

Human anti-ErbB2 immunoagents – immunoRNases and compact antibodies

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Immunotherapy, based on mAbs specifically directed against cancer cells, is considered a precious strategy in the fight against cancer because of its selectivity and lack of multidrug resistant effects. However, there are obstacles to the complete success of current immunotherapy such as immune responses to nonhuman or even humanized antibodies and the large size of the antibodies, which hinders their diffusion into bulky tumors. Fully human, small immunoagents, capable of inhibiting tumor growth may overcome these problems and provide safe, highly selective and effective antitumor drugs. An attractive target for immunotherapy is ErbB2, a transmembrane tyrosine kinase receptor, overexpressed on tumor cells of different origin, with a key role in the development of malignancy. An anti-ErbB2 humanized monoclonal (Herceptin[®]) is currently used with success for breast cancer therapy; however, it can engender cardiotoxicity and a high proportion of breast cancer patients are resistant to Herceptin[®] treatment. Anti-ErbB2 immunoagents of human origin, with potentially no or very low immunogenicity have been engineered to assemble 'compact', i.e. reduced size, antibodies, one consisting of a human single-chain antibody fragment (scFv) fused to a human RNase to construct an immunoRNase and the other made up of two human scFv molecules fused to the Fc region of a human IgG1. By choosing a human antibody fragment as the immune moiety and a human RNase as the effector moiety, an immunoRNase would be both nonimmunogenic and nontoxic, as it becomes toxic only when the scFv promotes its internalization by target cells. The alternative strategy of compact antibodies was aimed at producing therapeutic agents with an increased half-life, prolonged tumor retention and the ability to recruit host effector functions. Moreover, the bivalency of compact antibodies can be exploited to construct bispecific antibodies, as well as for other therapeutic applications.

In recent years, significant advances in antitumor therapy have been achieved. However, the lack of selectivity for tumor cells seen in both antitumor drugs and radiotherapeutic protocols, and the frequent occurrence of the multidrug resistant phenotype upon treatment with antineoplastic agents,

necessitate the search for novel anticancer therapies. Among the newly acquired tools in the anticancer arsenal, immunotherapy represents a sound and effective strategy in the fight against cancer, based mainly on mAbs specifically directed against selected cancer cells [1,2].

Abbreviations

ADCC, antibody-dependent cell-mediated cytotoxicity; cAb, compact antibody; ErbB2-ECD, extracellular domain of ErbB2 receptor; Erb-hcAb, human compact antibody against the ErbB2 receptor; Erb-hRNase, human anti-ErbB2 immunoRNase with erbinin fused to human pancreatic-RNase; IL, interleukin; IR, immunoRNase(s); NK, natural killer cells; scFv, single-chain antibody fragment; TAA, tumor-associated antigen.

Clear progress in immunotherapy has come with the use of phage display technology [3–5], a powerful technology, which allows the isolation of a variety of human single-chain variable fragments (scFv) [6–8] directed towards different tumor-associated antigens (TAA). Of these, ErbB2, is a good candidate for a tumor target, as it is a transmembrane tyrosine kinase receptor highly expressed on breast, ovary and lung carcinomas [9,10], as well as in salivary glands and gastric tumor-derived cell lines [11,12], with a key role in the development of malignancy [13]. Because of its preferential expression in tumor cells [14] and its extracellular accessibility, the ErbB2 transmembrane tyrosine kinase receptor is an attractive target for immunotherapy.

Furthermore, activated ErbB2 receptor is readily internalized, an event which can be mimicked by an antibody directed towards the receptor. Thus, an anti-ErbB2 immunoagent can deliver a toxic payload into ErbB2 overexpressing tumor cells.

Human scFvs specific for ErbB2 have been produced using its isolated recombinant extracellular domain [15,16] and subsequently breast tumor cells [17]. Given their high affinity for the receptor, these immunoagents may be considered precious tools as delivery vehicles for specifically directing cytotoxic agents towards antigen-bearing tumor cells. However, none of these has exhibited antitumor activity.

A novel human anti-ErbB2 single-chain variable fragment was found to have biological properties [18] not described for other previously isolated anti-ErbB2 scFvs. For its isolation, the Griffin.1 phage library [3] and an innovative selection strategy, performed on live cells, were used. This novel anti-ErbB2 scFv, Erbicin, specifically binds to ErbB2-positive cells with high affinity and is internalized upon specific antigen recognition by ErbB2-expressing target cells; it strongly inhibits receptor autophosphorylation and displays strong inhibitory activity on the growth of ErbB2-positive cell lines. In addition, a clear cytotoxic effect was evidenced toward ErbB2-hyperexpressing SKBR3 cells in which apoptotic death was induced [18].

Therefore, Erbicin represents an ideal immunobullet for ErbB2-positive cancer cells. Also, because of its capacity to be effectively internalized by target cells, Erbicin should provide a useful vehicle for delivering drugs or toxins into tumor target cells.

ImmunoRNases

ImmunoRNases (IRs) as anticancer immunoagents are variations on the theme of immunotoxins [19]. The latter are fusion proteins made up of an antibody fragment fused to a toxin, whereas in IRs the toxin is

replaced by an RNase. In either case, the immune moiety targets the fusion protein to an antigen on the surface of a cancer cell, a TAA, so that the antibody fragment is internalized and the RNase is tethered inside the cell. When it reaches the cytosol, the RNase can exert its RNA-degrading activity on RNA(s), seriously damaging the protein biosynthetic machinery through cell death.

Immunotoxins have had limited success in therapy, particularly because of their large size, which obstructs facilitated penetration into solid tumors, and the immune response to the toxins, which are generally of bacterial or plant origin. By contrast, RNases are small, stable proteins of mammalian and possibly human origin (see below).

The potential of IRs was first understood by Rybak, Youle and co-workers in the early 1990s [20,21]. In these first IRs the RNase was RNase A; bovine pancreatic RNase and full monoclonals were used in the fusions, predominantly directed towards the transferrin receptor. The transferrin receptor is an expedient tumor-associated cell target because of its higher expression on cell surfaces, but is inconvenient because of the propensity of the targeting immunoagent to cross the blood–brain barrier. Fusion was obtained through classical protein chemistry.

Later, scFv, often humanized to reduce the immune response, and fused to the RNases via genetic engineering were used [22–25]. Human enzymes, including angiogenin [26] or EDN (an eosinophile-derived RNase) were used as RNases [27] to minimize the immune response, alternatively onconase, an RNase endowed with antitumor activity, was used [28].

More recently, lymphocytes markers, such as CD22 and CD30, have been selected as TAAs so that the IR obtained could be aimed at hematologic pathologies [29,30]. Some of these CD-directed IRs had a dimeric structure [31,32], because dimeric IRs have been found to be consistently more active than monovalent IR [33].

Human immunoRNases

The availability of both Erbicin, a human scFv (see above) directed towards the ErbB2 receptor [18], and human pancreatic RNase (RNase 1 or HP-RNase) has led to the construction of a fully human IR (Fig. 1). The expediency of Erbicin, an immune moiety selectively cytotoxic for ErbB2-positive cells and ErbB2 as a TAA, are described above. HP-RNase, the human homolog of RNase A, the prototype for the vertebrate RNase superfamily, is an abundant, physiological component of human fluids and is apparently secreted by endothelial cells [34].

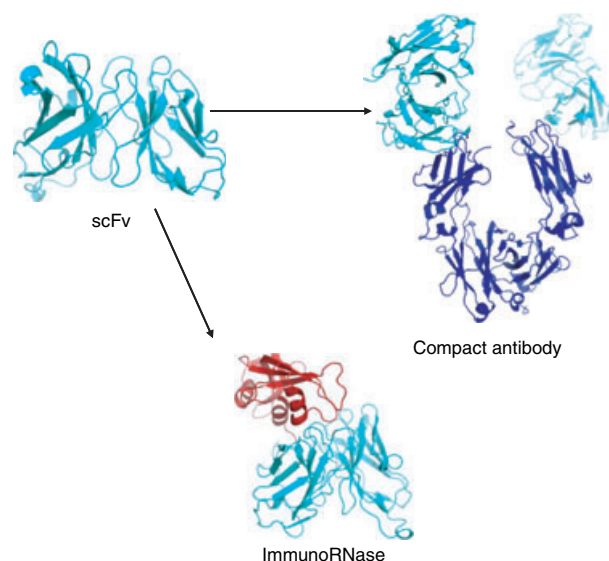


Fig. 1. Models for anti-ErbB2 scFv (Erbicin), a compact antibody (hcAb) and an immunoRNase (IR). A ribbon representation of hypothetical models of an immunoRNase and a cAb is given, with the scFv in cyan, the ribonuclease in red and the Fc fragment in blue. The scFv and RNase models were obtained by homology modeling, using as templates the crystal structures of a phage library-derived single-chain fragment 1F9 (PDB code 1DZB chain A) and that of a mutant of human pancreatic ribonuclease (PDB code 1DZA). The reciprocal orientation of Erbicin and human pancreatic ribonuclease structures in the immunoRNase molecule is based on rigid docking calculations performed using the rigid docking software FTDOCK. The spacer region was built manually. The hcAb model was obtained using the structure of the intact human antibody B12 (PDB code 1HZH) as a template.

The resulting construct, called Erb-hRNase [35] was found to bind selectively to ErbB2-positive cells with high affinity ($K_d = 4.5$ nM), and to kill target cells *in vitro* at low concentrations. Upon administration of five doses of $1.5 \text{ mg}\cdot\text{kg}^{-1}$ of Erb-hRNase to mice bearing an ErbB2-positive tumor, a remarkable reduction (86%) in tumor volume was induced.

More than 90% of the RNase activity of the free, native protein was found to be conserved in the fused HP-RNase. Furthermore, this RNase activity, obviously exerted in the cytosol where there are potential RNA substrates, is essential for expression of anti-tumor activity by the IR. Thus, it was surprising to find that HP-RNase fused in Erb-hRNase was inhibited, like free HP-RNase [36], by the cytosolic RNase inhibitor [37]. An investigation into the antitumor action of Erb-hRNase confirmed that its action is based on its RNase activity, naturally exerted in the cytosol of internalized cells, reached by Erb-hRNase directly from the endosomal compartment. The apparent inconsistency was resolved by the finding that the

amounts of IR entering the cytosol are greater than the amounts of endogenous inhibitor protein present in that compartment [36].

Based on the concern that the fraction of Erb-hRNase sequestered by the cytosolic inhibitor could not exert its antitumor activity, and that bivalent IRs are more powerful than monovalent ones, a new IR has been produced [38]. In this strategy, a dimeric variant of human pancreatic RNase [39] was fused to two Erbicin molecules, one per subunit. This novel immunoagent, called Erb-HHP2-RNase, was found to selectively bind to ErbB2-positive cancer cells with an increased avidity with respect to monomeric Erb-hRNase, and to exert a more powerful cytotoxic activity, possibly because of its resistance to the cytosolic ribonuclease inhibitor.

Of interest is the finding that, as it determined for an Erbicin-based compact antibody (cAb) (see below), Erb-hRNase is also virtually free of the cardiotoxic effects of Herceptin[®] (G. Riccio, G. Esposito, E. Leoncini, R. Contu, G. Condorelli, M. Chiariello, P. Laccetti, S. Hrelia, G. D'Alessio & C. De Lorenzo, unpublished results). Furthermore, ErbB2-positive cells resistant to Herceptin[®] were found to be susceptible to the cytotoxic action of Erb-hRNase (C. De Lorenzo, V. Damiano, T. Gelardi, R. Bianco, G. Tortora, P. Laccetti & G. D'Alessio, unpublished results).

Recently, a powerful anti-CD30 IR, both bivalent and fully human, was reported by Dübel *et al.* [40]. It is composed of a CD30 lymphoma-specific human scFv obtained from a semisynthetic human antibody library, linked to an IgG Fc segment, which in turn is fused to human pancreatic RNase. The scFv-Fc-RNase molecules were found to be homodimers, readily internalized and effective on CD30+ lymphoma cells at nM concentrations.

Compact antibodies

Antibodies represent the most important group of molecules used to target therapeutic compounds, but their large molecular mass is probably the cause of their inefficient delivery into solid tumors.

Recombinant antibody technology can be used to develop novel antibody and drug formats [41] with improved antigen-binding properties, pharmacokinetic and effector function. The choice of the optimal antibody format depends strongly on the intended therapeutic application. In this regard, molecular size, valency and the introduction of additional domains must be carefully balanced to ensure optimal targeting, pharmacokinetics and therapeutic efficacy. In general, small antibody fragments show better

penetration and distribution in solid tumors than larger molecules [42].

However, for therapeutic applications a monovalent antibody fragment of < 50–60 kDa would have a relatively limited half-life in the bloodstream and reduced tumor retention [43,44], as suggested by comparative studies revealing that small antibody fragments showed faster clearance from the circulation [45,46]. Therefore, it has been proposed that therapeutic molecules of 60–120 kDa provide the ideal balance between tumor penetration, retention and clearance [47]. Moreover, bivalent antibodies showed remarkably better tumor retention than their monovalent counterparts [48,49].

Finally, a reduced version of an IgG, named a 'compact antibody' (cAb) [50], in which two scFv molecules are fused to the immunoglobulin Fc moiety should have the advantages of being bivalent and have a molecular size better suited to therapeutic applications than either a small scFv or a full-size IgG-like molecule. Furthermore, the presence of the Fc portion should provide the cAb with a half-life similar to that of an intact antibody due to the interaction with the FcRn Brambell receptor [51,52].

The first report in this direction has shown the feasibility of cloning single gene constructs encoding fusion proteins made up of murine scFv and Fc fragments [53]. This chimeric scFv–Fc was a single gene product expressed in a homodimeric form, and had the advantages of higher stability and easier production with respect to whole tetrameric IgGs. Although the construct lacked the CH1 and CL domains, and hence had a reduced size (105 kDa), it was bivalent and all functionally relevant antibody regions were preserved.

The cAb format is expected to have a more protracted half-life and higher tumor retention than the parental scFv [54,55], but also improved penetration properties in solid tumors with respect to full-size IgGs. It has been shown [54,55] that in an immunoagent of ~ 100 kDa the prolonged half-life of an intact antibody is combined with increased extravascular diffusion, both very expedient features for targeting solid tumors.

In order to overcome several of the drawbacks of mouse antibodies, such as immunogenicity, a mouse–human chimeric scFv–Fc was obtained by fusing a murine scFv derived from mAb A21 directed against ErbB2, and human Fc [56]. The fusion molecule was expressed in mammalian cells and showed an antigen-binding site and activity identical to that of the parental antibody. Further studies showed that it was able to target human ovarian carcinoma cells (SKOV3) overexpressing ErbB2 both *in vitro* and *in vivo*; it may therefore be useful for diagnostic applications [57].

Given the expediency of a cAb with both scFv and Fc moieties of human origin, strategies leading to the preparation of fully *human*, and hence nonimmunogenic, antibody constructs were implemented. cAbs were reconstructed by fusing the available human scFvs, previously isolated using phage-display technology [5,6], to a human Fc antibody segment.

A recombinant, human scFv–Fc antibody specific for ErbB2 has been reported [58] to mediate *in vitro* antibody-dependent cell-mediated cytotoxicity (ADCC) and have a much longer serum half-life *in vivo* than its parental scFv. However, the protein was produced in yeast with yeast-controlled glycosylation; furthermore, it was found to be heterogeneous and was obtained at very low yields.

A significant addition to the arsenal of anticancer treatments has been the construction of a new anti-ErbB2 immunoagent from a human, namely cytotoxic, scFv and a human Fc domain. This fully human antitumor Ig was designed to be a compact, reduced version of an IgG, with the antiproliferative effect of the scFv moiety on tumor target cells combined with the ability of the Fc moiety to induce both antibody-dependent cellular and complement-dependent cytotoxicity.

To construct this immunoagent, the anti-ErbB2 scFv Erbicin [18] was fused to CH2, CH3 and hinge regions from a human IgG1 (Fig. 1) to obtain an antibody-like molecule [50]. The engineered antibody was called Erbicin-human compact antibody (Erb-hcAb) because of its 'compact' size (105 kDa) compared with a natural IgG (155 kDa).

It should be noted that Erb-hcAb was prepared in CHO cells, a mammalian model closer to human cells than yeast. Thus, it was not surprising that the glycosylation profile of Erb-hcAb was found to be virtually superimposable on that of a human IgG [59].

It has been reported [50] that Erb-hcAb is capable of selective binding to malignant cells that express ErbB2, and of inhibiting their growth *in vitro*, with no effects on ErbB2-negative cells. Moreover Erb-hcAb has both ADCC and CDC effects. When administered peritumorally or systemically to mice bearing breast tumors it strongly inhibits tumor growth [50,59]. Furthermore, an investigation into its mode of action has revealed that Erb-hcAb promotes downregulation of the receptor, inhibiting progression from the G0/G1 phase of the cell cycle, and induces apoptosis of ErbB2-positive cells [59].

Herceptin[®], currently used to treat advanced breast cancer [60,61], is a humanized version of a murine anti-ErbB2 monoclonal. Its antitumor activity is based mainly on its ability to downregulate ErbB2 and induce ADCC [62], but, as reported previously [63], it does not elicit CDC. The new immunoagent Erb-hcAb

by contrast displays a strong CDC effect, and is smaller (105 kDa) than Herceptin[®] (155 kDa).

Furthermore, in 40–60% of all patients with ErbB2-overexpressing tumors, Herceptin[®] has little or no effect on tumor regression [64]. For these patients, the prognosis is poor and the disease progresses more aggressively. To increase the response rate, treatments combining Herceptin[®] with anthracyclines have been performed, but unfortunately this leads to heart failure and cardiomyopathy. In fact, 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 28% when it is combined with anthracyclines [65–67].

It is of interest that the ErbB2-derived cAb recognizes on ErbB2-positive cells an epitope different from that targeted by Herceptin[®] [68] and does not affect the basal cardiomyocyte survival pathway (G. Riccio, G. Esposito, E. Leoncini, R. Contu, G. Condorelli, M. Chiariello, P. Laccetti, S. Hrelia, G. D'Alessio & C. De Lorenzo, unpublished results). Thus, it is not surprising that Erb-hcAb was found to exert no cardiotoxicity *in vitro* on rat cardiomyocytes, whereas Herceptin[®] was strongly toxic under identical conditions. *In vivo* studies on a mouse model showed that, unlike Herceptin[®] or doxorubicin, Erb-hcAb did not significantly alter cardiac function as measured by heart echocardiography performance, velocity of contraction, extent of cardiac fibrosis and apoptosis (G. Riccio, G. Esposito, E. Leoncini, R. Contu, G. Condorelli, M. Chiariello, P. Laccetti, S. Hrelia, G. D'Alessio & C. De Lorenzo, unpublished results).

Finally, Erb-hcAb binds the soluble extracellular domain of ErbB2 (ErbB2-ECD) with a lower affinity than that for the native receptor inserted in tumor cells. Herceptin[®], by contrast, shows a higher affinity for soluble ErbB2-ECD. Accordingly, ErbB2-ECD abolishes the *in vitro* antitumor activity of Herceptin[®] with no effects on the activity of Erb-hcAb [69]. Thus, the fraction of immunoagent neutralized by free, bloodstream extracellular domain is much higher for Herceptin[®] than for ErbB2-derived immunoagents, with suggestive effects on therapeutic dosage of the immunoagents.

Taken together, the data suggest that Erb-hcAb is a promising new anticancer agent which may fulfil the therapeutic need of patients ineligible for Herceptin[®] treatment due to cardiac dysfunction or the occurrence of resistance, and supports the concept that, after humanized monoclonals and scFvs, a new generation of immunoagents, human cAbs, may represent the format of choice for the therapy of solid tumors.

This hypothesis was further confirmed when a cAb derived from Herceptin[®] was made by fusing a

Herceptin[®]-derived scFv fragment (hu4D5v8) with the Fc portion (CH2–CH3 region) to achieve rapid clearance kinetics [70]. This antibody format, when evaluated by microPET, exhibited improved tumor targeting and reduced kidney uptake with respect to other mini-body formats.

The cAb format may also represent the ideal antibody moiety for other therapeutic applications such as those of immunoconjugates with RNase (as described above), cytokines or bispecific antibodies to selectively target tumor cells, or to activate immune effector cells, as described below.

One possible mechanism to enhance the therapeutic efficacy of cAb-based treatment is obtained by the use of bispecific antibodies able to bind two different TAAs, to improve tumor uptake and targeting selectivity over normal tissue that expresses only one target antigen (or low levels of both). Proof of this concept has been obtained with a bispecific, full antibody directed against CEA and ErbB2, in double-positive tumor-bearing nude mice [71], thus suggesting that targeting two distinct TAAs on the same cell may improve tumor localization.

An alternative experimental approach to increase the efficacy of cAb-based therapy has been aimed at enhancing effector cell functions, particularly in mediating ADCC. This could be achieved by activation of the immune response, as determined by interleukin-2 (IL-2), a cytokine which induces the proliferation of T cells, supports the growth of antigen-specific T-cell clones and enhances the activity of T- and natural killer (NK) cells [72,73].

To combine IL-2 activity with a tumor-specific antibody, an anti-ErbB2, scFv–Fc–IL-2 fusion protein, named HFI, was developed [74]. This construct, consisting of a murine anti-ErbB2 scFv, the Fc fragment of human IgG1 and IL-2, was obtained by fusing IL-2 to the C-terminus of the anti-ErbB2 scFv–Fc. The fusion protein retained ErbB2 specificity and IL-2 biological activity and was found to kill tumor cells by ADCC and to inhibit the growth of ErbB2-positive tumors in mice [75]. However, *in vivo* comparison of the HFI fusion protein and the parental anti-ErbB2 scFv–Fc showed only a slightly improved efficacy for HFI, as its benefit was in part offset by the hepatotoxic effects of IL-2.

The engagement of NK cells through the use of bifunctional cAbs able to bind both tumor and effector cells has also been exploited for cancer therapy. A new form of bispecific cAb that consists of two scFvs, one for ErbB2 and the other for CD16, was constructed using a 'knobs-into-holes' heterodimerization device from the CH3 domains of the human IgG1 Fc fragment

[76]. *In vitro* experiments demonstrated that the anti-ErbB2::anti-CD16 cAb was able to recruit human peripheral blood mononuclear cells to kill SK-BR-3 tumor cells more effectively than the commercial anti-ErbB2 IgG Herceptin®.

In a different experimental approach, the ligand of NKG2D, an activating receptor expressed on NK and T cells, was fused to anti-tumor IgG fragments to specifically coat this ligand on tumor cells and to induce their lysis by NK cells. An anti-ErbB2 bifunctional protein (scFv4D5/rH60)-Fc was obtained by assembling the Herceptin®-derived scFv (4D5) with the mouse NKG2D ligand H60 and human Fc fragment in a cAb format. The new bifunctional protein was found to be specific for targeted TAAs and capable of stimulating NKG2D-dependent tumor cell lysis by murine NK cells [77].

Conclusions

Given the recent success of immunotherapy in hospitals, it is imperative that a discussion is held on the promising results of new strategies for the construction of novel immunoagents. The IR strategy has long been implemented and tested, mainly *in vitro* and *in vivo* on mouse models. Third-generation IR are increasingly proposed and tested, as discussed above. The more recent strategy of cAbs is also very promising, given their optimal size, bivalency and the possibility of exploiting bispecificity. However, only judicious selection through clinical trials will decide on the most appropriate immunoagents of the future.

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