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Molecular modification of a recombinant, bivalent anti-human CD3 immunotoxin (Bic3) results in reduced in vivo toxicity in mice

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Abstract

A novel bivalent single chain fusion protein, Bic3, was assembled consisting of the catalytic and translocation domains of diphtheria toxin (DT_{390}) fused to two repeating sFv molecules recognizing human CD3 epsilon of the human T-cell receptor. Historically, problems with these constructs include low yield, toxicity, and reduced efficacy. Instead of using conventional Gly_4Ser linkers to connect heavy/light chains, aggregation reducing linkers (ARL) were used which when combined with a new SLS-based refolding method reduced aggregation and enhanced the yield of final product. Toxicity was reduced at least 25-fold by repeating the two sFv molecules and adding a portion of the hinge-CH2–CH3 human constant regions. The resulting Bic3 was just as cytotoxic to HPB-MLT.UM T leukemia cells in vitro (IC_{50} = 4 pmol) as a monovalent construct made with the same DT and sFv. In vivo, Bic3 was effective in a new and aggressive therapy model in which it significantly prolonged survival of scid mice with established human T-cell leukemia ($p < 0.0001$ compared to controls). Importantly, no toxicity measured by weight loss, enzyme function, or histology was observed at the highest dose of Bic3 tested (2000 ug/kg). Bic3 warrants investigation as a new drug for treating T-cell malignancy and other T-cell related disorders.

Keywords: Anti-human T cell; Immunotoxin; diphtheria toxin; Fusion protein; Leukemia; Human anti-cancer agent; Animal model; Anti-CD3 sFv

1. Introduction

Immunotoxins are synthesized by coupling an antibody or cytokine to a potent, mostly catalytic toxin, capable of inhibiting protein synthesis [1]. As originally proposed by Erlich in the early 1900s [2], the purpose of these labeled agents is to target cells responsible for numerous human diseases. CD3ε, a component of the T-cell receptor has been chosen as a target by a number of investigators in order to eliminate T cells [3]. Successful T cell depletion therapy would be useful for tolerance induction, diabetes prevention, treatment of T-cell malignancies, and prevention of organ rejection and graft-versus-host disease.

Diphtheria toxin is a good choice for IT production since it has first order Michaelis–Menten kinetics and a single

Abbreviations: ARL, aggregation reducing linker; AML, acute myeloid leukemia; Bic3, DT_{390} fused to two anti-CD3 sFv with portion of immunoglobulin constant region; CD3ε, CD3 epsilon; CH domain, constant-heavy chain domain; DMEM, Dulbecco's minimal essential media; DT, diphtheria toxin; DTCD22, DT_{390} fused to single anti-CD22 sFv; DTCD22CD22, DT_{390} fused to two anti-CD22 sFv; DTe23, DT_{390} fused to single anti-ErbB2 sFv; IC_{50}, concentration at which 50% response is inhibited; FITC, fluorescein isothiocyanate; GVHD, graft-versus-host disease; HAMA, human anti-mouse antibody; IT, immunotoxin; MTD, maximum tolerated dose; Mo3, DT_{390} fused to single anti-CD3 sFv; PBMC, human peripheral blood mononuclear cells; PHA, phytohemagglutinin; SLS, sodium *N*-lauroyl-sarcosine; TCR, T-cell receptor

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molecule in the cytosol is sufficient for cell killing [4]. Intact DT contains two fragments, A and B. The A fragment catalyzes the ADP-ribosylation of elongation factor 2 (EF-2) leading to protein synthesis inhibition and cell death [5,6]. Fragment B contains the native binding domain binding all human cells and must be replaced by an appropriate ligand (such as anti-CD3ε sFv) to render it a cell specific IT. DT was chosen because Williams et al. [7] described a series of internal frame deletion mutations that established 389 as the optimum site for genetic fusion of DT and target ligands. The native DT binding domain was replaced by anti-CD3ε sFv because pre-clinical studies have shown that CD3ε efficiently mediates entry of DT-based IT [8]. Anti-CD3ε IT were first used in our 1984 ex vivo clinical studies to deplete MHC-compatible donor bone marrow grafts of T cells prior to allogeneic bone marrow transplantation in matched sibling donors [9]. In vivo, the use of anti-CD3ε IT have been limited, in part due to the activation of the TCR by anti-CD3ε binding. In early studies, this problem was solved in mice using a non-activating recombinant IT encoding the sFv region of anti-mouse CD3ε [10] fused to DT_{390}. However, this agent showed unacceptable in vivo renal toxicity [11]. Later studies revealed that mutating this construct with the placement of a c-terminal cytseine, resulted in a highly effective bivalent anti-GVHD agent with reduced renal toxicity [12].

More recently, investigators have cloned high avidity single chain bivalent anti-CD3ε IT [13] which were effective against human T cells, but limited in expression and yield. Optimizing protein expression and production in a bacterial inclusion body model would be ideal because of the convenience and low cost of production. However, yield is often poor and refolding is complicated by the high number of intramolecular cysteines in sFvs which render molecules more susceptible to aggregation during refolding. In this report, to improve yield and purity of protein in a bacterial expression system, we synthesized a bivalent multi-domain anti-CD3ε IT called Bic3 which incorporated aggregation reducing linkers [14] and an optimized refolding procedure which enhanced its yield.

Another, major dose-limiting problem with all IT is their toxicity to non-target organs as previously observed with the monomeric form of an anti-mouse CD3ε IT described above [11]. As might be anticipated, the monomeric form of an anti-human CD3ε IT synthesized in this study also caused renal toxicity in mice. This toxicity was significantly reduced by incorporating a portion of the human IgG1 constant region into the design of Bic3. Bic3 was capable of inhibiting the growth of the systemic T-cell leukemia HPB-MLT.UM in a scid mouse model of T-cell leukemia and therefore shows potential as a therapy for eliminating normal and malignant T cells in vivo in humans. This is the first report of this agent and the human HPB-MLT.UM T-cell leukemia mouse model used to study it.

2. Materials and methods

2.1. Construction of Bic3

First, a monovalent IT, Mo3, was constructed. This hybrid immunotoxin gene encoded an ATG initiation codon, the first 389 amino acids of diphtheria toxin, a seven EASGGPE amino acid connector, and a single chain sFv gene derived from the murine anti-human CD3ε hybridoma, UCHT-1 [15] (kindly provided by Dr. Peter Beverley, The Edward Jenner Institute for Vaccine Research, Compton, Berkshire, UK). The UCHT-1 variable genes were individually amplified and fused by overlap PCR to produce sFv fragments. The anti-CD3ε sFv was orientated as VL-linker -VH and the linker was $(G_4S)_3$. The resulting 1947 bp *Nco*1/*Xho*I immunotoxin gene fragment was spliced into the pET21d expression vector (Novagen, Madison, WI, USA) under the control of the IPTG inducible T7 promoter creating the plasmid Mo3.pET21d (Fig. 1A). The DNA sequence was verified (University of Minnesota, Advanced Genetic Analysis Center, Minneapolis, MN) to confirm the gene had no mutations.

The construction of the single chain bispecific IT, Bic3, was completed in multiple steps. First overlap PCR was used to orient the sFv as VL-linker-VH with an aggregation reducing linker GSTSGSGKPGSGEGSTKG [14] placed in between the VL and VH segments. Next, the upstream primer 5′ GAAGCTTCCGGAGGTCCCGAGGACATCCAGATGACCCAGACCAC 3′ was used to add the EASGGPE linker to the 5′ end of VL-linker-VH and the downstream primer 5′ GGATCCGCCTCCGCCTGAGGAGACGGTGACGGTGGTCC 3′ was used to add (Gly_4Ser) to the 3′ end. This EASGGPE-VL-linker-VH-Gly_4Ser construct was ligated into the TA cloning vector, pGEMT.easy vector (Promega, Madison, WI, USA).

Next, a second vector was assembled. Standard DNA shuffling techniques were used to construct a truncated single chain human IgG1 constant region domain consisting of a hinge region (in which all Cys were mutated to Ala) and a separate downstream CH2–CH3 constant region. The upstream primer 5′ GGCGGAGGCGGATCCGACATCCAGATGACCCAGACCACCTCCTCCCTG 3′ was used to add G_4S linker to the 5′ end of the UCHT-1 VL-linker-VH construct and the downstream primer 5′ GTTTTGTCTGGAGATTTGGGCTCTGCTGAGGAGACGGTGACGGTGGTCCC 3′ was used to attach the hinge to the 3′ end creating Gly_4Ser-VL-linker-VH-hinge. The primers 5′ GGCGGAGGCGGATCCGACATCCAGATGACCCAGACCACCTCCTCCCTG 3′ and 5′ CAGCTCGAGTCATTTACCCGGAGACAGGGAGAGGCTC 3′ were used to overlap the Gly_4Ser-VL-linker-VH-hinge fragment and the hinge-CH2–CH3 fragment resulting in a Gly_4Ser-VL-linker-VH-hinge-CH2–CH3 fragment. This *Hin*dIII/*Xho*I fragment was ligated into a pET21d plasmid containing the DT_{390} gene so that the sFvs were positioned downstream of DT_{390} resulting in an expression vector, Bic3.pET21d.