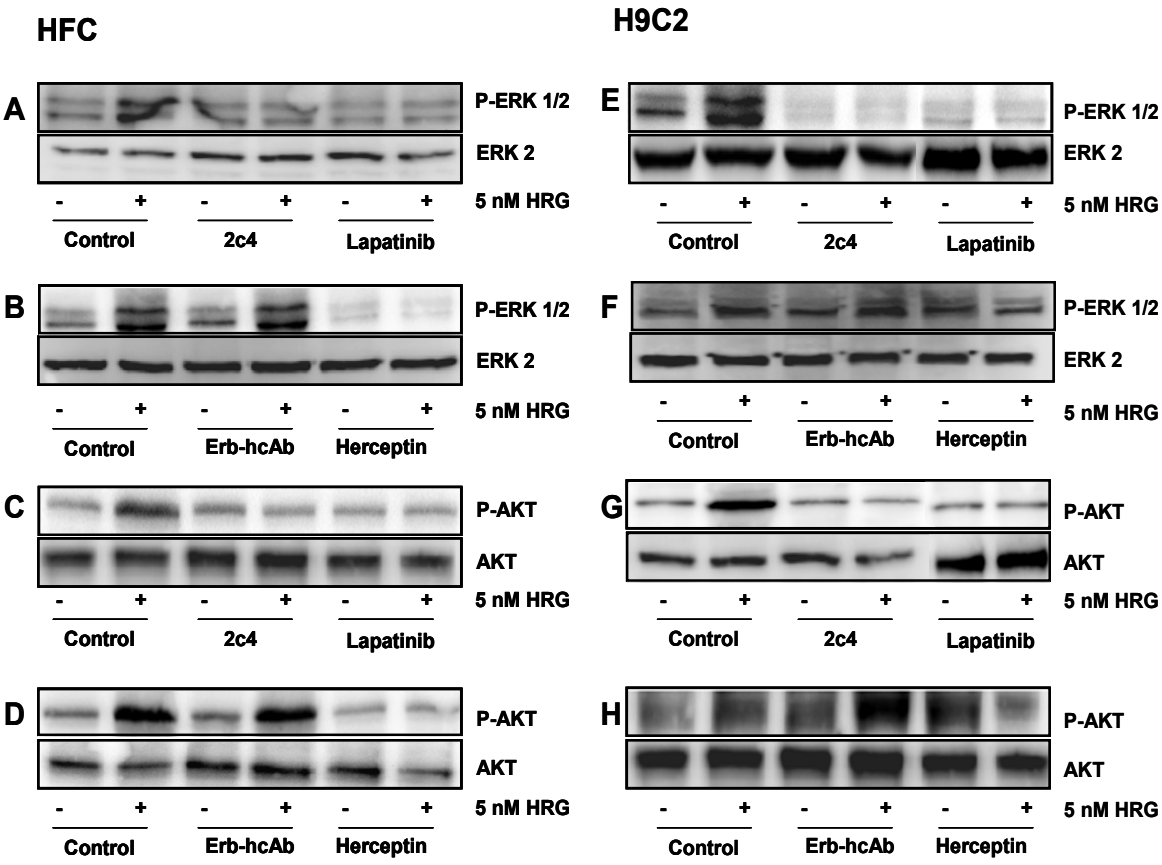

Figure 8: Effects on MAPK and Akt activation in HFC (A-D) and H9C2 (E-H), as exerted by the indicated drugs in the absence or in the presence of the ligand HRG.

3.6 Effects on cell survival of Erbicin-derived immunoagents in combination with doxorubicin

Large-scale clinical studies with Herceptin have shown that up to 7 % of patients suffer from cardiac dysfunction when Herceptin is used in monotherapy, and this percentage raises to 28% when it is combined with anthracyclines [9-11].

Thus, we performed experiments to evaluate the effects of the combination of Erb-hcAb or Erb-hRNase with doxorubicin on HFC and on a rat H9C2 cardiomyoblast cell line.

Cytotoxicity assays were carried out on cells treated for 24 h at 37°C with either doxorubicin (0.12-0.5 μM), or Erb-hcAb (0.25-2 μM) or with a combination of the chemotherapeutic drug and Erb-hcAb at the same concentrations. Results of control tests run with Herceptin and doxorubicin are presented for comparison. We found that the toxicity was clearly superior when the chemotherapeutic drug was given in combination with Herceptin (see Fig. 9), whereas no additive effects were observed for the combination of Erb-hcAb with doxorubicin.

Figure 10 shows the morphologic changes of cells treated for 24 h at 37°C with Herceptin (2 μM), Erb-hcAb (2 μM), doxorubicin (0.5μM), or with the combination of each immunoagent with doxorubicin at the same concentrations. The combinatorial treatment of Herceptin and doxorubicin clearly caused an increased cell death and more marked changes in cell morphology than each drug alone, as cells appeared to lose their typical features and assume distorted, round shaped forms. No such effects were observed for the cells treated with Erb-hcAb alone or in combination with doxorubicin.

Similar experiments were performed with the immunoRNase Erb-hRNase. Human fetal cardiomyocytes and rat H9C2 cardiomyoblasts were treated for 24 h at 37°C with either doxorubicin (0.12-0.5 μM), or Erb-hRNase (0.25-2 μM) or with a combination of doxorubicin with Erb-hRNase at the same concentrations. As shown in Fig. 11, doxorubicin displayed a similar cytotoxic effect when used alone or in combination with Erb-hRNase, thus suggesting that also this immunoagent, differently from Herceptin, does not increase the cardiotoxicity of the chemotherapeutic treatment.

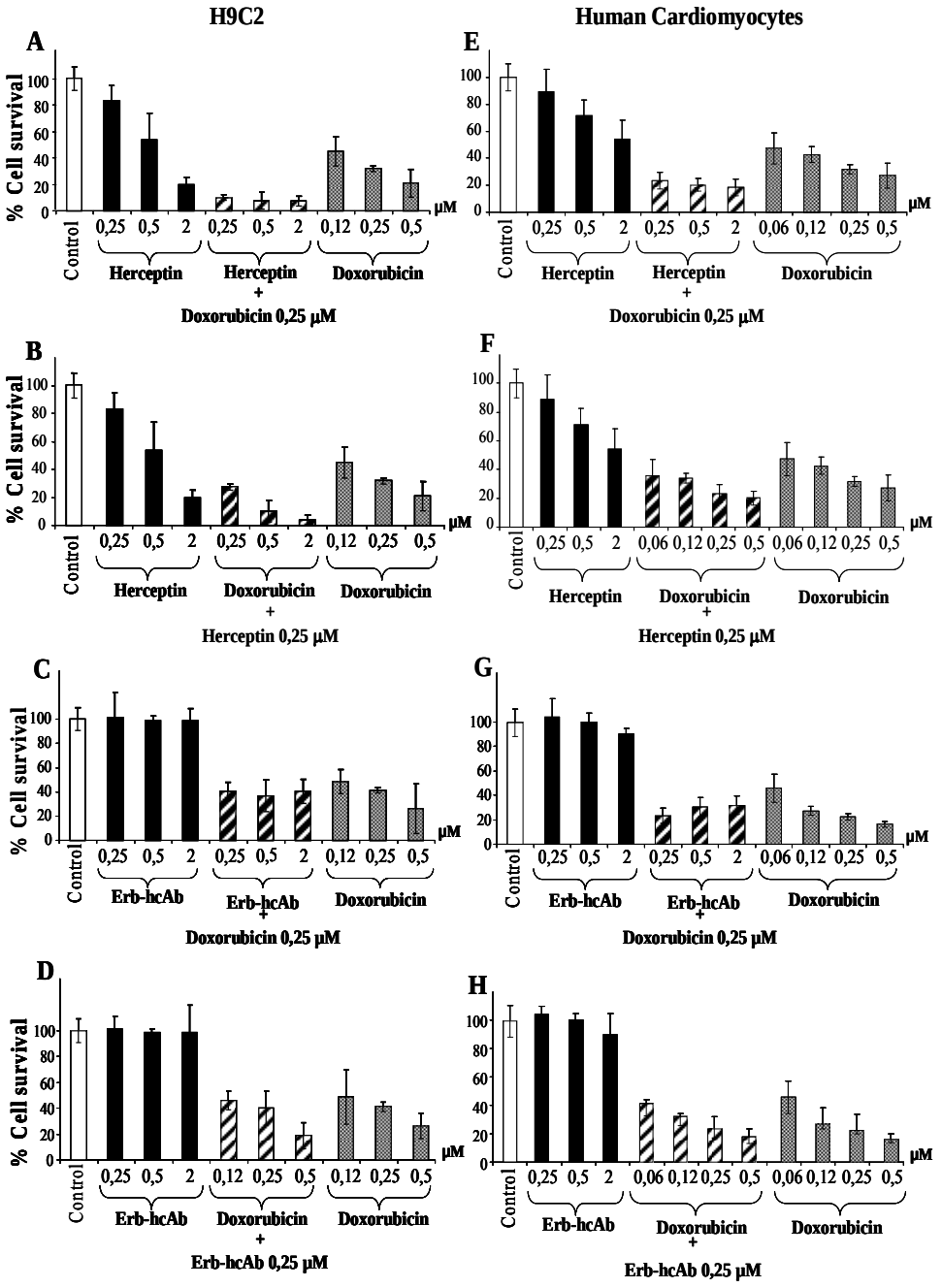

Figure 9: Effects of combination treatment of Herceptin (A, B, E, F) or Erb-hcAb (C, D, G, H) with doxorubicin on human fetal cardiomyocytes (HFC) and H9C2 cardiomyoblasts. Dose-response tests of the indicated cells treated for 24h with each antibody alone (black bars), doxorubicin alone (grey bars) or with the combination of each antibody and doxorubicin (striped bars).

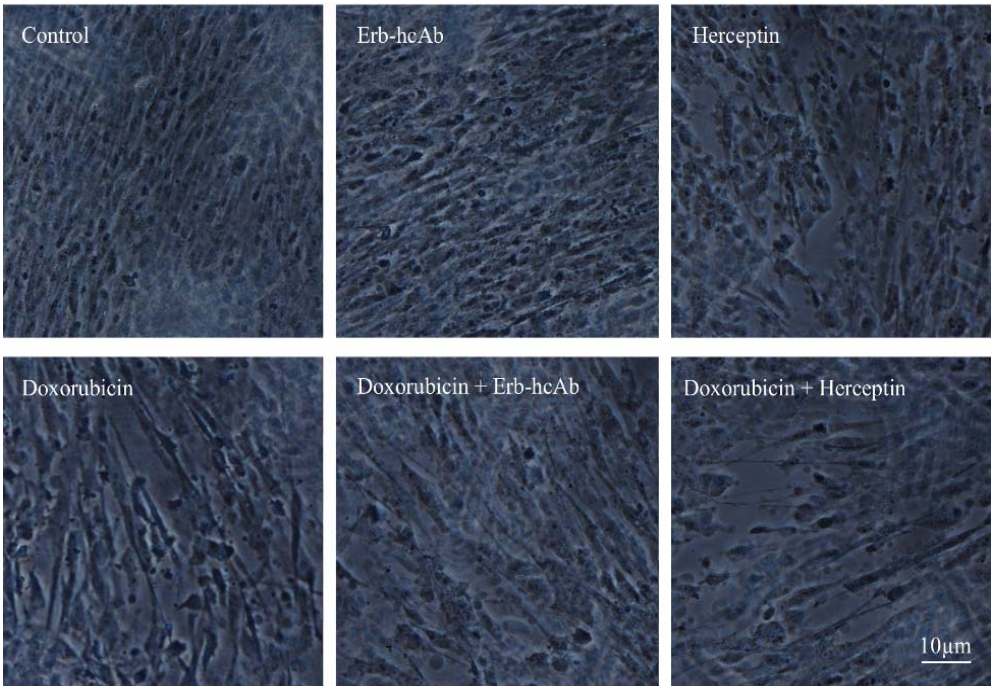

Figure 10: Effects on cell morphology of human fetal cardiomyocytes (HFC) grown for 3 days in the absence (control) or in the presence of Herceptin, Erb-hcAb, doxorubicin or with the combinations of each antibody with doxorubicin. Bar, 10 µm.

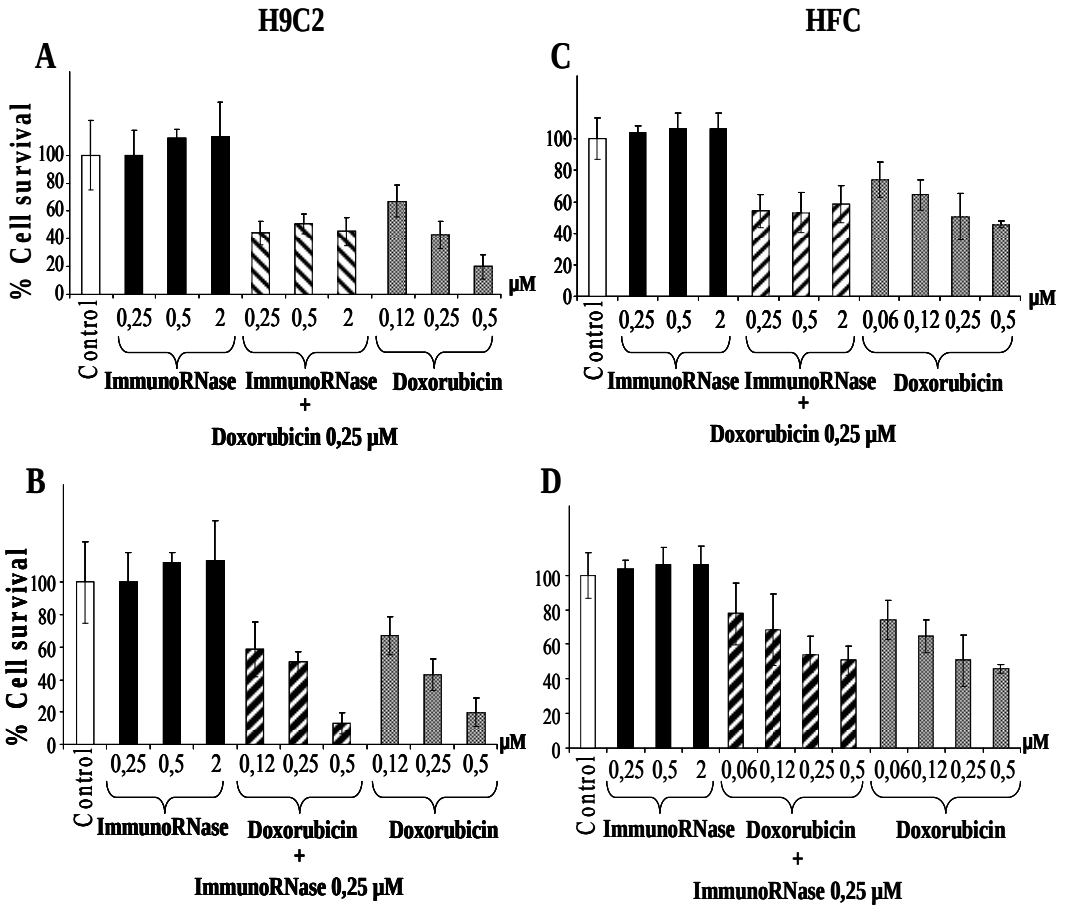

Figure 11: Effects of combination treatment of Erb-hRNase with doxorubicin on human fetal cardiomyocytes (HFC) and H9C2 cardiomyoblasts. Dose-response tests of the indicated cells treated for 24h with Erb-hRNase alone (black bars), doxorubicin alone (grey bars) or with the combination of Erb-hRNase and doxorubicin (striped bars).

3.7 In vivo cardiotoxic effects

We then tested the effects of combination of Herceptin, Erb-hcAb, or Erb-hRNase with doxorubicin, on a mouse model. For these experiments, groups of 6-10 mice were injected with equimolar doses of Erbicin-derived immunoagents or Herceptin, used either as single agents or in combination with doxorubicin. Echocardiography measurements were performed on mouse hearts before and after treatment. Erb-hcAb and the immunoRNase, each used in monotherapy, had no effects on cardiac function: FS (Fig. 12, panel A) and radial strain RS (Figure 12, panel B) were not affected; only a slight reduction of radial strain in the group treated for 7 days with Erb-hcAb is shown but the difference was found to be not statistically relevant. When combined with doxorubicin, EDIA showed no additive effects with respect to those of doxorubicin alone. Indeed, FS was reduced to 52±0.2% early (after 2 days) also in mice treated with doxorubicin in monotherapy (2.17 mg/kg/day). On the other hand, mice treated with a combination of doxorubicin (2.17mg/kg/day) and Herceptin (2.25 mg/kg/day) showed a drastic reduction of FS (49±2%, vs 60±0.4% in sham), and in mice treated with Herceptin alone (2.25 mg/kg/day) FS decreased at 7 days (49±1.5% vs 60±0.5%, p=0.002). In contrast, after 2 days, myocardial strain was already reduced not only in groups of mice treated with doxorubicin or doxorubicin combined with Herceptin, but also in groups treated with Herceptin alone (43± 1% vs 66±0.6% in sham).

To further confirm that radial strain is an early predictor of future onset of cardiac dysfunction, we tested the *in vivo* effects on this parameter of Lapatinib and 2C4 (the mouse antibody version of Pertuzumab), previously reported [15, 16] as less cardiotoxic ErbB2-inhibitors. As shown in Fig. 13 panel A, both FS and radial strain were reduced by treatment with 2C4. After 2

days of treatment with 2C4 (2.25 mg/kg/day), myocardial strain was already decreased compared to sham: 40±8% vs 66±0.6%, (p=0.02), with FS also significantly reduced: 58±1% vs 60±0.4, (p=0.01). LV dysfunction was exacerbated after 7 days of treatment, with strain further decreasing to 31±7%, and FS to 39±5% (Fig. 13, panel A).

In mice treated with Lapatinib (100mg/kg/day) a decrease of FS was observed after 7 days (56±2% vs 60±1% for sham animals, p=0.04), while after 2 days FS was unaffected. At this time point radial strain, evaluated by *speckle tracking,* was able to identify cardiotoxicity at a very early stage (34±7% vs 59±1% in sham, p=0.008) (Fig. 13, panel B).

After treatment, mice were sacrificed, and the hearts were removed and subjected to weight measurement, histological examination, and processing for detection of myocardial stress. Cardiac fibrosis, induced by 2C4 and Lapatinib, was examined by the Sirius red staining for collagen on 5-µm-thick tissue sections, as previously described for the treatment with Erb-hcAb or Herceptin [33]. The red-stained sections were assessed for the presence of fibrosis (collagen) in the heart tissues using computer-assisted image analysis to evaluate the percentage of the red staining. All analyses were carried out in parallel experiments on control untreated mice. As shown in Fig. 14, cardiac fibrosis was significantly increased in mouse hearts treated either with Lapatinib or 2C4 for 7 days with respect to control untreated mice.

As previously reported [33], also Trastuzumab and doxorubicin were found to induce cardiac fibrosis at higher extent than that shown in mice untreated or treated with EDIA.

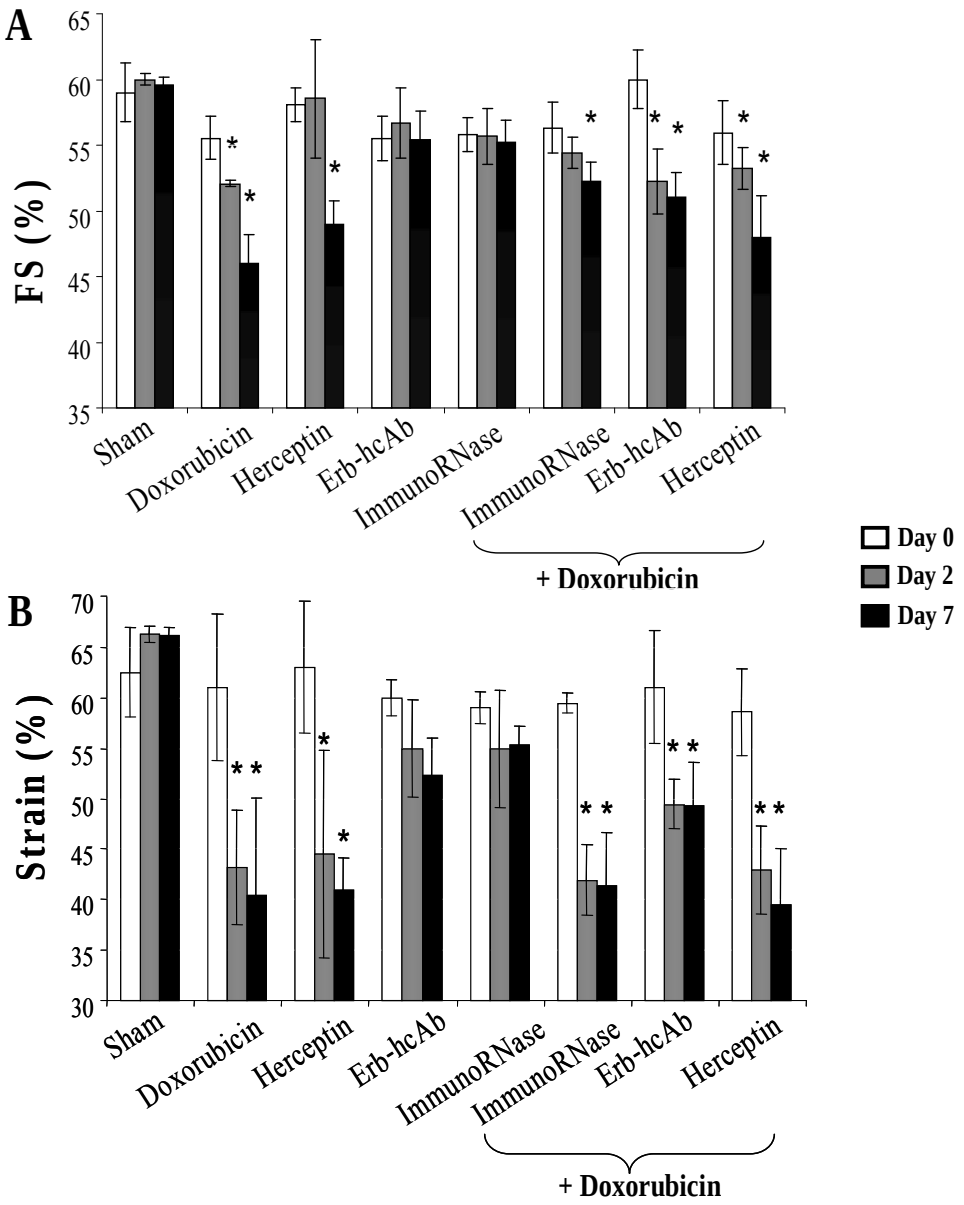

Figure 12: In vivo effects of combinations of anti-ErbB2 immunoagents with doxorubicin on heart function. Relative FS and radial strain are reported before or after the treatment of mice for 2 or 7 days with immunoRNase, Herceptin, or Erb-hcAb used as single agents or in combination with doxorubicin. *P≤ 0.05.

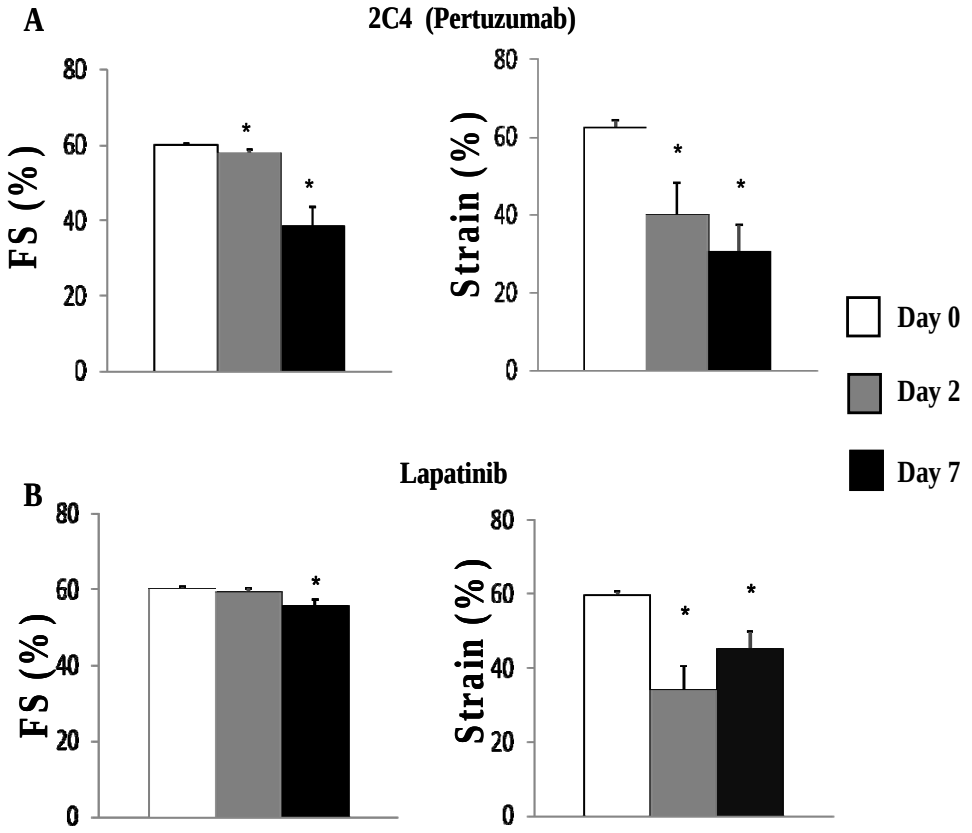

Figure 13: In vivo effects of 2C4 (Pertuzumab) and Lapatinib on heart function. Relative FS and radial strain are reported before or after the treatment of mice for 2 or 7 days with the anti-ErbB2 2C4 mAb antibody (panel A, *P≤ 0.02) or with Lapatinib, the Tyrosine Kinase inhibitor (panel B, *P ≤ 0.04).

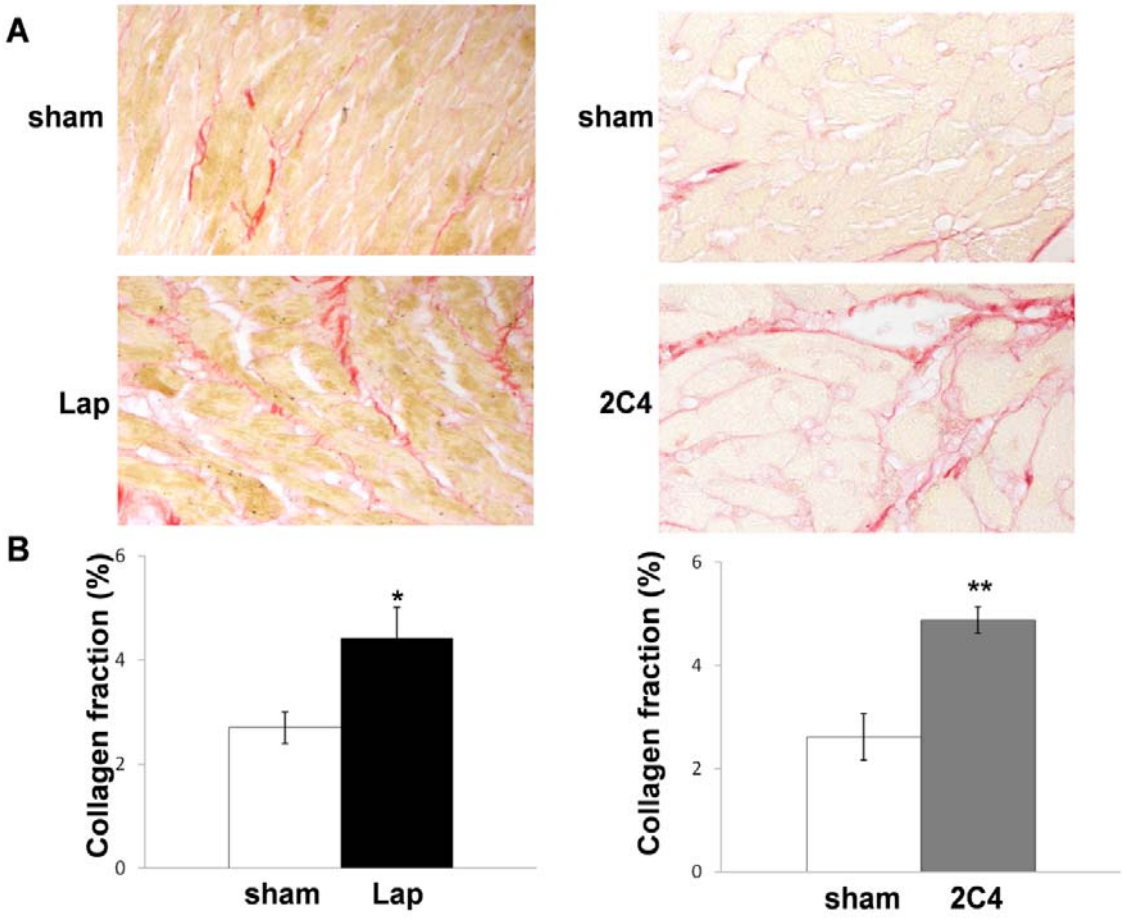

Figure 14: Analysis of cardiac fibrosis in mice treated with Lapatinib (Lap) or 2C4. Panel A: representative photomicrographs of LV sections from mice treated with the indicated drugs. Panel B: quantification of the interstitial fibrosis expressed as relative percentage of color intensity. (*p=0.02; **p=0.008 vs sham).

4. DISCUSSION/CONCLUSIONS

In the last decades, novel anticancer drugs have been successfully developed that effectively induce tumor regression or growth delay, thus prolonging patients survival time.

However, cardiovascular obnoxious side effects of the new anticancer drugs have been shown that can lead to therapy-related heart failure. Although cardiac problems were predictable for anthracyclines, they were instead totally unexpected from highly targeted anticancer agents, such as Herceptin or tyrosine kinase inhibitors.

Since its registration by the Food and Drug Administration (FDA) in 1998, Herceptin (Herceptin, Genentech, San Francisco, CA) has been used to treat more than 450,000 women with breast cancer worldwide [38]. As a monoclonal antibody directed against the human epidermal growth factor receptor-2 (HER-2 or ErbB2), Herceptin was initially shown to prolong the survival of women with HER-2 positive advanced breast cancer [10]. In 2005, landmark adjuvant studies demonstrated that adjuvant Herceptin either following or in combination with chemotherapy reduced the risk of relapse by approximately 50% and the risk of death by 33% for women with HER-2 positive early breast cancer [39].

Cardiac toxicity was recognized as an important side effect at an early stage in the development of Herceptin. Manifested as symptomatic congestive heart failure (CHF) or asymptomatic left ventricular ejection fraction (LVEF) decline, Herceptin-induced cardiotoxicity has been attributed to blockade of HER-2 signaling in cardiac myocytes. The cardiac safety of anti-HER-2 therapy is likely to be agent specific, as the early clinical experience with Lapatinib, a dual tyrosine kinase inhibitor of the EGFR and HER-2 receptors, suggests that

it may produce less cardiotoxicity compared with Herceptin. However, more studies are needed to better characterize the cardiac effects of Lapatinib, since the patient population studied was heterogeneous and highly selected, limiting the conclusions that can be drawn from this early data [40].

A new anti-ErbB2 monoclonal antibody which recognizes an epitope distant from that of Herceptin (in the extracellular portion of ErbB2), Pertuzumab, does not seem to be significantly cardiotoxic [15], but data are still very preliminary, since it is still being tested in pivotal clinical trials.

This awareness has led on one hand to search for novel and safe antitumor drugs devoid of cardiac side effects, and on the other to the development of novel non-invasive but more sensitive methods for the early screening of cardiac dysfunction.

Here we report on a comparative analysis of the cardiotoxic effects of EDIA, the novel human immunoagents targeting a novel epitope of ErbB2 [31, 32], different from those of other anti-ErbB2 antibodies or inhibitors currently in clinical use or trials for breast cancer therapy.

We show here for the first time that EDIA do not show *in vitro* toxic effects on human fetal cardiomyocytes in stark contrast to the prototypical antibody Herceptin. Surprisingly, also 2C4 (Pertuzumab) and Lapatinib, an anti-ErbB2 drug which inhibits directly its intracellular tyrosine kinase domain, were found to be toxic in a dose dependent fashion on HFC. Similar results were obtained for EDIA when they were tested in combination with doxorubicin, as they did not show additive toxic effects. Different results were obtained with Herceptin, which instead increased the toxicity of doxorubicin, according to previous studies [10].

It was also previously reported in literature [18] that Herceptin is toxic to cultured human atrial cardiac microfragments maintained by co-culture with

feeder cells from newborn rat heart, as it caused a complete loss of their healthy beating phenotype and tissue structure. Antibody-mediated inhibition of ErbB2 might regulate mitochondrial integrity through the BCL-X proteins, leading to ATP depletion and contractile dysfunction without profound changes in myocyte ultrastructure [14].

As for the different effects on cardiomyocytes of not toxic Erb-hcAb, and strongly toxic Herceptin, our previous data on rat cardiomyocytes indicate that it can be ascribed to their different apoptotic properties. Herceptin induces apoptosis in cardiac cells, as it lowers the level of Bcl-XL and activates caspase 3, whereas Erb-hcAb does not induce apoptosis [33].

In the present work we provide direct evidence that the cardiotoxic effects of Herceptin and Pertuzumab are based on their ability to prevent the assembly of HRG/ErbB2/ErbB4 complex, required for cardiomyocyte survival. On the contrary, Erb-hcAb does not inhibit the ErbB2-ErbB4 heterodimerization induced by HRG. This difference is probably due to the different epitopes of ErbB2 recognized by the three antibodies.

Consequently, Herceptin and Pertuzumab, differently from Erb-hcAb, affect also the ErbB2-ErbB4 signaling pathway by inhibiting MAPK (Erk1, Erk2) and Akt in a similar fashion to Lapatinib.

Mouse models were then used to monitor the *in vivo* cardiotoxicity of the anti-ErbB2 drugs and to validate speckle tracking echocardiography as a sensitive measure of myocardial function in animal models. EDIA did not impair cardiac function *in vivo* as they did not affect radial strain and fractional shortening. The latter parameter was evaluated here for the first time after the treatment of mice with EDIA to assess whether it could be used as a more sensitive method to predict cardiac dysfunction.

Indeed, in mice treated with Herceptin, 2C4 or Lapatinib alterations of RS preceded the reduction of conventional echocardiography markers, such as FS and LVEF. In particular, Herceptin in a fashion similar to doxorubicin reduced radial strain at two days and fractional shortening at seven days of treatment compared to the sham group.

Similar results are shown here for 2C4 (Pertuzumab) and Lapatinib, previously reported in literature [15, 16] as anti-ErbB2 drugs less cardiotoxic than Herceptin, thus confirming that limited number of clinical studies performed with these newer agents only provide us with partial and very preliminary results. Indeed radial strain, as evaluated by speckle tracking, was capable of identifying early cardiotoxicity after only two days of treatment with either 2C4 or Lapatinib.

As of today, traditional echocardiographic indexes of cardiac function such as FS and EF can identify patients in which Herceptin toxicity is already evident. Also, troponins proposed by other authors [41] can be already elevated following anthracyclines, therefore the secondary administration of Herceptin does not act as the cause of myocardial stress, but only as a modulator of anthracyclines'stress [42]. At the moment, besides the recognition of cardiac risk factors, there is not yet a bona fide none invasive tool that can discriminate patients who will develop cardiotoxicity by treatment with Herceptin. A very interesting study [43] has recently indicated that Doppler Tissue Imaging (DTI) can detect early LV dysfunction prior to alterations seen in conventional echocardiographic indices in an animal model of anthracycline and Herceptin–mediated cardiomyopathy. Of course, further studies will be needed to validate DTI and radial strain as useful tools in these settings.

In conclusion, the present *in vitro* and *in vivo* studies confirm that EDIA may fulfill the therapeutic need of patients ineligible to Herceptin treatment due

to cardiac dysfunction, and strongly indicate that Radial Strain, measured by Speckle Tracking echocardiography, could become a reliable marker for early detection of myocardial subtle changes, predicting cardiac dysfunction in advance.

Furthermore, the data on the mechanisms of action of the different ErbB2 inhibitors may have important implications for the treatment of anti-ErbB2-induced cardiotoxicity, as they provide for the first time the molecular basis of the cardiotoxic effects of Herceptin and the other available anti-ErbB2 drugs.

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