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A new RNase-based immunoconjugate selectively cytotoxic for ErbB2-overexpressing cells

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Abstract We report a new tumor-directed immunoRNase, a chimeric protein made up of an antibody fragment (single-chain Fv fragment) directed to ErbB2, a cell surface receptor, and a non-toxic, human ribonuclease, which upon cell internalization becomes cytotoxic. The immunoRNase is active as a ribonuclease, specifically binds and selectively kills ErbB2-positive cells. ErbB2 is one of the most specific tumor-associated antigens identified so far, overexpressed on tumor cells of different origin. Its choice as target antigen and that of a non-toxic, human RNase as the killer moiety makes this immunoRNase a new, potentially attractive anticancer agent.

Key words: Immunoconjugate; RNase; ErbB2; Antitumor; Immunotherapy

1. Introduction

Conventional anticancer treatments, such as radiation and systemic drugs, are characterized by the lack of tumor cell specificity. An alternative strategy is that based on immunotoxins, fusion molecules designed to kill only target cells. Immunotoxins have been shown to kill cancer cells with IC_{50} (the concentration for 50% cytotoxicity) in the 0.01–1 nM range [1,2]. However, problems have been encountered in clinical trials, especially for the toxicity and immunogenicity of the powerful bacterial or plant cytotoxins used for constructing immunotoxins [3,4].

A more recent strategy is that based on immunoconjugates, or 'immunoRNases' (IR), in which the toxin is replaced by an RNase molecule. The key to the IR strategy is that RNases, although per se not cytotoxic, become cytotoxic when they are internalized by the target cell, i.e. the cell that displays on its surface the epitope recognized by the antibody moiety [3]. This is why the most suitable epitopes for the selection of an antibody for IR are cell receptors, since the anti-receptor antibody can mimic the receptor ligand and be internalized. The first IR has been prepared with bovine pancreatic RNase A [5], fused to an anti-transferrin receptor antibody, and shown to be cytotoxic to tumor cells expressing the transferrin receptor in the 200–500 nM range.

The immunocompatibility of IRs as anticancer agents can be greatly improved by the use of human RNases, as they are physiologically present in extracellular fluids and tissues, and are expected to be much less immunogenic than xenogeneic proteins, if not immunogenic at all. On the other hand, in an immunotoxin or an IR antibodies can be replaced by single-chain variable fragments (scFv), which can easily penetrate solid tumors, fully preserving the specificity of the parental antibody. IRs, made up of human RNases fused to an anti-transferrin receptor scFv, have been prepared (see [3] for a review, and references therein). These IRs were cytotoxic to tumor cells with IC_{50} in the 5–20 nM range. However, the abundance of the transferrin receptor at the blood–brain barrier, which can result in the uptake of high amounts of these immunoconjugates by the brain, sets a limit to the use of these reagents as therapeutic drugs [6].

An attractive target for IR-based, directed tumor therapy is the ErbB2 transmembrane receptor. A member of the ErbB superfamily of growth factor tyrosine kinase receptors [7], ErbB2 is highly expressed on several tumor cells, especially in breast, ovary and lung carcinomas [8,9], and is implicated in the development of malignancy [10]. Conversely, in normal tissues it is expressed at low levels, and only in certain epithelial cell types [11]. Furthermore, ErbB2 is internalized upon ligand binding, an event which can be mimicked by an antibody directed towards the receptor, thus it is an appropriate target for delivering RNases into ErbB2-overexpressing tumor cells. Herceptin [12,13], an anti-ErbB2 monoclonal antibody, has proved to be an effective anticancer drug in clinical anticancer therapy.

In this report we describe the construction and characterization of a new, anti-ErbB2 IR, named ERB-HPR, constructed by fusing human pancreas RNase (HP-RNase) to an anti-ErbB2 scFv. We found that HP-RNase, by itself not cytotoxic, when fused to the anti-ErbB2 scFv acquires a selective cytotoxic activity towards antigen-bearing cell lines.

2. Materials and methods

2.1. Construction, expression and purification of ERB-HPR

The cDNA encoding the anti-ErbB2 scFv [14] was cloned in a pRSET-a vector (Invitrogen), which includes sequences encoding six histidines and the Xpress peptide, both positioned upstream of the scFv cDNA. The recombinant vector was linearized with *Eco*RI; protruding ends were then filled in by T4 bacteriophage DNA poly-

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Abbreviations: scFv, single-chain Fv fragment; HP-RNase, human pancreas RNase; IR, immunoRNase, or RNase-based immunoconjugate; ERB-HPR, immunoRNase made up of an anti-ErbB2 scFv fused to HP-RNase

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merase. By digestion with *Hin*dIII a linear blunt-ended/*Hin*dIII recombinant vector was generated.

The cDNA encoding HP-RNase [15], cloned in the *Nde*I/*Hin*dIII restriction sites of a pET 22b+ vector, was digested with *Nde*I and treated with the T4 bacteriophage DNA polymerase to create blunt ends; the linearized DNA was then digested with *Hin*dIII to generate a blunt-ended/*Hin*dIII fragment. This was inserted into the linearized vector downstream of the cDNA encoding the anti-ErbB2 scFv fragment described above. In the resulting chimeric DNA, a sequence encoding a spacer (GSPEFM) is interposed between the scFv and RNase moieties. The recombinant plasmid was used to transform *Escherichia coli* B834(DE3)pLysS (Novagen) competent cells.

Freshly transformed cells, grown in LB medium containing 0.5% glucose, 50 μg/ml ampicillin and 30 μg/ml chloramphenicol to an OD_{600} of 1.0, were centrifuged at 6000 rpm for 15 min and suspended in glucose-free medium to allow the expression of the fusion protein, induced with 0.4 mM IPTG for 16 h. Cells were harvested by centrifugation at 6000 rpm for 15 min at 4°C. The cell lysate was obtained according to the Xpress Purification System protocol (Invitrogen), and loaded on a ProBond™ resin (Invitrogen) to purify the recombinant protein via binding of the His tag to Ni^{2+} ions. Bound proteins, eluted following the manufacturer's instructions, were reduced with 0.1 M glutathione in the elution buffer, previously adjusted to pH 8.4. After 2.5 h at room temperature, the proteins were diluted with 20 volumes of 0.1 M Tris–acetate, at pH 8.4, containing 0.5 M L-arginine and 1 mM oxidized glutathione, and allowed to reoxidize for 24 h at room temperature. Proteins were then concentrated and dialyzed against 50 mM Tris–HCl, at pH 8.0, containing 0.25 M NaCl, 10% glycerol and 0.005% Tween 20.

Further purification was achieved by affinity chromatography using uridine 2′,5′- and 3′,5′-diphosphate agarose (pUp agarose, Sigma). Briefly, the sample was adjusted to pH 5.8 and loaded on the column, previously equilibrated in 0.1 M sodium acetate buffer at pH 5.8, containing 0.15 M NaCl, 10% glycerol and 0.005% Tween 20. After extensive washing with the equilibrium buffer, the protein was eluted in phosphate-buffered saline (PBS) containing 0.5 M NaCl, 10% glycerol and 0.005% Tween 20. The purified protein was analyzed by SDS–PAGE and quantitated by BCA assays (Pierce).

2.2. RNase activity assays

RNase activity was tested as previously described [16] on yeast RNA (Sigma) (8 mg/ml). Zymograms were performed as described in [17].

2.3. ELISA assays

SKBR3 and A431 cells (kindly provided by Menarini Research, Italy), grown in RPMI 1640 with 10% fetal calf serum on 96-well plates, were fixed with 2% formaldehyde in PBS (3.3×10^4 cells/well). After treatment with 3% bovine serum albumin (Sigma) in PBS, serial dilutions of the IR (in the concentration range 10–500 nM) were added in a final volume of 100 μl of PBS containing 3% bovine serum albumin, and the plates were incubated for 1 h at 37°C. Specifically bound ERB-HPR was detected after incubation either with a murine anti-Xpress peptide monoclonal antibody (mAb; Invitrogen), or with a rabbit anti-HP-RNase antibody (Igtech), followed by goat alkaline phosphatase-conjugated anti-mouse IgG, or by anti-rabbit IgG (both from Sigma), according to the source of the primary antibody. The phosphatase reaction product was measured as absorbance at 405 nm, and the values are expressed as the mean of at least three determinations (S.D.≤5%).

2.4. Western blot analyses

To prepare cell lysates, about 6×10^5 cells, detached from the plates with the Sigma cell dissociation solution, were washed three times in PBS and suspended in 0.3 ml of lysis buffer made up of 10 mM Tris–HCl, pH 7.4, 150 mM NaCl and 0.5% Nonidet P-40 containing Complete™ protease inhibitors (Boehringer Mannheim). After 20 min at 0°C, the extracts were clarified by centrifugation at 12 000 rpm for 15 min. Protein concentration was determined by a colorimetric assay (Bradford, Sigma), and aliquots of 20 μg were run on 7.5% SDS–PAGE, followed by electroblotting onto polyvinylidene difluoride membranes (Millipore). The ErbB2 protein was detected using the anti-ErbB2 MgR6 mAb [18], followed by rabbit anti-mouse HRP-conjugated IgG (NEN). The signal intensity of reactive bands was quantitatively measured with a phosphorimager (GS-710, Bio-Rad). The data were normalized to those obtained for the same extracts using an anti-actin mAb.

2.5. Cytotoxicity assays

SKBR3 and MDA-MB453 cells (a gift of H.C. Hurst, ICRF, London, UK) were seeded in 96-well plates at a density of 1.5×10^4/well in 150 μl; A431, at a density of 5×10^3/well. Proteins under test were added, and after 72 h cell counts were determined in triplicate using the trypan blue exclusion test. In parallel experiments, cells were pulsed for 3 h with [^{3}H]leucine (Amersham Pharmacia Biotech) prior to harvesting, and the incorporated radioactivity was measured. Cell survival is expressed as percent viable cells in the presence of the protein under test relative to control cultures grown in the absence of the protein. Typically, standard deviations were below 5%.

3. Results and discussion

3.1. Construction and purification of ERB-HPR

The cDNA encoding an anti-ErbB2 scFv fragment [14], obtained from mAb MgR6 [18], was fused to the 5′-end of the cDNA encoding HP-RNase [15]. A scheme of the protein chimera is shown in Fig. 1. At its N-terminus it contains a short peptide (Xpress peptide, Invitrogen) linked downstream to an affinity tag of six histidine residues. Between V_H and V_L chains of the scFv a 15-residue linker, made up of glycine and serine $(G_4S)_3$, is interposed, and a six-residue spacer is inserted between the antibody fragment and the ribonuclease to minimize hindrance between the two moieties of the chimeric protein. The scFv was linked to the N-terminus of HP-RNase as this is more solvent-accessible than the C-terminus [19].

The chimeric cDNA was fully sequenced, cloned in a T7 promoter-based *E. coli* expression vector (pRSET-a), and expressed at high levels as an insoluble protein sequestered in inclusion bodies. The inclusion bodies were denatured and the recombinant fusion protein was purified under denaturing conditions on an immobilized-metal affinity chromatography by using a nickel-chelating resin. After refolding, carried out in the presence of a glutathione redox buffer, RNase activity was recovered in suitable amounts (170 U/ml of culture medium).

The IR, named ERB-HPR, was further purified by a second affinity chromatography using pUp agarose (see Section 2). Analysis by SDS–PAGE indicated that the recombinant protein was homogeneous (see Fig. 2). The yield was approximately 5 mg/l culture.

3.2. Characterization of ERB-HPR

The purified fusion protein was analyzed by Western blotting with either an anti-Xpress peptide or an anti-HP-RNase antibody. By both analyses a band of the expected size (approximately 46 kDa) was visualized (Fig. 2).

By a zymogram assay (see Fig. 2), and a standard RNase assay in solution, ERB-HPR was found to be active, with a specific activity of 800 U/nmol, i.e. more than 70% of the activity of the original RNase molecule. These results, when compared to previous reports on RNase activity of other IRs [3,20], appear to be highly satisfactory.

The ability of ERB-HPR to specifically recognize ErbB2 was measured by ELISA assays using SKBR3 cells from human breast cancer, which overexpress ErbB2, and A431 cells from human epidermoid carcinoma, which express the receptor at very low levels. The results of these experiments (see Fig. 3) indicate that the IR effectively binds SKBR3 cells,

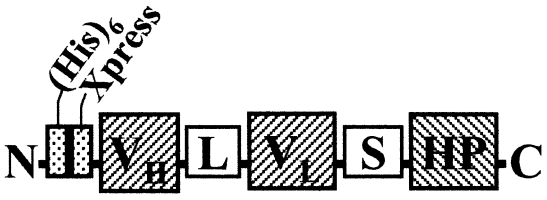

Fig. 1. Schematic representation of the IR ERB-HPR. $(His)_6$ is the six-residue His tag; Xpress is the peptide recognized by the anti-Xpress mAb; V_H and V_L, the variable domains of the heavy and light chains, respectively, of the anti-ErbB2 scFv; L, the 15-residue linker $(G_4S)_3$; S, the six-residue spacer (GSPEFM); HP, HP-RNase.

whereas it binds poorly to A431 cells. The apparent binding affinity of ERB-HPR for the ErbB2 receptor, i.e. the concentration corresponding to half-maximal saturation, was found to be 15 nM. In parallel experiments, this value was found to be comparable to that measured per single antigen binding site on the parental MgR6 mAb (see Fig. 3).

It is not easy to explain how monovalent ERB-HPR and bivalent MgR6 have comparable binding affinities. Besides the possibility that the anti-ErbB2 scFv has an intrinsic high affinity for the antigen, we cannot exclude that the presence of the RNase in the chimeric molecule induces further stabilizing interactions between the scFv and the antigen.

By size exclusion fast liquid chromatography on a Superdex 200 column, ERB-HPR was found to be stable as a monomer (data not shown), with a relative molecular mass of approximately 46 kDa, in line with the value obtained by SDS–PAGE (see above).

3.3. Cytotoxic effects on tumor cells

The finding that ERB-HPR is endowed with both RNase activity and antigen binding specificity led us to investigate whether it could also inhibit the growth of cells overexpressing ErbB2. ERB-HPR was tested for its effects on antigen-positive (SKBR3 and MDA-MB453 cell lines, both derived from breast carcinomas) and antigen-negative cell lines (A431 cells). Cells were plated in the absence or in the presence of increasing concentrations of ERB-HPR, and incubated for 72 h. Cell survival was measured either by counting trypan blue-excluding cells, or by measuring protein synthesis. In parallel experiments, the effects on cell survival of recombinant wild-type HP-RNase and of the parental anti-ErbB2 mAb MgR6 were also tested.

As shown in Fig. 4A, ERB-HPR was found by trypan blue exclusion test to be selectively cytotoxic for ErbB2-overexpressing cells, and in a dose-dependent manner. The IC_{50} values were found to be 50 and 120 nM for SKBR3 and MDA-MB453 cells, respectively. No effects on the growth of A431 cells were detected (see Fig. 4A). Moreover, no effects on cell survival were detected when either free HP-RNase (see Fig. 4A) or the parental MgR6 mAb (see Fig. 5) was tested on SKBR3 cells. These findings indicate that the IR ERB-HPR is a powerful cytotoxic agent, selective for ErbB2-bearing cells. When the experiments were repeated by measuring protein synthesis of treated versus untreated cells, identical results were obtained (data not shown).

To investigate the higher cytotoxicity of ERB-HPR on the SKBR3 cell line, compared to that on MDA-MB453 cells, and the lack of cytotoxicity on A431 cells, a quantitative analysis by Western blotting was performed on lysates from the three cell types, tested for their ErbB2 levels with the parental 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. The results of these experiments (Fig. 4B) show that the level of ErbB2 determined in MDA-MB453 cells is about six-fold lower than that measured in SKBR3 cells. No positive band corresponding to ErbB2 was detectable instead in A431 cells (see Fig. 4B). These results indicate that there is a strict

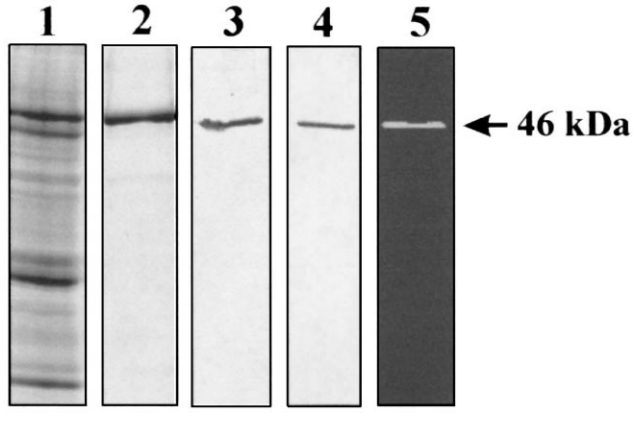

Fig. 2. Analyses by SDS–PAGE of crude and purified ERB-HPR. Lane 1, cell extract from inclusion bodies; lane 2, ERB-HPR (10 μg) eluted from the affinity chromatography on pUp agarose; lanes 3 and 4, Western blot analyses of ERB-HPR (100 ng), using the anti-Xpress mAb (lane 3), or the anti-HP-RNase IgGs (lane 4); lane 5, a zymogram of ERB-HPR (120 ng), using yeast RNA as a substrate.

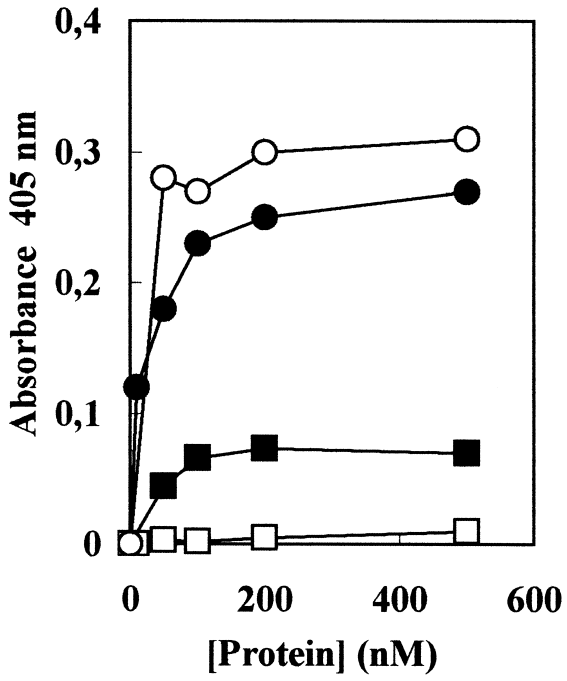

Fig. 3. Binding curves of ERB-HPR to ErbB2-positive (SKBR3) and -negative (A431) cell lines. SKBR3 cells were tested by ELISA assays with ERB-HPR (black circles), or with the anti-ErbB2 MgR6 mAb (white circles), as a control; A431 cells were tested with ERB-HPR or MgR6 mAb (black and white squares, respectively).

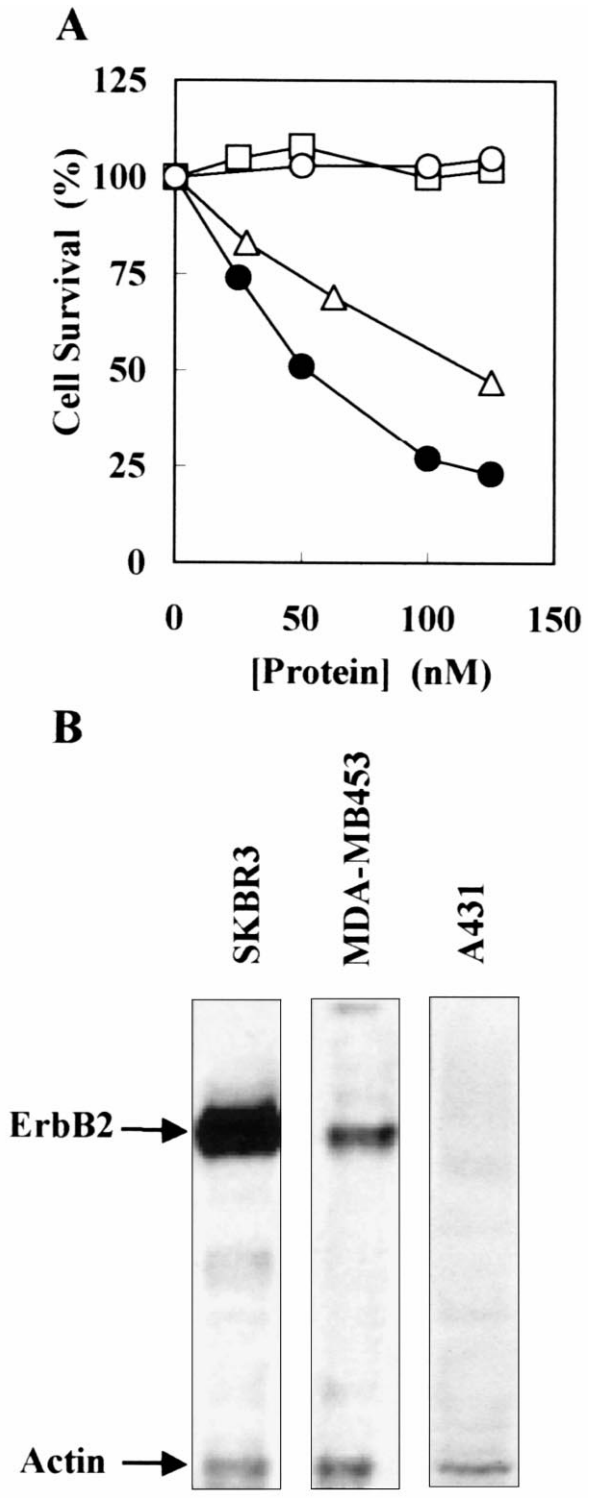

Fig. 4. Effects of ERB-HPR on cell survival. A: Dose–response curves determined for SKBR3 (black circles), MDA-MB453 (triangles), and A431 cell lines (squares), upon treatment for 72 h with ERB-HPR. SKBR3 cells were also tested with native HP-RNase (white circles). B: Western blot analyses of the expression levels of ErbB2 in the indicated cell lines, determined using the anti-ErbB2 MgR6 mAb. To normalize the band signals, an anti-actin mAb was used in the same experiments.

correlation between levels of expression of ErbB2 on a cell surface and sensitivity of that cell to ERB-HPR.

The specificity of ERB-HPR for target cells was confirmed when its cytotoxic activity was determined on SKBR3 cells in the presence of the parental anti-ErbB2 MgR6 mAb. As shown in Fig. 5, MgR6 was found to reverse the inhibitory effect of ERB-HPR on SKBR3 cell growth. When the molar excess of MgR6 (290 nM) over ERB-HPR (150 nM) was about two-fold, more than 50% of ERB-HPR cytotoxicity was lost. When a four-fold molar excess was reached, by lowering the ERB-HPR concentration to 75 nM, the toxicity of the IR was found to be fully suppressed (see Fig. 5). These results demonstrate that ERB-HPR and the parental anti-ErbB2 mAb compete for the same site on SKBR3 cells, and confirm that the cytotoxic action of the IR depends on its specific recognition of ErbB2.

These results also indicate that ERB-HPR interacts with the antigen and is internalized even in its monovalent form. This, however, may not be surprising, since it has been reported that the parental mAb is effectively internalized by ErbB2-overexpressing cells [18]. Furthermore, Poul and colleagues have recently isolated a panel of monovalent scFv that can be internalized, including scFv specific for ErbB2 [21].

To our knowledge, ERB-HPR is the first IR directed to ErbB2 to be reported to date. Based on the effectiveness and selectivity of its cytotoxic action on target cells, it represents a potentially valuable tool in cancer immunotherapy.

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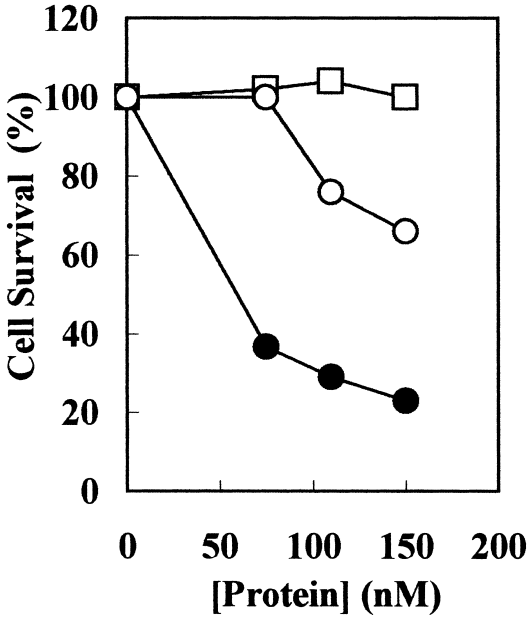

Fig. 5. Dose–response curves of SKBR3 cells treated with ERB-HPR in the absence (black circles) or in the presence of 290 nM anti-ErbB2 MgR6 mAb (white circles). The dose–response curve obtained with the anti-ErbB2 MgR6 mAb alone (squares) is also shown.

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