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# Re-engineering and evaluation of anti-DNA autoantibody 3E10 for therapeutic applications

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ABSTRACT

A key challenge in the development of novel chemotherapeutics is the design of molecules capable of selective toxicity to cancer cells. Antibodies have greater target specificity compared to small molecule drugs, but most are unable to penetrate cells, and predominantly target extracellular antigens. A nuclear-penetrating anti-DNA autoantibody isolated from the MRL/lpr lupus mouse model, 3E10, preferentially localizes to tumors, inhibits DNA repair, and selectively kills cancer cells with defects in DNA repair. A murine divalent single chain variable fragment of 3E10 with mutations for improved DNA binding affinity, 3E10 (D31N) di-scFv, has previously been produced in P. pastoris and yielded promising preclinical findings, but is unsuitable for clinical testing. The present study reports the design, expression and testing of a panel of humanized 3E10 (D31N) di-scFvs, some of which contain CDR substitution. These variants were expressed in a modified CHO system and evaluated for their physicochemical attributes and ability to penetrate nuclei to selectively cause DNA damage accumulation in and kill cancer cells with DNA repair defects. Secondary structure was conserved and most variants retained the key characteristics of the murine 3E10 (D31N) di-scFv produced in P. pastoris. Moreover, several variants with CDR substitutions outperformed the murine prototype. In conclusion, we have designed several humanized variants of 3E10 (D31N) di-scFv that have potential for application as monotherapy or conjugates for targeted nuclear drug delivery.



## 1. Introduction

Challenges associated with the low therapeutic index and associated off-target toxicity of small molecule chemotherapy drugs has given rise to an ever-expanding portfolio of antibodies targeted against cancer antigens. The past decade has seen an exponential growth of antibodies used in the treatment of malignancies, and more recently, with gains in linker chemistry and an understanding of antibody properties, antibody-drug conjugates [1] have emerged as a viable platform to improve chemotherapeutic outcomes. While such antibody therapeutics have been successful, their utility in targeting intracellular antigens has been limited due to their inability to directly penetrate cells and access intracellular compartments [2]. Hence, most antibodies in clinical development are against extracellular target antigens [3].

Autoantibodies reactive to intracellular host antigens are associated with inflammation and poor prognosis in autoimmune diseases [4,5]. Most cell-penetrating autoantibodies are toxic, but 3E10, an anti-DNA autoantibody isolated from the MRL/lpr lupus mouse model, has been found to penetrate nuclei in a manner that is non-toxic to normal cells [6]. 3E10 inhibits DNA single and double-strand break (DSB) repair and is synthetically lethal to cancer cells defective in DNA repair machinery (e.g. BRCA1/2 and PTEN mutations), but spares DNA repair-proficient cells [7,8]. Additionally, 3E10 is attracted to DNA released by necrotic cancer cells, and preferentially accumulates at tumor sites *in vivo* suggesting its use in cancer therapy [7].

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Previously a divalent 3E10 single chain variable fragment with improved DNA binding affinity (3E10 (D31N) di-scFv), hereafter referred to as di-scFv was evaluated [9]. Di-scFv lacks the unnecessary 3E10 Fc region that has potential to contribute to non-specific toxicity. Di-scFv has yielded promising pre-clinical results both as a monotherapy against DNA repair-deficient tumors and as a drug delivery ligand [9,10], but due to C-terminal Myc and $His_6$ tags is unsuitable for human use. Herein, we report the design, expression and evaluation of a panel of novel humanized di-scFv variants that lack any tags with a view to utilizing them as novel anticancer agents or drug delivery ligands.

## 2. Material and methods

### 2.1. Reagents

Unless otherwise stated, all cell culture reagents, protein L, and primary and secondary antibodies were obtained from Invitrogen (Carlsbad, CA, USA).

### 2.2. In silico humanization of di-scFv

Murine di-scFv with C-terminal Myc and $His_6$ tags was previously designed and evaluated using a combination of *in vitro* and *in vivo* studies [9]. Di-scFv lacks an Fc, and therefore efforts to humanize di-scFv focused on VL and VH domains. Sequence alignments between the murine di-scFv VL and VH, and human germline sequences identified the optimal acceptor framework for di-scFv VL and VH. J-segments were also selected. *In silico* CDR-grafting was subsequently performed wherein amino acids in the di-scFv framework sequence were changed to that of the human sequence. Subsequently, a series of back-mutations were selected to maintain di-scFv function by preserving the VL-VH interface and Vernier zone. Following completion of CDR-grafting and selection of back-mutations, the resulting sequences were immunoprofiled against 85 HLA class II allotypes to identify Th epitopes. Sequences were also screened for potential post-translational modification (PTM) sites. In some cases, substitutions within CDRs were permitted to reduce immunogenicity and screen for effects on cellular penetration. Ultimately, a panel of six distinct humanized VL (three containing a CDR change, and three without CDR changes) and six VH (three containing a CDR change and three without CDR changes) were selected, and 16 different combinations of these fragments were selected for expression (Supplementary Information Table S1).

### 2.3. Protein expression & purification

The murine (yeast) di-scFv prototype was expressed in *P. pastoris* and purified using approaches described elsewhere [9]. For novel di-scFv prototype expression, cDNAs encoding the murine di-scFv sequence or each of the 16 humanized variants were synthesized and sub-cloned into a proprietary CHO expression vector. Stable CHO cell pools expressing the antibody fragments were generated in CD-CHO media (Invitrogen) and a base feed utilized for the expression culture (bolus feed was added on day four, and the cultures harvested at day eight). The medium was subsequently centrifuged to remove cellular matter, and filtered using a 0.22 μm pore-sized filter. Purification of di-scFv variants was achieved using a two-step process including ion exchange chromatography and NaCl gradient elution (over 20 column volumes) on an Äkta system equipped with a HiTrap Capto S column at a (5 mL/min flow rate) (GE Healthcare). Following purification, samples were concentrated using Amicon Ultra-15 filters (Millipore) and subjected to analysis of monomeric purity using SEC-HPLC (see supplementary information, Table S2).

### 2.4. Cell lines

A matched pair of isogenic BRCA2-proficient and -deficient DLD-1 colon cancer cells (Horizon Discovery Ltd) were grown in RPMI-1640 supplemented with 10% *v/v* FBS. PTEN-deficient U251 and PTEN-proficient U251-PTEN glioma cells were a gift from Peter Glazer and grown in high glucose DMEM supplemented with 10% *v/v* FBS. PTEN induction in U251-PTEN was achieved by the addition of 400 μg/mL G418, 2 μg/mL blasticidin, and 1 μg/mL doxycycline as described elsewhere [9]. PTEN-deficient U87 glioma cells were grown in high-glucose DMEM containing 10% *v/v* FBS.

### 2.5. Sample preparation

For phospho-53BP1 foci formation and trypan blue exclusion assays, di-scFv variant samples were exchanged into respective growth media (i.e. DMEM or RPMI 1640) and sample concentration was quantified using the Bradford assay. Subsequently, SDS-PAGE with Coomassie staining (Invitrogen) was performed to confirm di-scFv stability in the samples. All samples were filter sterilized using a 0.22 μm pore-sized syringe filter, and the concentration of di-scFv in PBS was quantified using absorbance at 280 nm with a Nanodrop 1000 (Thermo Fisher Scientific).

### 2.6. In silico prediction of secondary structure and disorder tendency

Sequence-based prediction of di-scFv secondary and higher order structure was performed using Phyre 2 [11]. To predict short and long-range disorder, sequence data for murine and humanized di-scFv variants E and J was inputted into IUPRED, and the disorder tendency score for each residue was generated [12,13].

### 2.7. Circular dichroism

The secondary structure of murine di-scFv and variants E and J was examined using a Chirascan spectrafluorimeter (Applied Photophysics). Samples analyzed by CD were buffer exchanged into a 10 mM pH 6 potassium phosphate buffer (MWCO 10 kDa) prior to analysis. Individual CD spectra were collected between 260 and 190 nm at 20 °C using a 1 mm path length quartz cuvette. A sampling time of one second, step size of 1 nm and bandwidth of 1 nm was used for each measurement. Buffer spectra were also collected using the same data acquisition parameters, and subtracted from sample spectra. The resultant data was converted to molar ellipticity and mean residue ellipticity. Additionally, raw CD data (mdeg) was inputted into CAPITO [14] and BESTSEL [15] to determine the approximate secondary structure content of samples examined.

### 2.8. Cell penetration assays

DLD-1 cells cultivated in 96-well plates were treated either with control vehicle or 10 μM di-scFv variant(s) for 60 min at 37 °C. Subsequently, treated cells were washed with PBS twice, fixed with pre-chilled 100% ethanol. Fixed cells were blocked, incubated with protein L (Invitrogen) and probed with a chicken anti-protein L primary antibody (Invitrogen). Subsequently, cells were treated with an Alexa Fluor 555-conjugated goat anti-chicken antibody (Invitrogen) for one hour at ambient temperature. A DAPI counterstain was applied to aid qualitative assessment of di-scFv localization. All timings and reagents were kept constant for analysis of cell penetration.

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