Chapter 18
Design, Development, and Characterization of Recombinant Immunotoxins Targeting HER2/neu

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Background

The human epidermal growth factor receptor 2 (HER2), also known as ErbB2, c-erbB2, or HER2/neu, was initially discovered in 1985 by two independent laboratories [1, 2]. HER2/neu is a 185 kDa (1,255 aa) transmembrane receptor encompassing an intracellular tyrosine kinase domain and an extracellular ligand binding component [3–5]. Extensive clinical studies have shown that overexpression of HER2/neu is found in 20–40% of patients with breast, ovarian, endometrial, gastric, bladder, prostate, and lung cancers. Studies clearly demonstrate that HER2/neu overexpression correlates with the prevalence of metastatic spread of many tumors and is generally considered to be a poor prognostic indicator [6–9].

Since HER2/neu overexpression by tumor cells is quite specific, therapies directed against this receptor have rapidly gained recognition for their selectivity and efficacy in the clinical setting. While targeting of HER2/neu with humanized antibodies such as trastuzumab (Herceptin; Genentech) has proven to be an effective approach for the treatment of HER2/neu-overexpressing breast cancers, there are a significant number of patients with HER2/neu-positive tumors who do not respond or who acquire resistance to this therapy [10–13]. Therefore, there is a need for novel therapeutic approaches using HER2/neu not only as a target for interfering with the growth factor signaling component but also for receptor-mediated delivery of cytotoxic agents.

Immunotoxins are a novel approach for the development of highly specific, targeted agents and which generally employ a powerful class of protein toxins [14, 15]. These include plant toxins such as ricin [16–25], saporin [26–29], and gelonin [30–32], which inactivate ribosomes, and single-chain bacterial toxins

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Diphtheria toxin (DT) [33] and *Pseudomonas* exotoxin (PE) [34–43], which ADP ribosylate elongation factor 2 (EIF2). Anti-HER2/neu immunotoxins have been created initially by chemically conjugating an antibody to a whole protein toxin or, for more selective activity, using a protein toxin devoid of its natural binding domain [19, 23, 30, 44]. Technical advances in antibody engineering now enable us to produce various antibodies or antibody fragments in *Escherichia coli*, and as a result, HER2/neu-specific antibodies and engineered fragments thereof have been developed to deliver various toxins to HER2/neu-positive tumor cells [45–51]. Various anti-HER2/neu immunotoxins which have been developed or are currently under evaluation are described in Table 18.1.

Antibody–Drug Conjugates: Promise and Problems

Antibody-based therapeutics is of growing significance for cancer therapy. To date, two of the most promising strategies to enhance the antitumor activity of antibodies are antibody–drug conjugates (ADCs) and antibodies (or fragments) chemically conjugated or genetically fused to various toxins (immunotoxins).

One successful application of the ADC approach is Trastuzumab–DM1. This is a covalent conjugation of trastuzumab with the maytansinoid DM1—a highly toxic derivative of the antimitotic drug maytansine. The therapeutic potential of Trastuzumab–DM1 has been extensively investigated in both in vitro and in vivo models of trastuzumab sensitive and insensitive breast cancers [52–56]. It has demonstrated remarkable activity in phase I and II studies in which it was given to patients harboring trastuzumab-insensitive breast tumors [57, 58]. Furthermore, several other ADCs using anti-HER2/neu antibodies have been developed and have shown potent antitumor activity [59, 60]. Despite successful reports, it is important to note that this strategy has some limitations. The first is the limited reproducibility of chemical conjugation due to the fact that there are numerous coupling sites on an antibody molecule. Secondly, chemically modified antibodies have demonstrated a greater tendency to aggregate, especially when multiple drug molecules are conjugated to a single antibody. Furthermore, it is challenging to remove remaining unconjugated antibodies from the ADC mixture. Finally, the emergence of multidrug resistance (MDR and MRP) mechanisms in tumors from heavily treated patients may engender cross-resistance to ADCs.

With the development of recombinant DNA technology, anti-HER2/neu immunotoxins composed of antibodies (or fragments) and protein toxins have become a promising alternative approach for HER2/neu-positive tumors. Compared to the ADC approach, one attractive advantage of immunotoxins is that the targeting antibody and antitumor toxin can be produced directly as a single molecule, thus avoiding laborious chemical conjugation steps. In addition, the linkage between the toxin and targeting antibody is identical and exactly defined in a given preparation of recombinant immunotoxin, thereby promoting homogeneity of the final product. Compared with chemical conjugates, genetically engineered immunotoxins

Table. 18.1 Immunotoxins developed for HER2/neu targeted therapy

Toxin source	Toxin	Targeting device	Production	Tumor type
Plant	Gelonin	Humanized anti-HER2 mAb	Chemical conjugation	Ovarian cancer [30]
		Human anti-HER2 scFv	Recombinant protein	Ovarian cancer [31], breast cancer [32]
	Saporin	Murine anti-HER2 mAb	Chemical conjugation	Breast cancer [26, 29], Melanoma [27]
		Murine anti-HER2 mAb	Indirect bridge-linking	Ovarian cancer [28]
	Ricin	Murine anti-HER2 mAb	Chemical conjugation	Breast cancer [17, 20, 21, 23, 24], ovarian cancer [16, 18, 19], gastric cancer [22]
		Murine anti-HER2 Fab′	Chemical conjugation	Lung cancer [25]
Bacteria	Pseudomonas exotoxin	Murine anti-HER2 scFv	Recombinant protein	Ovarian cancer [36, 122], epidermoid cancer [40, 183], prostate cancer [34, 184], lung cancer [185–187], gastric cancer [84, 123, 188–190], schwannoma cancer [191], breast cancer [42, 50, 124]
			Recombinant gene delivery	Gastric cancer [152], ovarian cancer [192]
		Murine anti-HER2 disulfide-stabilized Fv fragments (dsFv)	Recombinant protein	Gastric cancer [38, 43], epidermoid cancer [49], breast cancer [48]
		Murine bivalent anti-HER2 dsFv $(dsFv)_2$	Recombinant protein	Epidermoid cancer [41, 51]
		Murine-bispecific scFv (anti-HER2 and anti-EGFR)	Recombinant protein	Epidermoid cancer [37, 46]
		Humanized anti-HER2 Fab′	Liposome-mediated chemical conjugation	Breast cancer [35, 39, 151]
		Humanized anti-HER2 mAb	Indirect bridge-linking	Epidermoid cancer [40, 193]
	Diptheria toxin	Murine-bispecific scFv (anti-HER2 and anti-EpCAM)	Recombinant protein	Colon cancer [33]
	Staphylococcal enterotoxin	Murine anti-HER2 mAb	Chemical conjugation	Colon cancer [44]
		Human anti-HER2 scFv	Recombinant protein	Breast cancer [47]
	Bacillus Cyt2Aa1 toxin	Human anti-HER2 scFv	Recombinant protein	Breast cancer [45]

can be easily designed to enhance antitumor efficacy. Finally, data suggests that the emergence of MDR cellular protection mechanisms in heavily pretreated patients may not impact cytotoxic effects of immunotoxins.

We have provided general principles for development of anti-HER2/neu immunotoxins, and current strategies to employ these molecules for directed cancer therapy are discussed focusing mainly on design optimization to improve antitumor efficacy and off-target toxicity.

Anti-HER2/neu Immunotoxins

Construction of Recombinant Immunotoxins

HER2/neu-overexpressing cancers are a model of disease for the development of rationally designed targeted therapies. The scientific advances in understanding the role of HER2/neu function, the structural aspects of HER2/neu function, and the signaling partners and circuitry underlying tumorigenic HER2/neu signaling have afforded unique opportunities for rational drug design to target these pathways. The development and application of various HER2/neu-targeted therapies has benefited greatly by a more advanced understanding of HER2/neu function and biology [61, 62].

Overall, strategies to enhance anti-HER2/neu immunotoxin potency include improvements to the affinity and specificity of targeting moiety, identification and incorporation of new and better toxins, reengineering known toxins for reduced immunogenicity, and designing novel linkers between toxins and targeting moieties to optimize toxin translocation to the cytosol [63–66]. Numerous excellent reviews have previously compared the advantages and disadvantages of a variety of cytotoxic proteins including bacterial, plant, and mammalian toxins successfully employed for the construction of immunotoxins [67, 68]. This review will address how linker design and antibody affinity affect immunotoxins in tumor-specific targeted therapies.

Peptide Linker Designs

The development of various linkers which bridge disparate molecules such as small drugs conjugated to tumor-targeting carriers has been the subject of numerous studies for the past few years [69, 70]. Based on numerous prior studies, the incorporation and design of linkers is critical to the success of ADCs. Conceptually, an ideal linker must be stable in systemic circulation, while being efficiently cleaved to allow rapid release of an active form of the drug once the construct has been internalized into the tumor cell target. To this end, a variety of linkers have been designed with different chemical structures and stabilities [71, 72]. Selection of an appropriate linker depends on the type of cancer and the required cytotoxic agent. Furin is a

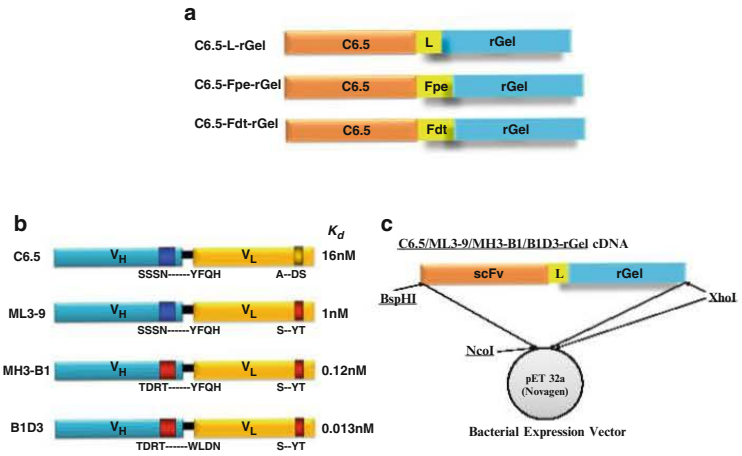

Fig. 18.1 Construction and preparation of scFv/rGel immunotoxins. (**a**) Schematic diagram of immunotoxin constructs containing scFv C6.5, peptide linker (L, Fpe or Fdt), and rGel toxin. (**b**) Amino acid mutations and affinity parameters of the C6.5 and its mutants, ML3-9, MH3-B1, and B1D3. The listed amino acids for each scFv indicate mutations to the sequence and the substituting amino acids. *Dashes* indicate no changes from the original sequence. (**c**) Diagram of immunotoxin constructions containing scFv (C6.5, ML3-9, MH3-B1, or B1D3) and rGel

cellular endoprotease and has been implicated in proteolytically activating large numbers of secreted proteins such as prohormones, growth factors, receptors, and viral glycoproteins. These proteins are synthesized as inactive precursors and must be proteolytically cleaved to become functionally mature. In previous studies, the inclusion of furin-cleavable linkers into fusion constructs containing ribotoxins, caspase3, or granzyme B (GrB) has demonstrated a significant improvement in specific toxicity compared to constructs containing stable linkers [73, 74].

The incorporation of cleavable linkers for immunotoxins is essential since, in general, the toxin components are enzymatically inactive in the construct until intracellular release from their cell-targeting carriers [75, 76]. For recombinant gelonin (rGel)-based constructs, the enzymatic (*N*-glycosidase) activity of the toxin is preserved in the intact fusion constructs, eliminating the absolute necessity for intracellular release of the rGel component. Nevertheless, we explored a variety of different linker strategies to determine whether intentionally cleavable linkers offered an advantage over linkers which were designed for flexibility only. Illustrations of various immunotoxin constructs are shown in Fig. 18.1a. The initial rGel-based immunotoxins consisted of a flexible linker (GGGGS, "L") tethering the C-terminus of the human anti-HER2/neu single-chain antibody (scFv) C6.5 to the native rGel N-terminus. The C6.5/rGel construct was further engineered by incorporating two different enzymatically sensitive furin cleavage linkers between

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