Optimizing a Lupus Autoantibody for Targeted Cancer Therapy

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Abstract

The specificity of binding by antibodies to target antigens is a compelling advantage to antibody-based cancer therapy, but most antibodies cannot penetrate cells to affect intracellular processes. Select lupus autoantibodies penetrate into cell nuclei, and the potential for application of these antibodies in cancer therapy is an emerging concept. Here, we show that a divalent lupus anti-DNA autoantibody fragment with enhancing mutations that increase its ability to penetrate cell nuclei and bind DNA causes accumulation of DNA double-strand breaks in and is highly and selectively toxic to cancer cells and tumors with defective homology-directed repair of DNA double-strand breaks. These findings provide proof of principle for the use of optimized lupus autoantibodies in targeted cancer therapy.

Introduction

Rational design of targeted cancer therapies requires development of safe and specific modulators of tumor targets. The unrivaled specificity of binding by antibodies to their antigens gives them a compelling therapeutic advantage over other molecules that have significant off-target effects. However, most antibodies do not cross plasma membranes and cannot directly affect intracellular processes. Select lupus autoantibodies have the unusual capacity to penetrate into cells, and we now recognize a potential new paradigm in antibody-based cancer therapy in which cell-penetrating lupus autoantibodies are used to disrupt key intracellular processes to selectively affect cancer cells. The nuclear-localizing lupus anti-DNA autoantibody 3E10 is the prototype that exemplifies this approach. 3E10 penetrates into cell nuclei, binds DNA, and inhibits both base excision repair (BER) and homology-directed repair (HDR) of DNA double-strand breaks (DSB; ref. 1). The degree to which 3E10 inhibits these DNA repair pathways is not sufficient to kill normal cells, but cancer cells with preexisting defects in HDR due to BRCA2 deficiency are somewhat sensitive to 3E10 (1). These findings revealed the potential to apply 3E10 as a targeted therapy for HDR-deficient tumors. However, an optimized 3E10 derivative with increased effect on HDR-deficient cancer cells is needed to allow translation of this discovery into a clinically relevant therapy.

In seeking to optimize 3E10 for targeted therapy of HDR-deficient malignancies, we have chosen to focus on 3E10 fragments that are missing Fc regions and carry less risk of nonspecific Fc-mediated activation of complement or antibody-dependent cell-mediated cytotoxicity (ADCC). A 3E10 single-chain variable fragment (hereafter referred to as scFv) that lacks an Fc region and has a D31N mutation in the V\textsubscript{H} region of the first complementarity determining region (CDR1) has previously been generated (2, 3). The D31N mutation significantly increases the DNA-binding affinity and efficiency of nuclear penetration by scFv, but despite this scFv still has only a modest effect on BRCA2-deficient cancer cells (1). We set out to build a more potent 3E10 fragment based on the scFv platform.

The primary two biochemical terms used to describe the quality of interaction between an antibody and its target antigen are the binding affinity and avidity. Affinity specifically quantifies the strength of the interaction between a single antigen–binding domain and the target antigen. Avidity, by contrast, refers to the strength of the overall interaction between the antibody and the antigen, and takes into account the exponential improvement in binding afforded by the presence of multiple antigen–binding sites in multivalent antibodies (4). The binding affinity of scFv to DNA has already been enhanced by the D31N mutation, but because scFv possesses only a single DNA–binding domain there remains room to build on the fragment’s binding avidity. It has previously been established that multivalent antibody fragments bind with much greater avidity to target antigens compared with monovalent fragments (5, 6), and we therefore hypothesized that a divalent 3E10 fragment with D31N mutations would have significantly increased avidity for DNA compared with scFv, and therefore yield more efficient accumulation of DNA damage in and have a more potent impact on the survival of HDR-deficient cancer cells. To test this hypothesis, a 3E10 di–single-chain variable fragment with D31N mutations (hereafter referred to as di-scFv) was generated and evaluated for potential as a targeted therapy for HDR-deficient malignancies.

Materials and Methods

Recombinant proteins

ScFv was generated as previously described (1). Di-scFv, a 3E10 di–single-chain variable fragment with D31N mutations, was
generated by recombinant fusion of two scFv fragments. Inter-
vening linker sequences were placed between scFv segments in di-
scFv as previously described (7). cDNA-encoding di-scFv was
ligated into the pPICZaA yeast expression vector, and pPICZaA-
di-scFv was transfected into X-33 cells. Di-scFv was expressed in
and purified from X-33 supernatant using the techniques as
previously described (8–10). Protein concentrations were deter-
mined by UV spectrophotometry using a NanoDrop 1000
(Thermo Fisher Scientific). Typical final yield of purified di-scFv
from a 1 L culture was 1 mg.

Cell lines

H1975, HCT116, SW48, and the matched pair of isogenic
BRCA2-proficient and -deficient DLD1 colon cancer cells were
obtained from Horizon Discovery Ltd., which performs cell line
validation by short tandem repeat profiling. PTEN-deficient
U251, U251 transfected with a tetracycline-inducible PTEN
expression vector system (U251-PTEN), and BRCA2-deficient
CAPAN-1 cells were a gift from Peter Glazer. U251 and U251-
PTEN cell lines were grown in DMEM (Life Technologies)
supplemented with 10% FBS (Sigma-Aldrich). U251, U251-
PTEN, and CAPAN-1 cells were allowed to adhere to glass coverslips
overnight at 37°C prior and then treated with control buffer or 5 μmol/L
scFv or di-scFv for 1 hour at 37°C. Cells were then extensively washed with PBS, fixed with chilled 100%
hanol for 5 minutes, washed again with PBS, and then probed
with an anti-Myc antibody (clone 9E10) overnight at 4°C. Cells
were then washed and then probed with Alexa488-conjugated
goat anti-mouse IgG antibody for 1 hour at 4°C (Cell Signaling
Technology). Cells were then washed and counterstained with
propidium iodide (PI; Sigma-Aldrich) for 30 minutes at room
temperature to allow visualization of cell nuclei. Nuclear pene-
tration by the antibodies and PI staining was then imaged using an
EVSOS fl digital fluorescence microscope (Advanced Microscopy
Group) using GFP and red fluorescent protein (RFP) filters (×40
magnification; Life Technologies). GFP and RFP images were
merged using ImageJ (NIH, Bethesda, MD).

Cell-penetration assays

DLD1 cells grown in 96-well plates were treated with control
buffer or 5 μmol/L scFv or di-scFv for 1 hour at 37°C. Cells were then extensively washed with PBS, fixed with chilled 100% ethanol for 5 minutes, washed again with PBS, and then probed
with an anti-Myc antibody (clone 9E10) overnight at 4°C. Cells
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merged using ImageJ (NIH, Bethesda, MD).

γH2AX and phospho-53BP1 foci

U251, U251-PTEN, and CAPAN-1 cells were allowed to adhere
glass coverslips overnight at 37°C prior and then treated with
control buffer, scFv, or di-scFv. Cells were then incubated at 37°C
for 4 or 24 hours and then washed with PBS before fixing with 4%
formaldehyde in PBS at 4°C for 15 minutes. Cells were then washed again and then blocked with 5% goat serum diluted in
PBS with 0.3% Triton X-100 for 60 minutes at 4°C. Cells were then
incubated overnight at 4°C with either anti-γH2AX or anti-
phospho-53BP1 (Ser1778) antibodies (Cell signaling Technolo-
yes). Cells were subsequently washed and probed with Alexa555-
conjugated goat anti-rabbit IgG (H+L; Life Technologies) for 60
minutes at 4°C. Next, cells were washed with PBS and mounted
onto microscope slides with ProLong Gold Antifade reagent with
DAPI for nuclear counterstaining (Cell Signaling Technology).
Slides were cured for 24 hours in the dark before imaging using an
Axiostar 200 microscope (Carl Zeiss MicroImaging, Inc.). Mean
number of foci per cell was determined from counts in at least 100
cells per condition.

Clonogenic survival assays

Surviving fractions of cells treated with control media or media
containing scFv or di-scFv were determined by colony formation
assay as previously described (1).

CAPAN-1 tumor study

CAPAN-1 tumors were established in athymic (NCr nu/nu)
males mice ages 5 to 6 weeks by s.c. injection of 5 × 10^6
CAPAN-1 cells in the right flank. Sixteen mice were injected with tumor cells,
and tumors with consistent growth were successfully established in
15 of the mice. One mouse tumor showed early stalling in
growth and was excluded from analysis. When tumors reached
volume of approximately 100 mm^3 mice were treated with i.p.
injection of di-scFv (40 mg/kg; n = 8) or an equivalent volume of
control PBS (n = 7) weekly for 3 weeks. Tumor volumes and
mouse body weights were tracked during the experiment, and at
closure of the experiment mice were sacrificed and tumors
were excised and masses recorded. All in vivo work was performed
in accordance with institutional guidelines under an Institutional
Animal Care and Use Committee-approved protocol.

Statistical analysis

P values were determined by the two-tailed Student t test. Error
bars in figures represent SEM.
Di-scFv is more toxic to BRCA2-deficient cancer cells than scFv

The impact of scFv and di-scFv on the clonogenic survival of BRCA2-deficient CAPAN-1 cells was compared. CAPAN-1 cells were treated with scFv or di-scFv (0–6.6 μmol/L) and surviving fractions were determined by colony formation assay as previously described (1). Di-scFv was observed to be significantly more toxic to the CAPAN-1 cells than scFv (Fig. 3A). For example, at a dose as low as 0.8 μmol/L, di-scFv reduced the surviving fraction of the cells to 0.51 ± 0.04, compared with 0.99 ± 0.13 with scFv (P = 0.05). At 6.6 μmol/L, di-scFv reduced the surviving fraction of the cells to 0.05 ± 0.02 compared with 0.20 ± 0.04 by scFv (P = 0.03). In these dose ranges the effect of di-scFv on the survival of the CAPAN-1 cells is similar to a previous report of the effect of PARP1 inhibition on CAPAN-1 cells (11).

To confirm that BRCA2 deficiency is associated with cellular sensitivity to di-scFv, we next compared the effects of scFv and di-scFv on the clonogenic survival of an isogenic pair of BRCA2-proficient and -deficient DLD1 colon cancer cells (12). The cells were treated with control media or 10 μmol/L scFv or di-scFv, and surviving fractions relative to control were determined by colony formation assay. As shown in Fig. 3B, neither scFv nor di-scFv were significantly toxic to the BRCA2-proficient cells (surviving fractions of 0.92 ± 0.05, P = 0.1, and 0.99 ± 0.06, P = 0.6, respectively), but both reduced the surviving fraction of the BRCA2-deficient cells (surviving fractions of 0.71 ± 0.06, P = 0.006, and 0.29 ± 0.09, P = 0.007, respectively). These data further demonstrate that di-scFv has a greater impact on BRCA2-deficient cancer cells than scFv and also show that di-scFv is not toxic to matched BRCA2-proficient cells.

Di-scFv is not toxic to MLH1-deficient cancer cells, but is synthetically lethal to PTEN-deficient U251 glioma cells

To further confirm that di-scFv is not simply universally cytotoxic, it was tested against additional cell lines. Specifically, H1975 lung cancer cells with intact DNA repair, SW48, and
HCT116 colon cancer cells with defective DNA mismatch repair due to MLH1 deficiency, and U251 glioma cells were treated with control media or media containing 3.3 μmol/L scFv or di-scFv, and surviving fractions were determined by colony formation assay. As expected, neither fragment was significantly toxic to the H1975 cells that have intact DNA repair mechanisms. Similarly, neither fragment had any significant impact on survival of the MLH1-deficient SW48 and HCT116 cells, indicating that mismatch repair defects do not confer sensitivity to the fragments. Consistent with our previous results, scFv was not toxic to the U251 cells (1). However, di-scFv was highly toxic to the U251 cells, with surviving fraction reduced to 0.06 ± 0.03 (P < 0.0001; Fig. 3C). The large impact of di-scFv on the U251 cells was somewhat surprising, particularly given that the scFv had no detectable impact on the cells. However, U251 glioma cells are known to be PTEN-deficient, and an association between PTEN status and HDR efficiency has been suggested (13–15). In addition, the observed effect of di-scFv on U251 cell survival is comparable with the previously reported impact of the PARP1 inhibitor olaparib on U251 cells (16). We hypothesized that PTEN deficiency was responsible for the observed sensitivity to di-scFv, and to confirm this, we obtained matched PTEN-proficient U251 cells (U251-PTEN) and tested their sensitivity to 3.3 μmol/L di-scFv. As shown in Fig. 3D, the U251-PTEN cells were not sensitive to di-scFv. To the best of our knowledge, this is the first evidence to suggest synthetic lethality resulting from exposure of PTEN-deficient cancer cells to a lupus autoantibody fragment.

**Di-scFv causes accumulation of DNA DSBs in PTEN- and BRCA2-deficient cancer cells**

BRCA2 deficiency is well known to result in defective HDR and to confer sensitivity to inhibition of both DNA single- and double-strand break repair mechanisms. The details of the association between PTEN status and HDR efficiency are less clear, but several groups have reported impaired HDR in and increased sensitivity of PTEN-deficient cells to genotoxic stress and to inhibition of PARP1 (13, 14, 17–19). We therefore hypothesized that the reason the PTEN- and BRCA2-deficient cells exhibit increased sensitivity to di-scFv compared with scFv is that the increased avidity and larger size of di-scFv yields greater inhibition of DSB repair and accumulation of DNA DSBs in these cells. To test this hypothesis U251 cells were treated with control media or 25 μmol/L scFv or di-scFv for 4 or 24 hours, and the number of DNA DSBs present were visualized by immunostaining for γH2AX foci. ScFv did not yield any increase in γH2AX foci at either time point, with 11.7 ± 1.3 and 11.2 ± 1.0 mean foci per cell in control cells at 4 and 24 hours, respectively, compared with 11.7 ± 1.2 and 8.8 ± 0.8 mean foci per cell in cells treated with scFv. Di-scFv similarly did not significantly increase the mean foci per cell after 4 hours of exposure (13.8 ± 1.5 mean foci per cell, P = 0.27), but 24 hours of exposure to di-scFv increased the mean number of foci per cell to 25.1 ± 1.2 (P < 0.0001), which is consistent with a time-dependent accumulation of DNA DSBs due to inhibition of BER and HDR in these PTEN-deficient cells (Fig. 4A and B). By contrast, 24 hours of exposure to di-scFv did not cause any significant increase in γH2AX foci in PTEN-proficient U251-PTEN cells (11.2 ± 1.1 mean foci per cell in control cells compared with 13.7 ± 1.2 and 11.7 ± 1.2 in cells treated with scFv and di-scFv, respectively; Fig. 4C). The impact of di-scFv on γH2AX foci in BRCA2-deficient CAPAN-1 cells was also evaluated, and as expected 24 hours of exposure to di-scFv resulted in a significant increase in the numbers of γH2AX foci (14.9 ± 2.0 foci in cells treated with di-scFv compared with 9.3 ± 1.8 foci in control cells; P = 0.04; Fig. 4D and E).

**p53-binding protein 1 (53BP1)** is phosphorylated in response to DNA damage and recruited to sites of DNA DSBs, and as a second test to confirm that di-scFv yields an increase in DNA DSBs in PTEN-deficient cells, we evaluated the effect of di-scFv on phospho-53BP1 foci in the U251 and U251-PTEN cells. The matched pair of cells was treated for 24 hours with control media or 20 μmol/L di-scFv and then phospho-53BP1 foci were visualized by immunofluorescence. Consistent with the results
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The effect of 3E10 on BRCA2-deficient tumors has not previously been tested in vivo, primarily because the modest effect of the original antibody on BRCA2-deficient cells in culture was not considered sufficient to be likely to yield an observable impact on gross tumors. However, as detailed above, di-scFv exhibited potent toxicity to BRCA2-deficient cancer cells, and we therefore hypothesized that di-scFv could be able to yield detectable suppression of the growth of BRCA2-deficient tumors in vivo, and thereby provide proof of principle for the use of a lupus autoantibody fragment as a single-agent against BRCA2-deficient tumors. BRCA2-deficient CAPAN-1 subcutaneous xenografts were generated in immunodeficient mice, and when tumors reached size of approximately 100 mm\(^3\), the mice were treated with three weekly i.p. injections of control buffer or di-scFv (40 mg/kg). Tumor volumes were followed, and masses were recorded at the end of the experiment. As predicted, di-scFv significantly suppressed the growth of the tumors, evidenced by decreased tumor volumes and masses (Fig. 6A and B). For example, at the midpoint of the experiment (2 weeks after the first treatment) mean tumor volume in mice treated with control buffer was 476 ± 54 mm\(^3\) compared with 277 ± 29 mm\(^3\) in mice treated with di-scFv (P = 0.005). At the end of the experiment (4 weeks after the first treatment) mean tumor volume in mice treated with control buffer was 1,102 ± 235 mm\(^3\) compared with 606 ± 57 mm\(^3\) in mice treated with di-scFv (P = 0.05; Fig. 6A). Mean tumor mass at the end of the experiment in mice treated with control buffer was 0.73 ± 0.2 g compared with 0.33 ± 0.03 g in mice treated with di-scFv (P = 0.03; Fig. 6B). Di-scFv was well tolerated by the mice with no observable toxicities. Mean body weights were indistinguishable between the mice treated with control buffer or di-scFv (Fig. 6C). To the best of our knowledge, this is the first demonstration of suppression of growth of an HDR-deficient tumor by a lupus autoantibody fragment.

**Discussion**

Tumor-specific targets sequestered inside cells and nuclei are inaccessible to most antibodies, which has been a limiting factor in antibody-based cancer therapy. Only seven antibodies are presently FDA approved for the treatment of solid tumors,

![Image](image-url)
and all target extracellular antigens (EGFR, HER2, CTA-4, or VEGF; ref. 20). The use of the cell-penetrating lupus autoantibody 3E10 to selectively affect cancer cells is a new approach. 3E10 penetrates cells through an equilibrative nucleoside transporter (ENT2) that is expressed in nearly all cells (9), and once inside the nucleus 3E10 appears to incompletely inhibit BER and HDR, which is not toxic to normal cells, but is synthetically lethal to cells that have preexisting defects in HDR due to BRCA2 deficiency (1). The targeted effect of 3E10 and its fragments on HDR-deficient cancer cells, therefore, is not necessarily due to selective penetration into cancer cells, but rather to the selective sensitivity of HDR-deficient cancer cells to further inhibition of DNA repair by 3E10. This is markedly distinct from nonpenetrating antibodies that target cancer cells by recognizing and binding to antigens that are overexpressed on the surface of certain cancer cells (20).

A further difference between 3E10 and nonpenetrating antibodies relates to respective methods of tumor toxicity. Nonpenetrating antibodies such as cetuximab that target extracellular receptors depend in part on Fc-mediated activation of ADCC and complement to exert an effect on tumors, and elimination of the Fc, therefore, diminishes the magnitude of their effect (21). By contrast, the ability of 3E10 to penetrate nuclei, bind DNA and inhibit DNA repair, and exert a synthetically lethal effect on BRCA2-deficient cells is independent of the 3E10 Fc region (1). This important difference between 3E10 and other antibodies is the primary reason that we have focused our efforts on optimizing 3E10 fragments that lack an Fc region for cancer therapy rather than making adjustments to the full 3E10 antibody. Specifically, 3E10 fragments that lack an Fc should be unable to activate ADCC and complement, and therefore will carry a lower risk of nonspecific side effects in clinical trials that may be considered in the reasonably near future.

As described above, even at high doses the magnitude of inhibition of DNA repair by scFv results in only a modest effect on cancer cells with defective HDR (1), and therefore the potential to use scFv by itself as a therapeutic agent targeted against HDR-deficient tumors is limited. However, we have now shown that di-scFv, a divalent 3E10 fragment with enhancing D31N mutations, has a greater effect on HDR-deficient cancer cells, but still remains nontoxic to cells with intact HDR. The increased potency of di-scFv is likely related to increased avidity for DNA due to its second binding site. The larger size of di-scFv also provides further advantages over scFv, including likely greater steric hindrance of DNA repair factors, decreased renal filtration in vivo and therefore a longer circulating half-life, and increased accumulation in tumor tissue due to the enhanced permeability and retention phenomenon (22, 23).

Another important finding that has emerged from the present study is an apparent association between PTEN deficiency and sensitivity to di-scFv. PTEN is linked to numerous cellular functions, including AKT signaling, cell-cycle regulation, HDR, and maintenance of genomic integrity (13, 15, 19, 24–26). We have previously shown that 3E10 inhibits BER and HDR, and have now found that di-scFv causes a selective increase in DSBs in PTEN-deficient cells. Taken together, these findings are most consistent with a preexisting impairment in DNA DSB repair in the PTEN-deficient cells, likely related to defective HDR, being responsible for their sensitivity to di-scFv. PTEN deficiency is associated with many malignancies, including breast, prostate, glioma, ovarian, endometrial, melanoma, and lung cancers (15, 27–33). Additional studies are needed to fully explore the potential to use di-scFv against PTEN-deficient tumors, but the finding that PTEN deficiency may be predictive of sensitivity to di-scFv significantly increases the number of malignancies that may be susceptible to 3E10-based therapy.

Disclosure of Potential Conflicts of Interest
G. Chan, R.H. Weisbart, and J.E. Hansen have ownership interest (including patents) as inventors on Yale University patent filing “Cell-penetrating anti-DNA antibodies and uses thereof to inhibit DNA repair.” No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.W. Noble, J.E. Hansen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.W. Noble, J.E. Hansen
Writing, review, and/or revision of the manuscript: P.W. Noble, M.R. Young, J.E. Hansen
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Other (developed the molecular construct used in these studies): R.H Weisbart

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