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In Vitro Evaluation of Humanized/De-immunized Anti-PSMA Immunotoxins for the Treatment of Prostate Cancer

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Abstract. *Background: We generated humanized/de-immunized immunotoxins targeting the prostate-specific membrane antigen (PSMA) and tested their cytotoxic activity against prostate cancer cells in vitro. Materials and Methods: The humanized/de-immunized version of our murine anti-PSMA single-chain antibody fragment (scFv) D7, termed hD7-1(VL-VH), was ligated to the 40-kDa toxin domain of Pseudomonas aeruginosa exotoxin A (PE40), and to the deimmunized 24-kDa toxin domains PE24 or PE24mut. The immunotoxins designated as hD7-1(VL-VH)-PE40, hD7-1(VL-VH)-PE24 and hD7-1(VL-VH)-PE24mut were bacterially expressed and purified by affinity chromatography. Binding and cytotoxicity were examined by flow cytometry and viability assay, respectively. Results: All immunotoxins revealed strong binding to prostate cancer cells expressing PSMA and specific cytotoxicity, with half-maximal inhibitory concentration values in the picomolar range. Conclusion: We successfully created powerful anti-PSMA immunotoxins with reduced immunogenicity for further clinical development and application against advanced prostate cancer.*

Current treatment against advanced prostate cancer still requires improved progressive concepts with enhanced efficacy. One possibility is the development of immunotoxins against the prostate-specific membrane antigen (PSMA), which has been demonstrated to be an excellent target in various preclinical and clinical trials against prostate cancer (1-5).

By our group, immunotoxins were constructed by recombinant fusion of the anti-PSMA single chain antibody fragment (scFv) D7 to the truncated toxin domain of *Pseudomonas aeruginosa* exotoxin A (PE), called PE40 (6, 7). The scFv D7 was derived from our murine monoclonal antibody (mAb) to PSMA 3/F11 (8, 9). The PE40 domain consists of the structural domains II, Ib and III (Figure 1). Domain II, designated as the transmembrane domain, is responsible for permeabilization through cellular membranes and cytosolic localization. Domains Ib and III cause ADP-ribosylation of the elongation factor 2 (eEF2) on the ribosomes, resulting in inhibition of protein biosynthesis and effective cell death (10).

Despite their powerful cytotoxic features, a major problem of PE-based immunotoxins is their high immunogenicity in patients, which is the result of the non-human origin of the antibody-binding domain and the bacterial background of the toxin domain. This makes such immunotoxins inappropriate for clinical application (11). Since multiple immunotherapy cycles are a prerequisite in achieving clinical responses, repeated applications of immunogenic immunotoxins might lead to an activation of the patient's immune system with consequent formation of anti-drug antibodies (ADA), thereby triggering severe side-effects (12, 13). Therefore, many attempts have been made to diminish the immunogenicity of immunotoxins by humanization/de-immunization of the antibody binding domains (14, 15), modification with macromolecules (16-19), or structural changes of the PE domain (20-22). Several preclinical studies described that deletions or mutations within the PE toxin domain led to the removal of immunodominant B- and T-cell epitopes and successfully contributed to diminished immunogenicity and reduced ADA production (20-22).

The goal of the present work was to generate less immunogenic anti-PSMA immunotoxins by use of a humanized/de-immunized anti-PSMA scFv as binding domain and de-immunized PE variants as toxin domains, and to test their binding and cytotoxic efficacy on prostate cancer cells.

Materials and Methods

Cell lines. The androgen-sensitive PSMA-expressing prostate cancer cell line LNCaP, its androgen-independent subline C4-2, and the PSMA-negative line DU145 (ATCC, Manassas, VA, USA) were

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cultured in RPMI 1640 medium (Gibco, Invitrogen, Karlsruhe, Germany) with 10% fetal calf serum (Biochrom, Berlin, Germany) with addition of streptomycin (100 mg/l) and penicillin (100 U/ml) at 37˚C with 5% CO_2. Cell lines were authenticated by short tandem repeats (STR) analysis (Cell Lines Services GmbH, Eppelhein, Germany). Anti-PSMA mAbs 3/F11 and J591 (ATCC, Manassas, VA, USA) were purified from hybridoma supernatant by protein G-sepharose chromatography (GE Healthcare Biosciences AB, Uppsala, Sweden) and dialyzed against phosphate-buffered saline (PBS). Protein content was determined using a Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific, Darmstadt, Germany).

Construction and purification of the humanized/de-immunized anti-PSMA scFv and immunotoxins. Humanized/de-immunized versions of the variable domains of the heavy (VH) and light (VL) chains of the murine anti-PSMA scFv D7 were designed by *in silico* modeling (Lonza Biologics Company, Cambrige, UK). Both chains were synthesized (Gene Art Gene Synthesis, Thermo Fisher Scientific, Regensburg, Germany) and cloned in a VL-VH orientation into the vector pHOG21 containing a flexible glycine-serine G_4S linker and *C*-terminal human c-myc and hexahistidine tags for protein detection and purification. The scFv was called hD7-1(VL-VH).

For the construction of the immunotoxin hD7-1(VL-VH)-PE40, the DNA sequence encoding *P. aeruginosa* exotoxin domain PE40 was fused onto the *C*-terminus of the scFv (Figure 1A). To reduce the immunogenicity of the PE domain, the transmembrane domain II (aa 251-364) was partially deleted, with exception of the furin-cleavable site (aa 274-284, RHRQPRGWEQL), resulting in a 24 kDa toxin domain, called PE24. Domain III of the PE24 domain was further de-immunized by the insertion of the following mutations: R427A, R458A, D463A, R467A, R490A, R505A, R538A (20). This variant was named PE24mut. The DNA of the PE24 and PE24mut domains was optimized for *Escherichia coli* expression, synthesized (Gene Art Gene Synthesis) and *C*-terminally cloned to the scFv hD7-1(VL-VH). This resulted in the immunotoxins hD7-1(VL-VH)-PE24 (Figure 1B) and hD7-1(VL-VH)-PE24mut (Figure 1C). Sequences of all immunotoxins were proved for correctness by Sanger sequencing (GATC, Konstanz, Germany).

The scFv and the immunotoxins were expressed in the periplasm of *E. coli* XL-1 blue and purified by immobilized metal affinity chromatography (IMAC), as described previously (23). Protein content was determined using a Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Purity and size of the anti-PSMA scFv and immunotoxins were determined by SDS-PAGE (Invitrogen, Carlsbad, CA, USA) as described earlier (6). The SDS gels were stained using Coomassie Brillant Blue (InstantBlue Protein Stain, Expedeon; Biozol Diagnostica Vertrieb GmbH, Eching, Germany).

Flow cytometry. Binding of the anti-PSMA scFv and immunotoxins was characterized utilizing PSMA-positive LNCaP and C4-2 cells, as well as PSMA-negative DU145 cells by flow cytometry. For this, 2×10^5 cells in PBS (with 3% fetal bovine serum and 0.1% sodium azide) were incubated with anti-PSMA scFv or immunotoxins at different concentrations for 1 hour at 4˚C. Then cells were washed three times with cold PBS, incubated with mouse anti-human c-myc antibody (Roche Diagnostics, Mannheim, Germany) for 1 hour at 4˚C, and labelled with secondary goat anti-mouse IgG-RPE antibody (Becton Dickinson, Mountain View, CA, USA) for 1 hour in the darkness at 4˚C. Cells were washed again and resuspended in 200 μl PBS containing propidium iodide (1 μg/ml) (Sigma Aldrich, St. Louis, MO, USA) to exclude dead cells. Mean fluorescence intensity (MFI) values were determined by flow cytometry (FACS Calibur) and the binding affinity (Kd), defined as the half-maximal saturation concentration, was calculated using the integrated CellQuest Pro software (BD Biosciences, Heidelberg, Germany).

To test competitive binding, LNCaP cells were pre-incubated with increasing concentrations of the murine anti-PSMA mAb 3/F11 or J591. Then 5 μg/ml of the humanized/de-immunized scFv hD7-1(VL-VH) was added to the cells for 1 hour on ice and detected using the secondary antibodies rat anti-human c-myc mAb (Bio-Rad AbD Serotec GmbH, Puchheim, Germany) and goat anti-rat IgG R-PE (Thermo Fisher Scientific) for an additional 40 minutes on ice before flow cytometry.

In vitro cytotoxicity. WST-1 cell viability assay (Roche Diagnostics, Indianapolis, IN, USA) was performed to determine the cytotoxic activity of the immunotoxins. 1.5×10^4 cells/well in a 96-well plate were treated with different concentrations of anti-PSMA immunotoxins alone for 24, 48, and 72 hours. After that, plates were incubated with WST-1 reagent and the optical density was measured at 450 nm (Ref. 690 nm; ELISA reader, BMG Labtech, Ortenberg, Germany). The IC_{50} values, defined as a 50% decrease in cell viability, were estimated utilizing non-linear regression fit with the equation: [log (inhibitor) *vs*. response (three parameters)] (GraphPad Prism 6 Software; GraphPad Inc., San Diego, CA, USA) as described elsewhere (6).

Results

Generation, purification and binding of the humanized/de-immunized anti-PSMA scFv. The humanized/de-immunized anti-PSMA scFv hD7-1(VL-VH) was cloned into the vector pHOG21, periplasmatically expressed in *E. coli* and purified via IMAC. The high-grade purity of the 32 kDa protein was assessed by SDS-PAGE (Figure 2A). The scFv in the VL-VH orientation exhibited a higher binding to PSMA-positive LNCaP and C4-2 cells (K_d=4.7 nM and 3.7 nM, respectively, Figure 2B) compared to the scFv in the VH-VL orientation (data not shown). No binding was detected to PSMA-negative DU145 cells (Figure 2C). Binding inhibition of the scFv hD7-1(VL-VH) with the parental murine anti-PSMA mAb 3/F11 proved that the scFv retained binding to the same PSMA epitope. No binding inhibition was seen with the anti-PSMA control mAb J591, which is known to bind to a different PSMA epitope (Figure 2D) (8). Due to its strong and specific PSMA binding, the scFv hD7-1(VL-VH) was identified as a suitable candidate for the construction of the immunotoxins.

Generation, purification and binding of the humanized/de-immunized anti-PSMA immunotoxins. Genes of native toxic domain PE40 and of the de-immunized variants PE24 and PE24mut were inserted C-terminally to the scFv hD7-1(VL-VH) in the vector pHOG21. All immunotoxins were

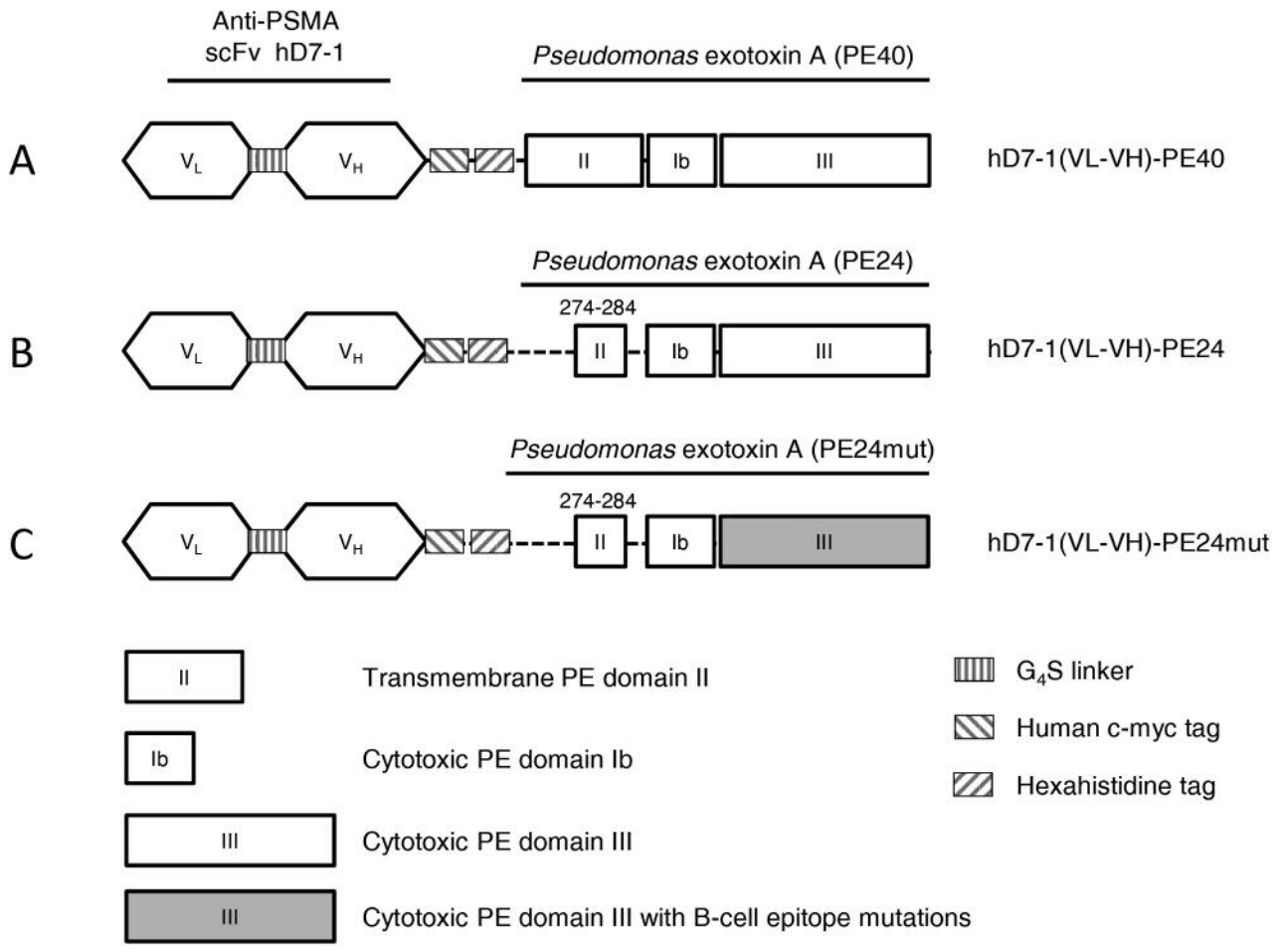

Figure 1. *Schematic representation of the de-immunized anti-prostate specific membrane antigen (PSMA) immunotoxin. A: hD7-1(VL-VH)-PE40 containing the original cytotoxic Pseudomonas exotoxin A domain (PE40) consisting of the domains II, Ib and III, B: hD7-1(VL-VH)-PE24 containing the toxic domain PE24 consisting of the deleted PE domain II (with exception of the furin-cleavable linker (amino acids 274-284), Ib and III, and C: hD7-1(VL-VH)-PE24mut with additional mutations in immunodominant B-cell epitopes within domain III (R427A, R458A, D463A, R467A, R490A, R505A, R538A). G_4S linker, protein linker (amino acids GGGGS); scFv, single chain; V_L, variable domain of the antibody light chain; V_H variable domain of the antibody heavy chain.*

bacterially expressed and purified in the same way as the scFv. The immunotoxin hD7-1(VL-VH)-PE40 with a molecular weight of about 72 kDa was characterized by SDS-PAGE. The immunotoxins hD7-1(VL-VH)-PE24 and hD7-1(VL-VH)-PE24mut showed an expected molecular size of about 55 kDa (Figure 3A).

The flow cytometric experiments demonstrated that all immunotoxins were capable of specific and efficient binding to PSMA-positive androgen-sensitive LNCaP and androgen-resistant C4-2 cells with comparable K_d values. With the immunotoxin hD7-1(VL-VH)-PE40, Kd values of 26.4 nM and 9.2 nM for LNCaP and C4-2 cells were yielded. With hD7-1(VL-VH)-PE24, Kd values of 10.1 nM and 6.1 nM and with D7-1(VL-VH)-PE24mut K_d values of 20.9 nM and 10.6 nM were achieved for LNCaP and C4-2 cells, respectively (Figure 3B). Flow cytometric analysis of the immunotoxins on PSMA-negative DU145 cells revealed no binding (Figure 3C).

Cytotoxicity of humanized/de-immunized immunotoxins. The immunotoxins hD7-1(VL-VH)-PE40, hD7-(VL-VH)-PE24 and hD7-1(VL-VH)-PE24mut were tested for specific cytotoxic activity against PSMA-positive LNCaP and C4-2 cells, as well as PSMA-negative DU145 cells. As demonstrated in Figure 4, IC_{50} values of 55 pM and 25 pM against LNCaP and of 37 pM and 6 pM against C4-2 cells for hD7-1(VL-VH)-PE40 were reached after 48- and 72-h incubation, respectively. In addition, high and specific

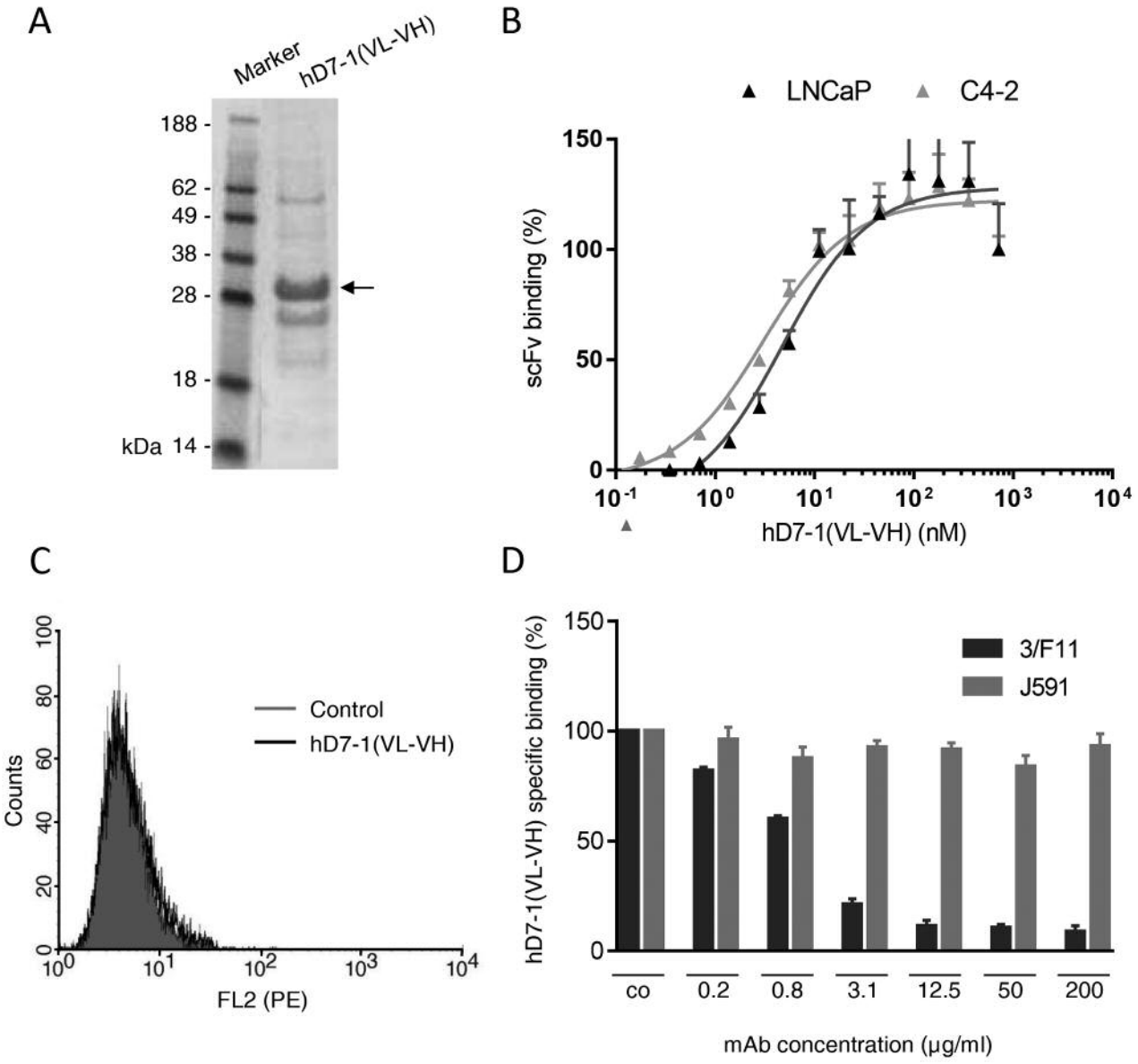

Figure 2. *A: Purified humanized/de-immunized single chain antibody fragment (scFv) hD7-1(VL-VH) by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis. B: Binding of hD7-1(VL-VH) to prostate-specific membrane antigen (PSMA)-expressing LNCaP and C4-2 prostate cancer cells by flow cytometry. Data represent three independent experiments±SEM. C: Binding of hD7-1(VL-VH) at saturation concentration to PSMA-negative DU145 cells by flow cytometry. D: Binding inhibition of hD7-1(VL-VH) by the parental murine monoclonal antibody (mAb) 3/F11 and the control mAb J591.*

cytotoxicity of hD7-1(VL-VH)-PE24 was achieved. With IC_{50} values of 121 and 82 pM against LNCaP and of 126 pM and 24 pM against C4-2 cells after 48 and 72 h, respectively, it showed about 2- to 5-fold reduced cytotoxicity compared to the immunotoxin hD7-1(VL-VH)-PE40 containing the complete transmembrane domain II. The immunotoxin hD7-1(VL-VH)-PE24mut, additionally containing mutated B- and T-cell epitopes within domain III, was shown to be less cytotoxic, with IC_{50} values of 180 pM and 138 pM against LNCaP and of 39 and 42 pM against C4-2 cells. No cytotoxicity was seen for any of the tested immunotoxins against DU145 cells (Figure 4C). Taken together, our findings show that de-immunization of the PE toxin domain led to a retention of specificity, however, it also slightly reduced cytotoxicity against PSMA-expressing prostate cancer cells.

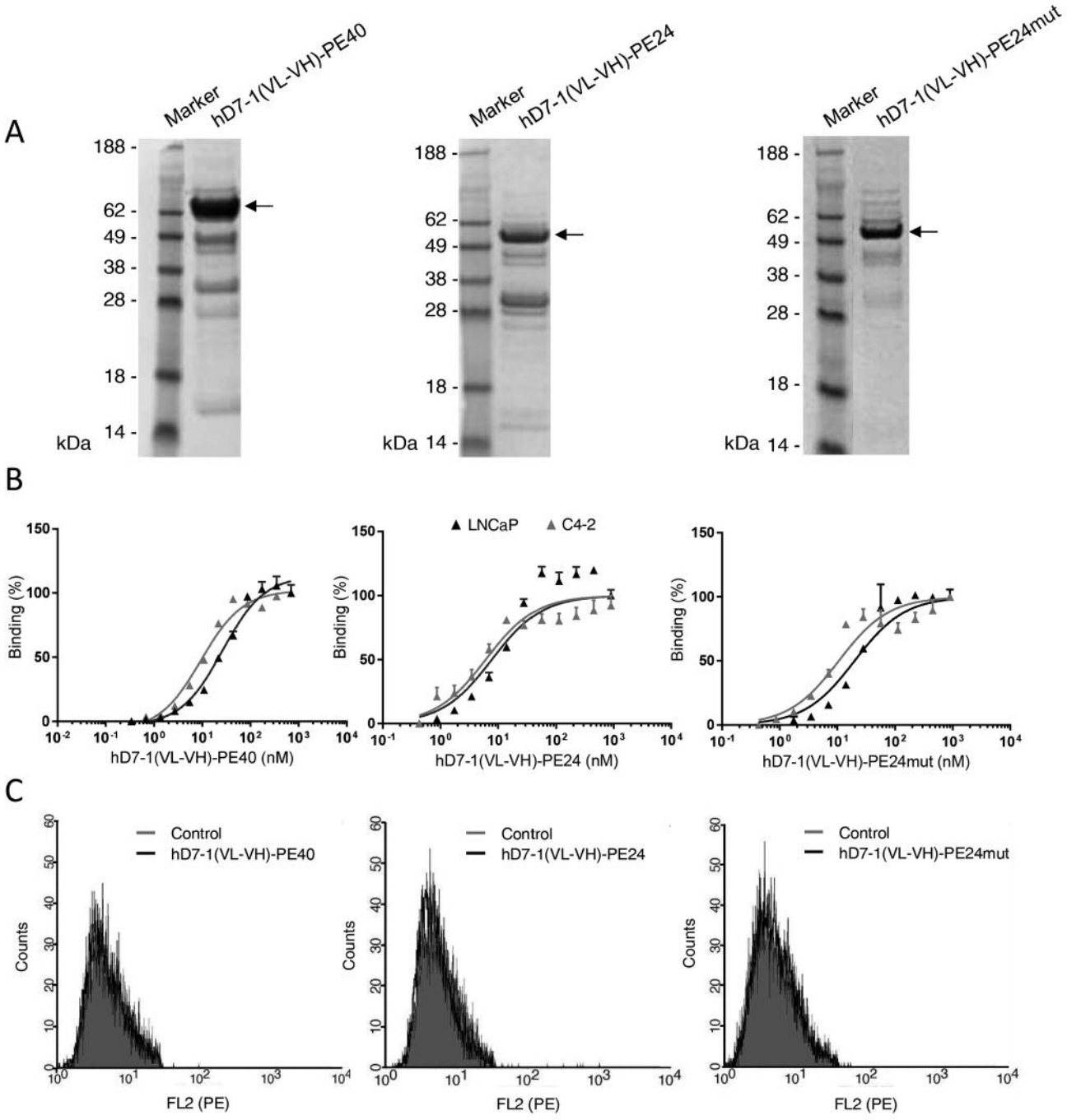

Figure 3. *A: High purity of the de-immunized immunotoxins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). B: Flow cytometric analysis of the immunotoxin-specific binding to LNCaP and C4-2 cells by flow cytometry, data represent three independent experiments±SEM. C: Binding of the immunotoxins to prostate specific membrane antigen (PSMA)-negative DU145 cells by flow cytometry.*

Discussion

The goal of the present work was the construction of anti-PSMA immunotoxins with low immunogenicity that could serve as future therapeutic agents against prostate cancer still remaining incurable in advanced stages. Firstly, we used a humanized/de-immunized variant of our murine scFv D7(VL-VH) (6, 23) to avoid immunogenicity of the PSMA-binding domain. The humanized/de-immunized scFv hD7-1(VL-VH) showed the same PSMA-epitope binding as its

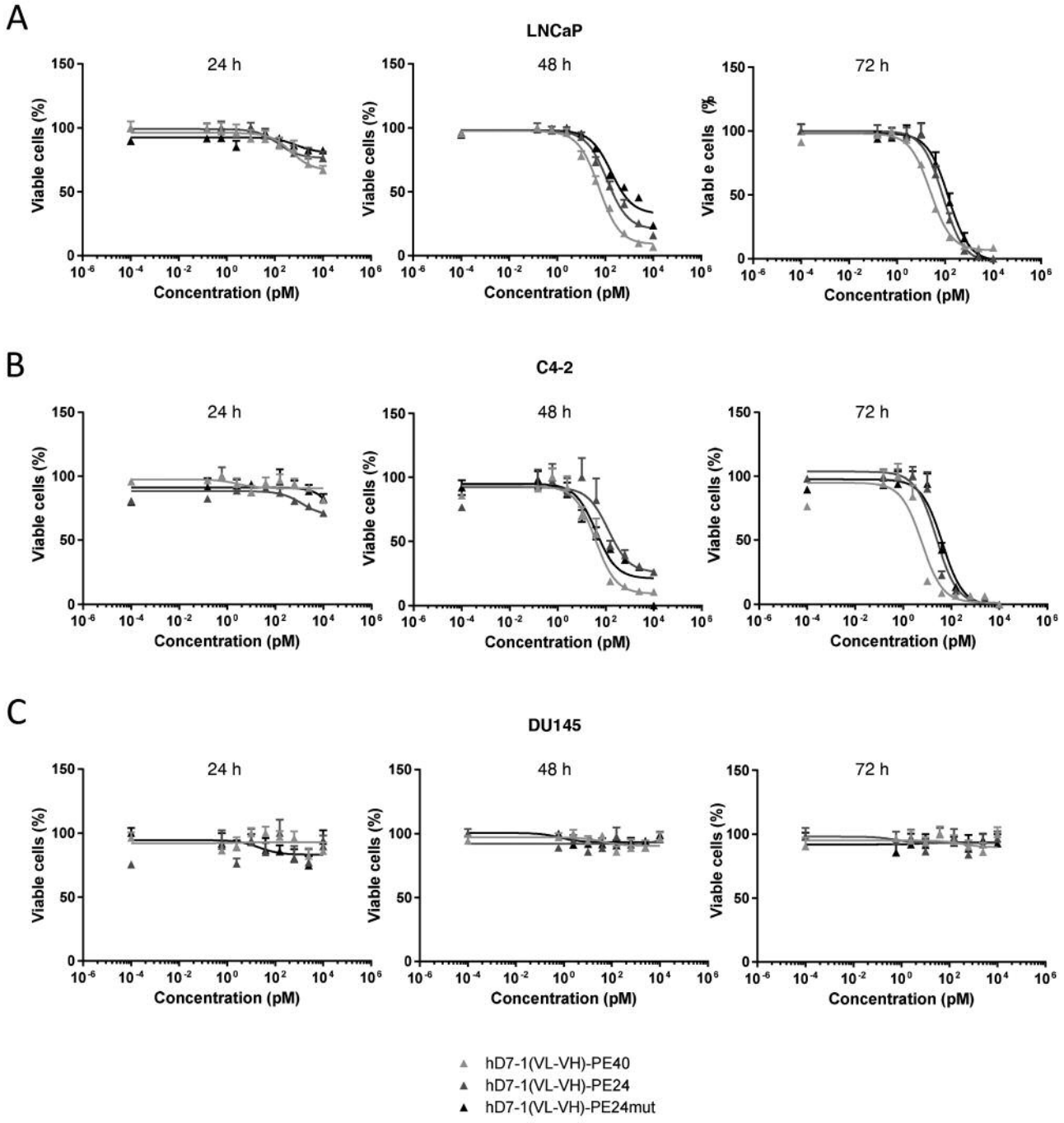

Figure 4. *Cytotoxic efficacy of the de-immunized immunotoxins on A: LNCaP, B: C4-2, and C: DU145 cells after 24, 48 and 72 h as measured by WST-1 viability assay.*

parental murine scFv D7(VL-VH). Moreover, our data demonstrate that this scFv binds very strongly, with Kd values in the low nanomolar concentration. Therefore, hD7-1(VL-VH) was proven to be particularly suitable for the development of targeted immunotoxins as it was shown that scFv with binding affinities in the nanomolar range have optimal tumor accumulation and penetration (24-26).

PE-based immunotoxins with truncated 40 kDa or 38 kDa domains have been previously used in preclinical and clinical trials against various tumors (27-29). There are currently

ongoing phase I and II clinical studies with PE38-based immunotoxins against hematological and solid tumors. Wayne *et al*. characterized the safety profile of CD22-PE38 (HA22, moxetumomab pasudotox) against acute lymphoblastic leukemia in 55 patients and demonstrated moderate immunogenicity. Continuous administration (10 doses of 50 μg/kg) of this immunotoxin, however, resulted in hemolytic uremic syndrome and thrombotic microangiopathy in six out of 14 patients (30). Kreitman *et al*. also showed that moxetumomab pasudotox was immunogenic in 10 out of 28 examined patients developing ADA during the study (31). The application of immunotoxins against solid tumors is much more complex than against hematological malignancies. ADA formation is present as early as after one therapy cycle due to the strong patient's immune system, which does not occur or is very rare in patients suffering from hematological tumors (32). In a phase I study, Hassan *et al*. demonstrated that the majority of patients (n=34) produced ADA with grade 3 toxicities (related to fatigue, back pain, hypoalbuminemia, hypotension) even after a single dose of SS1P immunotoxin (SS1-dsFv-PE38) against mesothelin-positive solid tumors (33, 34).

In order to diminish immunogenicity of the PE domains, structural analyses have been undertaken to identify and mutate immunodominant epotipes. Mazor *et al*. described that deletion of domain II can silence the immunodominant B- and T-cell epitopes, and thereby reduce the immunogenicity of the toxin domain and diminish the production of ADA (11, 35). Furthermore, it has been shown that structural and functional changes within the translocation domain II of PE result in enhanced stability (36, 37). Weldon *et al*. constructed an anti-CD22 immunotoxin (HA22-LR) against hematological malignancies (37) and an anti-mesothelin immunotoxin (SS1-LR-GSS) against solid tumors (38) with lysosome protease resistance. They demonstrated that deletion of domain II or insertion of specific mutations within domain III led to enhanced stability without loss of its activity (36-38). Moreover, they found that deletion of domain II, with exception of the furin-cleavable site (aa 274-284), resulted not only in higher stability but also in an approximately two-fold higher cytotoxicity compared to the initial construct (37). According to these publications, we generated the humanized/de-immunized anti-PSMA immunotoxin hD7-1(VL-VH)-PE24 with the deleted transmembrane domain II retaining the furin-cleavable site. The specific PSMA binding was maintained and high and specific cytotoxicity against PSMA-expressing prostate cancer cells was demonstrated. However, the partial deleted transmembrane domain II within the hD7-1(VL-VH)-PE24 immunotoxin resulted in reduced cytotoxicity, indicating that these changes might contribute to a diminished release of the toxin domain into the cytosol. Further experiments will be conducted to analyze the cellular fate of the immunotoxin in detail. The reduced cytotoxicity is in contrast to the publication of Weldon *et al*., where an approximately twofold enhanced cytotoxicity of the deimmunized immunotoxin HA22-LR compared to the immunotoxin with the PE38 domain was described (37).

Besides deleting B- and T-cell epitopes within the transmembrane domain II, it was shown that specific mutations within the PE domain III silenced additional B-cell epitopes and led to reduced immunogenicity (20, 21). Liu *et al*. found that mutations of seven immunodominant B-cell epitopes to alanine (R457A, R458A, D463A, R467A, R490A, R505A, R538A) led to partial or complete disappearance of binding of neutralizing antibodies (20, 39). We implemented these mutations in our immunotoxin hD7-1(VL-VH)-PE24mut and found that these mutations did not affect binding to prostate cancer cells. We also obtained specific cytotoxicity comparable with that of the immunotoxin hD7-1(VL-VH)-PE40 (IC_{50} values in picomolar range). Further work is needed to examine the antitumor activity in *in vivo* models.

In our present work, we generated highly specific and cytotoxic de-immunized anti-PSMA immunotoxins that might be suitable for a future improved targeted-therapy of advanced prostate cancer.

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