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Effects of a second-generation human anti-ErbB2 ImmunoRNase on trastuzumab-resistant tumors and cardiac cells

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The inhibition of ErbB2 by the use of human antibodies can be a valuable strategy for the treatment of breast and gastric cancer. Trastuzumab, a humanized anti-ErbB2 antibody in clinical use, is effective but can engender resistance as well as cardiotoxicity. ImmunoRNases, made up of a human anti-ErbB2 scFv and human pancreatic ribonucleases (HP-RNases), have been engineered to overcome the limits of other immunotoxins, such as immunogenicity and nonspecific toxicity. Here, we report that a novel anti-ErbB2 immunoRNase, called Erb–HPDDADD-RNase, obtained by fusing Erbicin, a human ErbB2-directed scFv, with an HP-RNase variant that resists the cytosolic inhibitor protein, binds with high affinity to a panel of ErbB2-positive gastric tumor cells and inhibits their growth more than does the parental immunoRNase, which is not resistant to the inhibitor. Moreover, Erb–HP-DDADD-RNase is endowed with antiproliferative activity for trastuzumab-resistant cancer cells both *in vitro* and *in vivo* that is more potent than that of the parental immunoRNase. Importantly, Erb–HP-DDADD-RNase does not show cardiotoxic effects *in vitro* on human cardiomyocytes and does not impair cardiac function in a mouse model. Thus, Erb–HP-DDADD-RNase could fulfil the therapeutic need of cancer patients ineligible for trastuzumab treatment due to primary or acquired trastuzumab resistance or to cardiac dysfunction.

Keywords: breast cancer/cardiotoxicity/ErbB2/gastric cancer/trastuzumab-resistance

Introduction

An attractive target for cancer immunotherapy is ErbB2, a tyrosine kinase receptor that is overexpressed in several types of human cancer (Fukushige *et al.*, 1986; Slamon *et al.*, 1989; Tagliabue *et al.*, 1991). High levels of ErbB2 are associated with a more aggressive clinical behavior and poor prognosis for breast cancer patients (Baselga and Albanell, 2001). Trastuzumab (Herceptin®), the humanized antibody currently used to treat mammary carcinomas, improves the survival of both metastatic and early breast cancer patients (Stebbing *et al.*, 2000; Romond *et al.*, 2005). Nevertheless, large-scale clinical studies with trastuzumab have shown that up to 7 or 28% of patients suffer from cardiac dysfunction when trastuzumab is used either in monotherapy or in combination with anthracyclines, respectively (Sparano, 2001; Burris *et al.*, 2005; Perez *et al.*, 2006). The mechanism of trastuzumab-related cardiotoxicity is associated with its ability to inhibit the ErbB2/ErbB4 heterodimerization mediated by neuregulin (NRG-1), thereby interfering with the myocyte protective pathway triggered by ErbB2 (Fedele *et al.*, 2012b).

Some carcinomas with high levels of ErbB2, such as prostate, breast and gastric tumors, are insensitive or even resistant to trastuzumab (Agus *et al.*, 1999; Gong *et al.*, 2004). A phase III trial (ToGA) of trastuzumab for ErbB2-positive advanced and inoperable gastric cancer showed a survival benefit when trastuzumab was added to chemotherapy in ErbB2-overexpressing gastric cancer patients (Bang *et al.*, 2010), but a large fraction of treated patients did progress (Bang *et al.*, 2010; Okines *et al.*, 2010). These data suggest that trastuzumab resistance can become a problem during the treatment of gastric cancer, as was also observed for breast cancer (Nahta *et al.*, 2006). More recently, in preclinical models of ErbB2-overexpressing gastric cancer cells, some success derived from the combination of trastuzumab with pertuzumab, another humanized anti-ErbB2 antibody that was approved recently by the FDA for metastatic breast cancer therapy (Chung and Lam, 2013; Yamashita-Kashima *et al.*, 2013). However, pertuzumab was also found to induce cardiac dysfunction, although it was not possible to assess whether the rate is similar to that of trastuzumab (Lenihan *et al.*, 2012), and other issues regarding efficacy have emerged. Accordingly, we have focused on alternative ErbB2-directed treatments.

A novel human ErbB2-specific scFv (single-chain variable fragment), named Erbicin (De Lorenzo *et al.*, 2002), recognizes an epitope distinct from that of trastuzumab and pertuzumab (Troise *et al.*, 2011). Erbicin is internalized through receptor-mediated endocytosis and inhibits the tumor-cell proliferation. The efficacy of antibody-based therapies can be improved markedly by conjugating toxins or radionuclides (Reiter and Pastan, 1998; Zhang *et al.*, 2007; Sievers and Senter, 2013). Immunogenicity that arises from use of a murine antibody or a non-human toxin, together with non-specific toxicity (*e.g.* vascular leak syndrome or hepatotoxicity) can limit the therapeutic efficacy of antibody–drug conjugates (Weiner *et al.*, 1989; Schindler *et al.*, 2001). To overcome these limits, a new type of an 'immunoRNase' was developed in which human pancreatic ribonuclease (HP-RNase or RNase 1) acts as the toxin. The enzyme manifests its RNA-degrading activity only

upon antibody-mediated internalization into target cells (Rybak and Newton, 1999; De Lorenzo and D'Alessio, 2008, 2009).

A first-generation anti-ErbB2 immunoRNase (Erb–HP-RNase) was obtained by fusing Erbicin with HP-RNase. The fusion protein binds selectively and with high affinity to ErbB2-positive cells, and inhibits specifically their proliferation both *in vitro* and *in vivo* (De Lorenzo *et al.*, 2004; De Lorenzo and D'Alessio, 2009; Gelardi *et al.*, 2010). Furthermore, Erb–HP-RNase did not show cardiotoxic side effects either *in vitro* or *in vivo* (Riccio *et al.*, 2009; Fedele *et al.*, 2012a). Unexpectedly, however, Erb–HP-RNase was found to be sensitive to the neutralizing action of the RNase inhibitor (RI), a cytosolic protein with sub-femtomolar affinity for HP-RNase (Haigis *et al.*, 2003; Dickson *et al.*, 2005; Johnson *et al.*, 2007).

Despite this limitation, Erb–HP-RNase still exerts its activity within ErbB2-positive tumor cells probably because enough immunoRNase enters the cytosol to overwhelm endogenous RI and manifest RNA-degrading activity (De Lorenzo *et al.*, 2007).

To obtain a more potent cytotoxic agent, we engineered a second-generation immunoRNase, named Erb–HP-DDADD-RNase, by fusing Erbicin with a variant of HP-RNase that resists RI. In this variant, five residues at the RI–HP-RNase interface are substituted (R39D/N67D/N88A/G89D/R91D), reducing affinity for RI by 6×10^9-fold but retaining high RNA-degrading activity (Johnson *et al.*, 2007). Erb–HP-DDADD-RNase is fully resistant to RI inhibition and kills mammary ErbB2-positive tumor cells more efficiently than does Erb–HPRNase (Riccio *et al.*, 2013).

Here, we compare the efficacy of the first- and second-generation immunoRNases as chemotherapeutic agents *in vitro* and *in vivo*. In our analyses, we use a variety of relevant cell lines, including gastric tumor cells expressing low levels of ErbB2 (which are relatively insensitive to trastuzumab treatment) and breast cancer cells resistant to trastuzumab. We also describe the effect of the immunoRNases on cardiac cells *in vitro* and *in vivo* to reveal their potential for cancer patients who suffer from cardiac dysfunction and are thus ineligible for trastuzumab treatment.

Materials and methods

Cell cultures

Cell lines SKBR3 (human breast cancer), A431 (human epidermoid carcinoma), NCI-N87 (human gastric carcinoma from a liver metastasis), MKN-7 (human gastric carcinoma) and AGS (adenocarcinoma of a Caucasian female) were cultured in RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, UK). Cell line JIMT-1, which was established from a pleural metastasis of a 62-year-old breast cancer patient clinically resistant to trastuzumab, was grown in Dulbecco's modified Eagle's medium (Gibco, BRL). Media were supplemented with 10% (7.5% for JIMT-1) heat-inactivated fetal bovine serum, penicillin (100 UI ml^{-1}), streptomycin (100 μg ml^{-1}) and 2 mM glutamine (all from Gibco, BRL) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Antibodies

The following antibodies were used for ELISA and western blotting: horseradish peroxidise (HRP) conjugated anti-His mouse monoclonal antibody (Qiagen GmnH, Hilden, Germany), anti-ErbB2 rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA), anti-actin rabbit polyclonal antibody (Sigma–Aldrich, St Louis, MO, USA), affinity-isolated IgGs from a rabbit anti-HP-RNase antiserum (Igtech, Salerno, Italy), and HRP-conjugated anti-rabbit immunoglobulins from goat antiserum (Thermo Scientific, Rockford, IL, USA).

Binding assays

Binding assays were performed by cell ELISA, as described previously (De Lorenzo *et al.*, 2004). Values of SD (≤5%) were calculated on the basis of results obtained in three independent experiments.

Cell lysis and western blotting

Cells were harvested in a non-enzymatic dissociation solution (Sigma–Aldrich) and lysed as described previously (Fedele *et al.*, 2014). Western blotting analyses were performed by using anti-ErbB2 mAb and anti-actin Ab.

In vitro *cytotoxicity assays*

Cells were seeded in 96-well flat bottom plates, ErbB2-positive SKBR3 control cells at a density of 1.5×10^4/well, trastuzumab-resistant JIMT-1 and A431 cells at 5×10^3/well, NCI-N87 and AGS cells at 1×10^4/well, and MKN-7 cells at 2×10^4/well. To test the effects of the immunoRNases on cell growth, SKBR3, A431, NCI-N87, MKN-7 and AGS cells were incubated at 37°C for 72 h in culture medium in the absence or presence of increasing concentrations (25–100 nM) of the immunoRNase.

JIMT-1 cells were treated as described previously (Gelardi *et al.*, 2010). The SD (≤5%) was calculated on the basis of the results obtained in three independent experiments.

Cardiotoxicity tests were performed with human fetal cardiomyocytes (HFCs) as described previously (Fedele *et al.*, 2012a).

Cell survival values were obtained from at least three independent experiments, and values of SD (≤5%) were calculated on the basis of the results obtained from all the experiments.

Cellular internalization assays

ErbB2-positive SKBR3 cells and JIMT1 cells resistant to trastuzumab were seeded in 6-well flat-bottom plates at a density of 6×10^5/well and grown at 37°C for 24 h in culture medium. After the addition of an immunoRNase at a concentration of 200 nM, cells were incubated for 2 h at 37°C. Cells were then washed extensively with a stripping solution of 50 mM glycine buffer, pH 3.0, containing NaCl (1.0 M), harvested with dissociation solution and treated as previously described (Borriello *et al.*, 2011).

Internalized Erb–HP-RNase or Erb–HP-DDADD-RNase was detected by using appropriate dilutions of the anti-HP-RNase antibody, followed by an HRP-conjugated anti-rabbit antibody. The signal intensity of the reactive bands was measured with a phosphorimager (45–710; Bio-Rad, Hercules, CA, USA).

In vivo *antitumor and cardiotoxicity assays*

The antitumor activity of Erb–HP-DDADD-RNase *in vivo* was determined with Balb/cAnNCrlBR athymic (*nu/nu*) mice (Charles River Laboratories, Calco, Italy). Trastuzumab resistant JIMT-1 cells were suspended in sterile PBS and injected subcutaneously into mice at a density of 2×10^6 cells/mouse

(Day 0). On Day 30, when tumors were clearly detectable, Erb–HP-RNase or Erb–HP-DDADD-RNase was administered intraperitoneally five times at 72-h intervals to groups of 5 mice at doses of 1.2 mg kg^{-1} of body weight. Another group of control mice was treated with identical volumes of sterile PBS to follow tumor development. Tumor volume (V) was calculated by the formula of a rotational ellipsoid: $V = A \times B^2$, where A is the axial diameter and B is the rotational diameter as measured with a caliper.

Cardiac function *in vivo* was assessed by transthoracic echocardiography in sedated 7-week-old WT C57Bl/6 mice (Harlan Italy, San Piero al Natisone, UD, Italy) using a Vevo 2100 high-resolution imaging system (40-MHz transducer, VisualSonics, Toronto, ON, Canada). Cardiac function was evaluated by non-invasive echocardiography in basal conditions and after intraperitoneal treatment with five doses (1.2 mg kg^{-1} of body weight) of the novel immunoRNase.

Fractional shortening (FS) and radial strain (RS) were evaluated as described previously (Fedele *et al.*, 2012a); ejection fraction (%EF) was calculated with the formula: $\%EF = (VTD - VTS/VTD) \times 100$. Data were the mean $\pm$ SD.

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

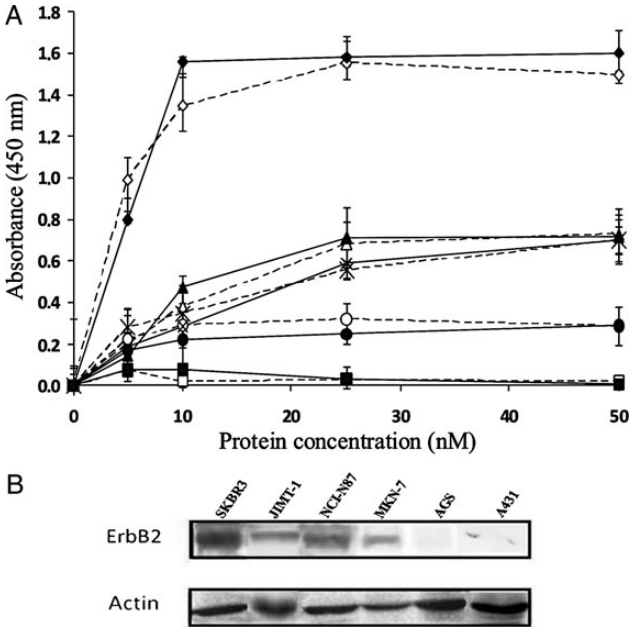

Fig. 1. Binding of immunoRNases to breast and gastric tumor cells. (**A**) Binding curves of Erb–HP-RNase (dashed lines) or Erb–HP-DDADD-RNase (black lines) to gastric NCI-N87 (triangles), MKN-7 (circles) and AGS (squares) cell lines, as well as to breast SKBR3 (rhomboids) and JIMT-1 (crosses) cell lines, which were positive controls. (**B**) Quantitative analysis by western blotting of ErbB2-receptor levels on gastric (NCI-N87, MKN-7 and AGS) cell lines and breast cancer (SKBR3 and JIMT-1) cell lines. The A431 cell line from human epidermoid carcinoma was a negative control.

Results

Binding assays of Erb–HP-DDADD-RNase to breast and gastric cell lines

The affinity of Erb–HP-DDADD-RNase for the ErbB2 receptor on gastric tumor cells was tested by cell ELISA assays, performed on NCI-N87, MKN7 and AGS cells. In a parallel assay, mammary SKBR3 or JIMT-1 cells, expressing high or moderate levels of ErbB2 receptor, respectively, were used as positive controls, and epidermoid A431 cells, expressing very low levels of ErbB2, as a negative control (data not shown).

The results, shown in Fig. 1A, indicate that Erb–HP-DDADD-RNase retains the specificity of the parental Erb–HP-RNase, binding with a similar high affinity to control SKBR3 and JIMT-1 cells. As for the gastric tumor cells, both immunoRNases bind to MKN7 and NCI-N87 with a high affinity, but do not recognize AGS cells.

To investigate whether the differential binding of the immuno RNases to the three gastric cell lines was due to a differential expression of ErbB2 in these cells, a quantitative analysis of their ErbB2 levels was performed on lysates by western blotting with a commercial anti-ErbB2 mAb. The positive bands were analyzed with a phosphorimager, and the corresponding signal intensities were normalized to those obtained in the same lysates by an anti-actin antibody.

As shown in Fig. 1B, ErbB2 is expressed in the tested gastric cell lines NCI-N87 and MKN7 at levels comparable to those found in JIMT-1 breast cancer cells, though lower with respect to those detected in SKBR3 cells. No significant signal corresponding to the receptor was detected in AGS tumor cells, suggesting that these cells express very low levels of ErbB2. These results validate a positive correlation between the ErbB2 levels on a cell and the affinity of an immunoRNase for that cell.

In vitro *cytotoxic effects of Erb–HP-DDADD-RNase on breast and gastric tumor cells*

The antitumor effects of the immunoRNase were tested on gastric tumor NCI-N87, MKN-7 and AGS cells in comparison with those observed on other ErbB2-positive tumor cells from different tissues, such as mammary SKBR3 (positive control), JIMT-1 cells resistant to trastuzumab and ErbB2-negative epidermoid A431 tumor cells (negative control; data not shown). After a 72-h incubation at 37°C, cell survival was measured by counting trypan blue-excluding cells, and cell survival was expressed as a percentage of viable cells in the presence of an immunoRNase with respect to control cells grown in the absence of the protein.

As shown in Fig. 2, Erb–HP-DDADD-RNase was found to inhibit the growth of the gastric tumor cells tested in a dose-dependent manner, showing a strong effect on the NCI-N87 cell line, even at low protein concentrations. This immunoRNase is toxic to MKN-7 cells, albeit at a higher protein concentration, and more so than the parental Erb–HP-RNase. Similar results were obtained for mammary JIMT-1 cells, which express ErbB2 levels comparable to those of NCIN87 and MKN-7 cells, but lower than those of SKBR3 cells, which were found to be equally sensitive to both immunoRNases. No cytotoxic effects were detected on AGS cells, as expected from the low affinity of both immunoRNases to these cells. Indeed, all of the cytotoxicity data indicate a correlation between the level of ErbB2 expression by a tumor cell and its vulnerability to an immunoRNase.

The different efficacy of the two immunoRNases is consistent with increased levels of ErbB2 receptor leading to increased internalization. Indeed, ErbB2-positive SKBR3 cells expressing high levels of ErbB2 receptor allow for the internalization of large amounts of the immunoRNases that exceed the quantity

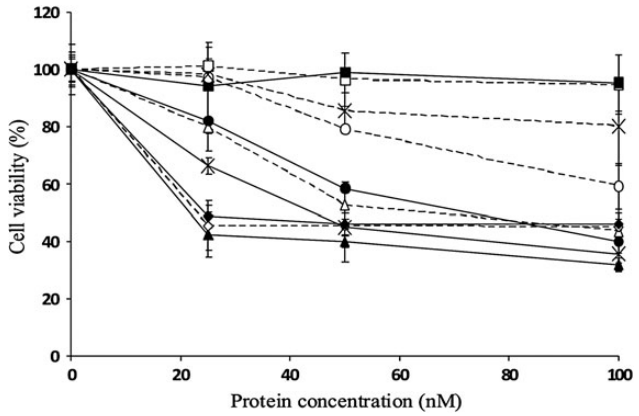

Fig. 2. Toxicity of immunoRNases for tumor cells *in vitro*. Dose–response curves for gastric NCI-N87 (triangles), MKN-7 (circles) and AGS (squares) cell lines, as well as to breast SKBR3 (rhomboids) and trastuzumab-resistant JIMT-1 (crosses) cell lines, treated for 72 h with Erb–HPRNase (dashed lines) or Erb–HP-DDADD-RNase (black lines).

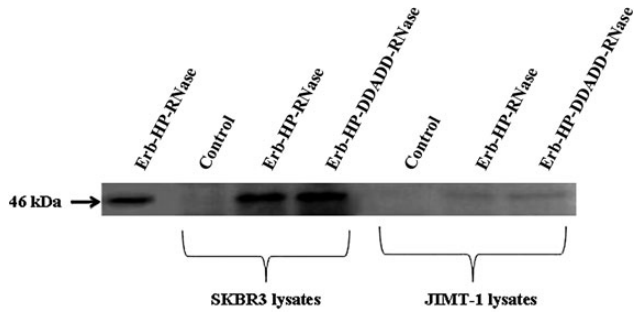

Fig. 3. Internalization of immunoRNases into tumor cells expressing different levels of ErbB2. Analysis by western blotting of lysates from SKBR3 cells (high ErbB2 expression) or JIMT-1 cells (low ErbB2 expression) untreated (control) or treated with Erb–HP-RNase or Erb–HPDDADD-RNase.

of endogeneous RI, leading to the inhibition of only a small fraction of each immunoRNase (De Lorenzo *et al.*, 2007). In contrast, lower levels of ErbB2 such as those detected in NCI-N87, JIMT-1 and MKN7 cells allow for less efficient internalization of the immunoRNases, leading to different behavior. In these lines, Erb–HPRNase is efficiently inhibited by RI and manifests poor cytotoxic activity, whereas the RI-resistant Erb–HP-DDADD-RNase can exert its activity fully.

To test this hypothesis, an internalization assay was performed by using SKBR3 cells expressing high levels of ErbB2 and JIMT-1 cells expressing low levels of the receptor, as described in the Materials and methods section. Briefly, cells were incubated for 2 h at 37°C with Erb–HP-DDADD-RNase or the parental Erb–HP-RNase, washed with low pH stripping buffer to remove the protein bound to the cell surface, lysed and analyzed by western blotting using a rabbit anti-HP-RNase antibody, followed by a HRP-conjugated anti-rabbit antibody. The results are shown in Fig. 3. The presence of a reactive band corresponding to the expected molecular weight (46 kDa) in both the lysates shows that Erb–HP-DDADD-RNase is internalized by ErbB2-positive cells, and its levels are comparable to those of the native immunoRNase. Still, as expected, the levels of both internalized immunoRNases in SKBR3 cells are higher than those detected in JIMT-1 cells. This result is in line with the hypothesis that higher levels of ErbB2 in SKBR3 cells allow for the internalization of the immunoRNases at higher levels than those detected in resistant JIMT-1 cells expressing lower levels of ErbB2.

In vivo *antitumor activity of Erb–HP-DDADD-RNase*

To evaluate the therapeutic effects of Erb–HP-DDADD-RNase, we used ErbB2-positive JIMT-1 cells, clinically resistant to trastuzumab, for studies *in vivo*. As shown in Fig. 4, Erb–HPDDADD-RNase reduces tumor volume substantially, whereas Erb–HP-RNase has a lesser effect. During treatment, the mice did not show signs of wasting or other visible signs of toxicity. Thus, we conclude that Erb–HP-DDADD-RNase exerts specific efficacy *in vivo* that is stronger than that of the parental immunoRNase, consistent with its resistance to RI.

In vitro *effects of Erb–HP-DDADD-RNase on human cardiac cells*

Anti-ErbB2 antibodies such as trastuzumab have been found to be cardiotoxic in a high percentage of cases. To evaluate the potential cardiotoxic effects of Erb–HP-DDADD-RNase, an analysis was carried out on human fetal cardiomyocytes (CFH) *in vitro*. Cells were incubated at 37°C for 72 h in the absence or presence of increasing concentrations (50–100 nM) of an immunoRNase, and then analyzed by MTT assay to evaluate cell viability. Doxorubicin, at a concentration of 0.1 and 0.25 μM, was used as a positive control. As shown in Fig. 5A, Erb–HP-RNase and Erb–HP-DDADD-RNase do not have cardiotoxic effects, whereas doxorubicin was toxic for cardiac cells, as expected.

In vivo *cardiotoxic effects of Erb–HP-DDADD-RNase*

The cardiotoxic effects of Erb–HP-DDADD-RNase were also tested with a mouse model. Echocardiography measurements were performed on mouse hearts before and after treatment.

Erb–HP-DDADD-RNase did not alter LVEDD and LVESD dimensions. The FS and the EF, which can signal myocardial failure, were not affected (Fig. 5B and D). This result is in line with results obtained previously for the

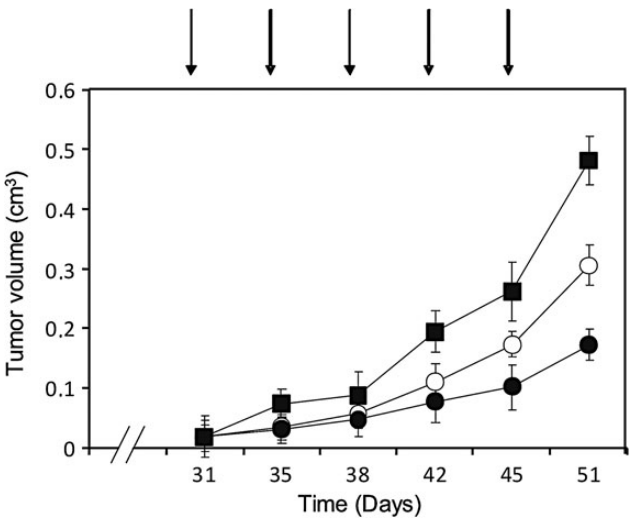

Fig. 4. *In vivo* effects of Erb–HP-DDADD-RNase or Erb–HP-RNase on trastuzumab-resistant JIMT-1 tumors induced in mice. Treated mice ($n = 5$) were injected with Erb–HP-RNase (empty circles) or Erb–HP-DDADD-RNase (black circles) at five doses (arrows) of 1.2 mg kg^{-1} of body weight. Control mice ($n = 5$) were treated with sterile PBS (squares).

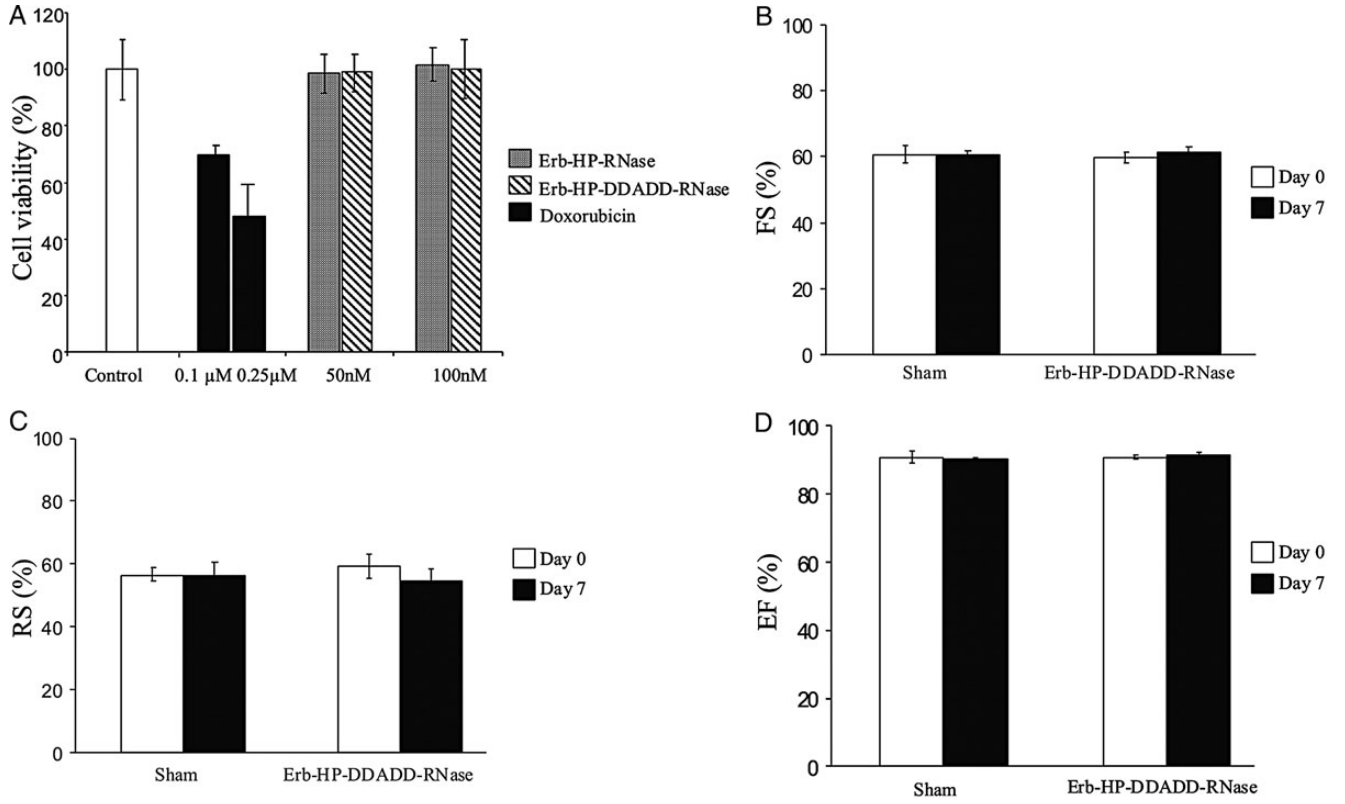

Fig. 5. Cardiotoxic effects of Erb–HP-DDADD-RNase *in vitro* and *in vivo*. (**A**) Effect of Erb–HP-DDADD-RNase on the viability of human fetal cardiomyocytes (HFC) *in vitro*. Cells were treated for 72 h with an immunoRNase at concentrations of 50 and 100 nM, treated with doxorubicin at concentrations of 0.1 and 0.25 μM or untreated (negative control). (**B–D**) Effects of Erb–HP-DDADD-RNase on heart function *in vivo*. Relative FS, RS and EF are reported before and after the treatment of mice ($n = 5$) with Erb–HP-DDADD-RNase (5×1.2 mg kg^{-1} of body weight over 7 days).

parental immunoRNase, which (unlike trastuzumab and doxorubicin) did not show cardiotoxic effects (Riccio *et al.*, 2009).

To validate this finding, we evaluated as an index of myocardial deformation, the RS, which is an early predictor of the future onset of cardiac dysfunction. As shown in Fig. 5C, RS was not reduced by the treatment with Erb–HP-DDADD-RNase compared with sham, confirming that this immuno RNase, unlike trastuzumab (Riccio *et al.*, 2009), does not impair cardiac function.

Discussion

The ErbB2 receptor is an attractive target for cancer immunotherapy because of its high expression levels on tumor cells of various origins, such as human breast and gastric cancer (Fukushige *et al.*, 1986; Slamon *et al.*, 1989; Tagliabue *et al.*, 1991). The humanized anti-ErbB2 antibody trastuzumab is used to treat these types of tumor with success, though many breast and gastric cancer patients are resistant to trastuzumab treatment and manifest side effects, such as cardiac dysfunction (Agus *et al.*, 1999; Sparano, 2001; Gong *et al.*, 2004; Burris *et al.*, 2005; Perez *et al.*, 2006). Pertuzumab, another humanized anti-ErbB2 antibody recently approved by the FDA for metastatic breast cancer therapy, has likewise been found to induce cardiac dysfunction, although it was not possible to assess whether the rate is similar to that of trastuzumab (Lenihan *et al.*, 2012), and other issues regarding the efficacy emerged.

We have characterized Erb–HP-DDADD-RNase, a fully human, inhibitor-resistant immunoRNase which targets the ErbB2 receptor. This immunoRNase was obtained by fusing a variant of human pancreatic RNase (HP-DDADD-RNase) with Erbicin, an anti-ErbB2 scFv that recognizes an epitope different from that of trastuzumab and pertuzumab (Troise *et al.*, 2011). On the basis of data *in vitro* and *in vivo*, we conclude that the novel immunoRNase retains the biological action of its component moieties, binding to a panel of ErbB2-positive gastric cells expressing different levels of ErbB2 with an affinity comparable to that of the previously reported parental immunoRNase, Erb–HP-RNase, and inhibiting the *in vitro* growth of gastric cancer cells more efficiently due to its resistance to RI.

The differential efficacy of the two immunoRNases is highlighted with cell lines expressing low levels of ErbB2 receptor. Less efficient internalization of the immunoRNases into the cytosol does not affect the cytotoxicity of the novel immuno RNase Erb–HP-DDADD-RNase, but leads to full inhibition of the parental Erb–HP-RNase. In addition, Erb–HP-DDADD-RNase inhibits the growth of trastuzumab-resistant breast cancer cells *in vitro* and *in vivo* with potency greater than that of the parental immunoRNase. Finally, in line with results previously obtained for the parental immunoRNase (Riccio *et al.*, 2009), Erb–HP-DDADD-RNase does not show cardiotoxic effects either *in vitro* or *in vivo*. Thus, Erb–HP-DDADD-RNase could be a promising candidate for the many patients ineligible for trastuzumab treatment due or to primary or acquired trastuzumab resistance or to cardiac dysfunction, as this immunoRNase manifests high antiproliferative activity without cardiotoxicity.

Conflict of interest: None declared.

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