International Journal of Pharmaceutics 511 (2016) 538–549

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Fusion of an albumin-binding domain extends the half-life of immunotoxins

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ARTICLE INFO

Article history:
Received 20 March 2016
Received in revised form 24 June 2016
Accepted 21 July 2016
Available online 22 July 2016

Keywords:
Immunotoxin
Affibody
Pseudomonas exotoxin
Albumin binding domain
Half-life extension

ABSTRACT

Immunotoxins have documented potential as a cancer treatment due to their extreme potency; a single toxin molecule delivered to the cytosol may be sufficient to kill a cell. However, their short half-life in the circulatory system may be one of the key problems associated with the clinical use of immunotoxins and may continue to limit their therapeutic activity. Herein, we genetically fused an albumin-binding domain (ABD) to the human epidermal growth factor receptor 2 (HER2)-specific immunotoxin Z_{HER2}-PE38 to extend the circulation time and thus improve the therapeutic outcome of this immunotoxin. Furthermore, the fusion of an ABD to the immunotoxin was found to promote non-covalent interactions between the immunotoxin and serum albumin, which rescue the immunotoxin from lysosomal degradation through a serum albumin-mediated interaction with the neonatal Fc receptor (FcRn). This manuscript reports the construction, purification, and characterization of the ABD-fused HER2-specific immunotoxin, ABD-Z_{HER2}-PE38, both *in vitro* and *in vivo*. Compared with non-fused Z_{HER2}-PE38, this new construct exhibits a clearly increased half-life in plasma (330.8 *versus* 13.5 min, approximately 24.4-fold extension) and remarkably improved antitumor effects in an NCI-N87 subcutaneous xenograft model. Therefore, the new construct represents a potentially attractive therapeutic modality, and the proposed strategy may also have useful applications for current immunotoxin designs.

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1. Introduction

Monoclonal antibodies (mAbs) that bind antigens on cancer cells provide effective clinical options for the management of malignancies (Henricks et al., 2015). However, an increasing body of evidence suggests that unconjugated mAbs have limited utility in many malignancy subtypes because their activities are generally suboptimal (Scott et al., 2012). Specifically, malignant cells are easily resistant to antibody-induced apoptosis, or patient immune systems are not sufficiently efficient for antibody- or complement-dependent cytotoxicity, which are the common mechanisms that antibodies use to kill cancer cells (Nahta et al., 2006). However, antibodies armed with cytotoxic agents, such as radioisotopes, cytotoxic drugs or protein toxins, can directly kill cells without relying on such mechanisms (Wu and Senter, 2005). Such molecules, which are referred to as radio-immunoconjugates, antibody-drug conjugates and immunotoxins, respectively, represent a second revolution in antibody-mediated cancer therapy (Kraeber, 2014; Dosio et al., 2014).

Immunotoxins are chimeric proteins that comprise a modified toxin linked to a tumor-specific ligand (antibody, antibody fragment or growth factor) and present special advantages due to their extreme potency, *i.e.*, a single toxin molecule delivered to the cytosol may be sufficient to kill a cell (Yamaizumi et al., 1978). Protein toxins are different types of protein synthesis inhibitors, and immunotoxins have documented potential for the treatment of cancers (Weidle et al., 2014). Denileukin diftitox (Ontak; Eisai Inc., Woodcliff Lake, NJ, USA) contains human interleukin-2 and truncated diphtheria toxin and is approved for use in cutaneous T-cell lymphoma (Gottlieb et al., 1995). Certain other immunotoxins are currently in different phases of clinical trials, which have revealed impressive clinical responses (Alewine et al., 2015). PE38, a truncated version of *Pseudomonas* exotoxin A (PE), is one of the

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http://dx.doi.org/10.1016/j.ijpharm.2016.07.046
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most widely used toxins for the construction of immunotoxins (Kreitman et al., 1992). It delivers a cytotoxic effect in the cytosol through the ribosylation of elongation factor 2 (EF-2) and prevents proper ribosomal function (Pastan and FitzGerald, 1989; Pastan et al., 2007). Many PE38-based immunotoxins directed against various surface antigens that are overexpressed in tumors have been constructed and tested in preclinical and clinical trials (Weldon and Pastan, 2011). For example, clinical trials have shown that an immunotoxin containing an anti-CD22 Fv and PE38 results in complete remission in a high proportion of hairy-cell leukemia cases (Kreitman et al., 2001). Furthermore, SS1P, which is composed of an anti-mesothelin antibody variable fragment (Fv) linked to PE38, was engineered for he targeted elimination of malignant cells that express the tumor-associated antigen mesothelin (Hassan et al., 2007).

However, the short half-life of immunotoxins in the circulation system may be one of the key problems associated with their clinical use and may continue to limit their therapeutic activity (Kreitman et al., 2001; Hassan et al., 2007). The toxin proteins are typically fused to a single-chain variable fragment of an antibody or antibody mimetic as the targeting molecule, which was originally designed as a small molecule to enhance penetration into solid tumor masses (Kuan et al., 2011; Kreitman and Pastan, 1998). However, these fused proteins show markedly shorter plasma half-lives in the circulation, an effect that is attributable to kidney clearance and more rapid distribution outside the vasculature (Traini and Kreitman, 2011; Thurber, 2008; Schmidt and Wittrup, 2009). The approved immunotoxin denileukin diftitox has a clinical half-life of only 70–80 min (Eklund and Kuzel, 2005). In addition, the maximum tolerated dose of immunotoxins is low (0.05 mg/kg for human use) (Kreitman et al., 2000) and is far below the dose of most therapeutic monoclonal antibodies that are clinically used for the treatment of solid tumors (*e.g.*, trastuzumab and cetuximab, 2–10 mg/kg weekly) (Vogel et al., 2001). This low dose requirement coupled with their rapid clearance likely limits the diffusion and localization of immunotoxins to solid tumors.

Extending the *in vivo* half-lives of immunotoxins is important for further improving their therapeutic outcomes. The albumin-binding domain (ABD) ABD_{035} is a 46-amino-acid moiety engineered from Streptococcal Protein G and has femtomolar affinity for human serum albumin as well as high affinity for serum albumin from rat, mouse and cynomolgus monkey (Nord et al., 1997, 2000; Nygren, 2008). The inclusion of this ABD in a fusion protein causes a strong non-covalent interaction with serum albumin after injection of the fusion protein into the blood, and this interaction may increase the serum half-life of the fusion protein by facilitating serum albumin-mediated interactions with the neonatal Fc receptor (FcRn), which would lead to its rescue from lysosomal degradation (Andersen et al., 2006; Andersen and Sandlie, 2009; Roopenian and Akilesh, 2007). For example, the terminal half-life of bispecific single-chain diabodies (scDBs) in mice was improved from 0.2 to 1.8 h through fusion with an ABD (Hopp et al., 2010).

To test the efficiency of the immunotoxin half-life extension strategy using ABD_{035}, we genetically fused the ABD_{035} molecule to a human epidermal growth factor receptor 2 (HER2)-specific immunotoxin, Z_{HER2}-PE38, which, as demonstrated in the literature, shows potent anti-tumor activity against a series of HER2-positive tumor cells both *in vitro* and *in vivo* (Zielinski et al., 2009, 2011). The immunotoxin Z_{HER2}-PE38 refers to the product of the fusion of PE38 with the anti-HER2 affibody molecule $Z_{HER2:342}$, a 58-amino-acid affinity protein (~7 kDa, almost 20-fold smaller than antibodies and four-fold smaller than scFvs) engineered from the triple helical B-domain of staphylococcal protein A that has an equilibrium dissociation constant of 60 pM for the HER2 receptor. Due to its ultra-small size, high affinity, and high specificity, the anti-HER2 affibody molecule $Z_{HER2:342}$ and analogs constitute a useful alternative to antibodies or scFvs as targeting agents for clinical and preclinical use (Baum et al., 2010). Moreover, compared with the commonly used single-chain Fv-based antibody as the targeted ligand, which usually must be either expressed in the periplasm or refolded after its expression in inclusion bodies, the expression of the immunotoxin with the affibody as the targeted ligand is simpler and easily achieved on a large scale (Löfblom et al., 2010). Despite its great clinical potential for cancer treatment due to its extremely high HER2 specificity and high cytotoxicity for cancer cells, the immunotoxin Z_{HER2}-PE38 has a half-life of only approximately 9 min (Zielinski et al., 2011), which markedly restricts its clinical application. By fusing this molecule with an ABD, the new recombinant protein ABD-Z_{HER2}-PE38 may exhibit an extended circulation time and thereby an improved therapeutic outcome. Moreover, this strategy may be used as a general method for extending the half-life of many other immunotoxins, even unrelated protein therapeutics. Liu et al. recently presented a tripartite immunotoxin that includes an anti-HER2 affibody, an albumin-binding domain and a modified PE38 × 8. The constructed immunotoxin has high affinity to HER2 or albumin protein and shows high cytotoxicity against HER2-positive cancer cells *in vitro* (Liu et al., 2015).

In this manuscript, we report the construction, purification, and characterization of the HER2-specific immunotoxin ABD-Z_{HER2}-PE38, which combines the HER2-specific affibody $Z_{HER2:2891}$ as the targeting moiety and PE38KDEL as the cytotoxic agent with ABD_{035} to achieve a half-life extension. In this study, the anti-HER2 affibody $Z_{HER2:2891}$ was used instead of $Z_{HER2:342}$ to obtain increases in the melting point, stability, and overall hydrophilicity of the immunotoxin (Feldwisch et al., 2010). Although $Z_{HER2:2891}$ has a slightly lower affinity than $Z_{HER2:342}$, its increased hydrophilicity is believed to be beneficial for biopharmaceutical applications, such as improving the tumor targeting and reducing the liver uptake of immunotoxins. Furthermore, the native REDL C-terminal sequence of PE38 was changed to KDEL to increase the cytotoxicity of the PE38 portion by increasing the efficiency of its Golgi-to-endoplasmic reticulum (ER) transport (Seetharam et al., 1991; Kreitman and Pastan, 1995). Compared with non-fused Z_{HER2}-PE38, the new construct showed a clearly increased plasma half-life (330.8 *versus* 13.5 min, corresponding to an approximately 24.4-fold extension) and exhibited remarkably improved antitumor effects in an NCI-N87 subcutaneous xenograft model. The new construct thus represents a potentially attractive therapeutic modality, and this strategy may also have useful applications for current immunotoxin designs.

2. Materials and methods

2.1. Cell lines and animals

The human breast cancer cell lines BT474, SKBR3 and MCF7 were obtained from the American Type Culture Collection. The human gastric cancer cell line NCI-N87 and human prostatic carcinoma cell line PC-3 were purchased from a typical culture preservation commission cell bank of the Chinese Academy of Sciences (Shanghai, China). The BT474 and NCI-N87 cells were grown in RPMI 1640 medium, SKBR3 cells were grown in McCOY's 5A medium, MCF7 cells were grown in DMEM, and PC-3 cells were grown in F-12 culture media supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin and 0.1 mg/ml streptomycin. A solution composed of 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS) was used for cell detachment. The cells were cultured in humidified incubators at 37 °C with 5% CO_2.

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