Efficient Cancer Therapy with a Nanobody-Based Conjugate

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ABSTRACT

Nanobodies are the smallest fragments of naturally occurring single-domain antibodies that have evolved to be fully functional in the absence of a light chain. Nanobodies are strictly monomeric, very stable, and highly soluble entities. We identified a nanobody with subnanomolar affinity for the human tumor-associated carcinoembryonic antigen. This nanobody was conjugated to Enterobacter cloacae β-lactamase, and its site-selective anticancer prodrug activation capacity was evaluated. The conjugate was readily purified in high yields without aggregation or loss of functionality of the constituents. In vitro experiments showed that the nanobody–enzyme conjugate effectively activated the release of ptylenediamine mustard from the cephalosporin nitrogen mustard prodrug 7-(4-carboxybutanamido) cephalosporin mustard at the surface of carcinoembryonic antigen-expressing LS174T cancer cells. In vivo studies demonstrated that the conjugate had an excellent biodistribution profile and induced regressions and cures of established tumor xenografts. The ease of generation and manufacturing yield of nanobody-based conjugates together with their potent antitumor activity make nanobodies promising vehicles for new generation cancer therapeutics.

INTRODUCTION

Carcinoembryonic antigen (CEA) is highly expressed on cancer cells of epithelial origin, such as colorectal, lung, breast, and ovarian carcinoma (1). It is not expressed in other cells of the body except for low-level expression in gastrointestinal tissue. This expression profile makes it an attractive target for tumor therapy.

Antibodies have become a rapidly expanding class of pharmaceuticals for treating cancer (2). Depending on the final use, antibodies are engineered to modify their biological properties. Core technologies are aimed at designing molecules with high specificity and functionality; antibodies are reduced in size, rebuilt into multivalent formats, and fused to several compounds to improve their efficiency for cancer therapy. Critical factors in the development of effective conjugates for cancer therapy are their homogeneity, absence of regions prone to aggregation or susceptible to proteolysis, affinity and specificity, high solubility, and stability.

The hunt for the smallest antibody fragment still capable of binding to antigens has progressed from full antibody molecules to Fab and recombinant single-chain Fv fragments. These smaller molecules have improved tumor penetration, faster blood clearance, and reduced immunogenicity compared with the complete antibody. Despite their beneficial properties, scFvs and their conjugates are still amenable for immunogenicity compared with the complete antibody. Despite their improved tumor penetration, faster blood clearance, and reduced immunogenicity, scFvs and their conjugates are still amenable for immunogenicity compared with the complete antibody.

Functional heavy-chain antibodies devoid of light chains are naturally occurring in nurse sharks (5), wobbegong sharks (6) and elidae (7). Their antigen-binding site is reduced to a single domain, the VHH domain. Because the variable domain of the heavy-chain antibodies is the smallest fully functional antigen-binding fragment with a molecular mass of only 15 kDa, we refer to this entity as a nanobody. Their small size and robustness (8, 9) make them particularly suitable for targeting antigens in obstructed locations, such as tumors, where tissue penetration is critical. Moreover, nanobodies derived from camelds show high homology with the human VH3 gene family (10).

We previously demonstrated that cAb-Lys3, a nanobody that inhibits lysozyme activity in vitro (8), effectively targets tumors and metastatic lesions transgenic for hen egg lysozyme in a scid mouse model (11). Here we report the isolation of nanobodies specific to CEA and their use as modular building units for the construction of immunoconjugates, using the hinge of llama heavy-chain antibodies as natural linker. Conjugates are readily purified in high yields without out aggregation or loss of functionality. Conjugate treatment in a targeting strategy known as antibody-dependent enzyme prodrug therapy (12) effected immunologically specific cell kill and led to regressions and cures of established tumor xenografts.

MATERIALS AND METHODS

Construction of the Nanobody Library and Retrieval and Purification of Binders. We constructed the nanobody library from the immunized dromedary as described (13). The phage-display library was used for panning on human CEA (Scripps, San Diego, CA) coated on microtiter plates (2 μg/ml). Selection of enriched clones was performed by ELISA, and clones were sequenced to remove doubles. Proteins of five positive clones were purified from periplasmic fractions (14) with use of Ni-NTA resin (Qiagen) followed by size-exclusion chromatography on Superdex 75HR 16/70 (Pharmacia, Gaithersburg, MD) in PBS.

Affinity Determination and Epitope Mapping. The kinetic binding parameters, k on and k off , were determined with an IAsys Biosensor (Affinity Sensors, Cambridge, United Kingdom) or a Biacore3000. CEA protein was coupled to the sensor through its carbohydrate moiety by periodate oxidation and reductive amination. Epitope recognition was assessed with an IAsys Biosensor. The nanobody was added at saturating concentrations (10 7–2 3 × 10 −7 M) to the cuvette containing immobilized CEA and allowed to bind. Thereafter, a second VHH was added, and binding of the latter was monitored.

Stability Measurements. The functional stabilities of the nanobodies were determined by incubating purified proteins in PBS at 37°C for 24 h before measuring CEA binding activity with the IAsys instrument.

VHH melting curves were recorded on a JM-715 spectropolarimeter (Jasco, Tokyo, Japan) in the range 30°C–90°C with a temperature gradient of 1°C/min at a fixed wavelength of 203 nm.

Fluorescence-Activated Cell-Sorting Analysis. We incubated 10 6 LS174T human colon adenocarcinoma cells with anti-CEA VH5 (5 μg/ml), washed them with Dulbecco’s Balanced Salt Solution, and incubated them with mouse anti-β-galactosidase antibody (Serotec, Bicester, United Kingdom). Cells were stained with fluorescein-conjugated sheep antimouse IgG (ICN Biomedicals, Irvine, CA), and analyzed on a FACSVantage fluorescence-activated cell sorter (Becton Dickinson, San Jose, CA). Monoclonal mouse anti-CEA IgG C6G9 (Sigma) was used as a positive control.

Cloning and Expression of cAb-CEA5: β-Lactamase (BL). The BL gene was first amplified from Enterobacter cloacae P99 strain (15) and cloned as a Neo-EcoRI fragment with use of primers βL forward (5′-CATGCGCAT- GGCACGGCATGTGGTAC AAAA-3′) and βL reverse (5′-GGGCTGAAT- TCD TATGATGTAGTAGTGATGCTGAGCGCGCCGAGG-3′; the Neo- EcoRI restriction sites are underlined). The cAb-CEA5 llama γc hinge was

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Note: V. Cortez-Retamozo and N. Backmann contributed equally to this work.

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then designed as a NcoI fragment by use of the sense primer 5'-CATGCCATGACTGGCGGAGGGTCG-3' and the antisense primer 5'-CATGCCATGACTGGCGGAGGGTCG-3' and the antisense primer 5'-CATGCCATGACTGGCGGAGGGTCG-3' and the antisense primer 5'-CATGCCATGACTGGCGGAGGGTCG-3'.

The conjugate was purified to homogeneity from periplasmic fractions of Top 10 F' Escherichia coli cells. The antilysozyme nanobody cAb-Lys3 conjugated to βL was also engineered and used as a non-CEA-binding control. The 29-mer llama IgG2a upper hinge was used as linker for this construct (17). All conjugate preparations were stored in PBS at 4°C.

Characterization and Activity of cAb-CEA5::βL. The binding characteristics of the VHH portion of the fusion protein were determined by use of a surface plasmon resonance biosensor (Biacore). Enzymatic activity assays for the βL portion of cAb-CEA5::βL were performed with nitrocefin as the substrate (18). The increase in the 490:630 nm absorbance ratio ($\Delta A_{490:630}$) was linear and directly proportional to the specific activities of cAb::βL-containing solutions.

In Vivo Cytotoxicity. LS174T cells (10⁵/well) were allowed to adhere overnight. For blocking experiments, cells were incubated with native cAb-CEA5 (0.1 mg/ml) before conjugate treatment. Cells were exposed to conjugate at 1, 5, and 10 nm. After 30 min at 4°C, cells were washed with RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum. Subsequently, different concentrations of produg 7-(4-carboxybutanamido) cephalosporin mustard (CCM) or the drug phenylenediamine mustard (PDM) were added (19). CCM and PDM were also added to cells that were not treated with conjugates. After 1 h at 37°C, cells were washed with EMEM and incubated for 24 h. Cells were then pulsed for 18 h with [3H]thymidine (1 Ci/well). Radioactivity was counted with a beta-plate counter. Another set of experiments was performed with cAb-Lys3::βL control.

Conjugate Localization. Subcutaneous LS174T adenocarcinoma tumors were established in female athymic nu/nu mice (8 weeks of age; Harlan, the Netherlands). Tumor-bearing mice received i.v. injections containing 1 mg/kg 125I-labeled cAb-CEA5::βL conjugate (Iodo-Gene; Pierce, Rockford, IL). At various times, cohorts of three mice were sacrificed. Blood, organs, and tumors were removed, and the radioactivity was counted in a gamma counter. cAb-Lys3::βL was used as the non-CEA-binding control.

In Vivo Therapy Experiments. LS174T tumor-bearing mice (five animals/group; average tumor volume, 150 mm³) received i.v. injections containing cAb-CEA5::βL at 1 mg/kg, followed 24 h later by CMM at different doses. Control mice were either untreated or received PDM (4 mg/kg) or cAb-Lys3::βL. This process was repeated weekly for a total of three rounds. Tumor volume was determined by use of the formula: $V = \text{length} \times \text{width} \times \text{depth}/2$. Mice were removed from the study before their tumor volumes reached 2000 mm³, at which point average tumor sizes from the remaining mice were no longer plotted.

RESULTS

Identification of Nanobodies against CEA. After immunizing a dromedary with CEA, we cloned its nanobody repertoire from 10⁷ peripheral blood lymphocytes in a phage-display vector. Panning of the resulting library with immobilized CEA yielded five CEA-specific nanobodies. Four nanobodies were produced in E. coli and purified.

### Table 1 Melting temperatures determined by circular dichroism and kinetic constants of anti-carcinoembryonic antigen antibodies as determined by IAsys biosensor

<table>
<thead>
<tr>
<th>Antibody fragment</th>
<th>$T_{209}$ (°C)</th>
<th>$k_{on}$ (1/μs)</th>
<th>$k_{off}$ (1/μs)</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAb-CEA5</td>
<td>45.4</td>
<td>1.2 x 10^9</td>
<td>3.3 x 10^9</td>
<td>40.4</td>
</tr>
<tr>
<td>cAb-CEA61</td>
<td>36.5</td>
<td>6.2 x 10^9</td>
<td>1.7 x 10^9</td>
<td>254.5</td>
</tr>
<tr>
<td>cAb-CEA72</td>
<td>10.5</td>
<td>4.1 x 10^9</td>
<td>3.9 x 10^9</td>
<td>254.5</td>
</tr>
<tr>
<td>cAb-CEA5::βL</td>
<td>10.5</td>
<td>3.9 x 10^9</td>
<td>3.9 x 10^9</td>
<td>310.5</td>
</tr>
<tr>
<td>cAb-CEA5::βL</td>
<td>40.34</td>
<td>6.2 x 10^9</td>
<td>1.7 x 10^9</td>
<td>254.5</td>
</tr>
<tr>
<td>cAb-CEA3::βL</td>
<td>ND</td>
<td>4.1 x 10^9</td>
<td>3.9 x 10^9</td>
<td>310.5</td>
</tr>
<tr>
<td>cAb-CEA5::βL</td>
<td>310.5</td>
<td>4.1 x 10^9</td>
<td>3.9 x 10^9</td>
<td>310.5</td>
</tr>
</tbody>
</table>

* $T_{209}$ melting temperature; CEA, carcinoembryonic antigen; βL, β-lactamase; ND, not determined.

* $K_D$ values for cAb-CEA5 and cAb-CEA5::βL were estimated by use of BiAcore biosensor.

The cAb-CEA antibodies were stable entities: at least 88% of the initial antigen-binding activity was retained after a 24-h incubation in PBS at 37°C, and the heat-induced unfolding revealed high melting temperatures for the different binders (63°C–78°C; Table 1).

All nanobodies recognized a distinct nonoverlapping epitope on the CEA molecule as seen from the association traces of two nanobodies added sequentially to the IAsys cuvette (Fig. 1). Irrespective of the order of nanobody addition, after the first complex reached its equilibrium (first plateau of each sensogram in Fig. 1), it could not prevent the binding of any of the subsequently added nanobodies.

By flow cytometry we analyzed the binding specificities of the cAb-CEA antibody fragments to CEA expressed on LS174T cells and compared them with that of the anti-CEA mouse monoclonal antibody C6G9 (Fig. 2). Significant mean fluorescence shifts were observed (Fig. 2, A–D) that were comparable to that of anti-CEA mouse monoclonal antibody C6G9 (Fig. 2E). The level of staining obtained on incubation of LS174T cells with cAb-Lys3, a lysozyme-specific VHH, was indistinguishable from that obtained for cells mixed with FITC-labeled antihuman IgG only.

The affinity of the cAb-CEA antibodies for their antigen ($K_D$) ranged from 0.34 (cAb-CEA5) to 55 nm (cAb-CEA3; Table 1). The values for the association rate constants ($k_{on}$) of the different binders were within an order of magnitude, whereas their $k_{off}$ values differed nearly by two orders of magnitude, with cAb-CEA5 having the highest $k_{on}$ and the lowest $k_{off}$ values. This nanobody was therefore chosen as fusion partner for β-lactamase, an enzyme with excellent catalytic properties for converting cephalosporin-based prodrugs into potent toxins (20).

Production and Characterization of cAb-CEA5::βL Conjugate. The cAb-CEA5::βL conjugate was obtained as a single-chain construct of cAb-CEA5 and the mature E. cloacae P99 βL spaced by the llama γ2c hinge. Protein purification from bacterial periplasmic extracts by Ni-NTA and gel-filtration chromatography resulted in the isolation of ~2 mg pure conjugate/liter of culture. The conjugate migrated on SDS-PAGE as a single band under reducing and nonreducing conditions, and reverse-phase chromatography showed that the cAb-CEA5::βL preparation was >95% pure.

The CEA-binding capacity of the conjugate was unaltered compared with the parental nanobody (Table 1). Moreover, Michaelis–
Menten kinetic analysis of the lactamase moiety revealed a $K_m$ of 26.18 ± 2 μM and a $k_{cat}$ of 296.3 ± 20 s⁻¹ on nitrocefin, indicating that the fusion protein fully retained the enzymatic activity of non-conjugated E. cloacae P99 β-lactamase (21).

**Biodistribution Studies.** The ability of cAb-CEA5::βL to localize selectively at tumor sites was assessed by biodistribution analysis in mice bearing s.c. LS174T xenografts. The retention of both cAb-CEA5::βL and cAb-Lys3::βL in the tumor, blood, and organs was determined 6, 24, and 48 h after i.v. inoculation of iodinated recombinant proteins. Specific accumulation at the tumor site was observed for cAb-CEA5::βL but not for cAb-Lys3::βL (Fig. 3). After 48 h, tumor:organ ratios of cAb-CEA5::βL ranged from ~9:1 to 53:1 except for the kidneys, which apparently also contained a specific amount of radioactivity (tumor:kidney ratio of 2.7:1). However, an enzymatic activity assay using nitrocefin as substrate revealed that only the tumor, and not the kidneys, accumulated biologically active β-lactamase (data not shown).

**In Vitro Cytotoxicity Assays.** The cytotoxic effect of cAb-CEA5::βL in combination with CCM prodrug was determined on LS174T cells. The prodrug CCM (IC₅₀ = 37 μM) was ~40-fold less toxic to LS174T cells than the active drug PDM (IC₅₀ = 0.9 μM). cAb-CEA5::βL effectively activated the prodrug, leading to a cytotoxicity equivalent in activity to PDM (Fig. 4A). Prodrug activation was immunologically specific; no prodrug activation was observed after cells were saturated with unconjugated cAb-CEA5 before conjugate exposure or on exposure of cells to the nonbinding control conjugate cAb-Lys3::βL before CCM treatment (Fig. 4B).

**Therapeutic Activity.** In vivo therapy experiments were performed in nude mice with established LS174T xenografts. Conjugate treatment was initiated once the tumors reached ~150 mm³. CCM was administered 24 h later, and the treatment protocol was repeated weekly for another two rounds. Therapeutic effects were compared with those of PDM at its maximum tolerated dose.

Therapeutic efficacy was dose dependent (Fig. 5). Tumor cure was obtained in all animals that received 200 mg/kg CCM per injection after treatment with cAb-CEA5::βL. In the dosing schedule, significant antitumor activity was obtained, including one cure in the group of five mice when the CCM dose was reduced to 150 mg/kg per injection. All other mice, including the group who received 100 mg/kg per injection had tumors that underwent partial regression but eventually began to grow 20 days after the last prodrug treatment. There was no apparent toxicity in any of the treated groups. In contrast, weekly administration of PDM for three rounds at the maximum tolerated dose of 4 mg/kg led to toxicity and resulted in >10% body weight loss. PDM had no persistent antitumor activity; delay in tumor outgrowth was observed only during treatment, after which tumors progressed. Administration of the non-tumor-specific conjugate cAb-Lys3::βL followed by treatment with 150 mg/kg CCM per injection had no effect on tumor growth and was comparable to the untreated group (Fig. 5).

**DISCUSSION**

Although antibodies are realizing their potential as anticancer therapeutics, they are still amenable for improvement in terms of functionality (e.g., stability, affinity, specificity, and size) and pharmacokinetic properties (2). Reducing the size of the conventional antibody to a Fab or scFv by cloning the corresponding gene fragments and expression in bacteria is becoming a standard technique because it facilitates the mutagenesis of the antigen binder for the above aims (14). In addition, the smaller size and absence of Fc dramatically
We isolated a panel of anti-CEA nanobodies from a phage-display library derived from in vivo-matured camel heavy-chain antibodies. Binding affinity to CEA ranged from 0.34 to 55 nM, which is within the affinity range of other single-domain antibodies (16, 27) and comparable to the mouse anti-CEA scFv MFE-23 (2.5 nM; Ref. 28) or human scFv CEA6 (7.7 nM; Ref. 29). The isolated anti-CEA cAbs recognize different nonoverlapping epitopes on the CEA molecule, creating the possibility of generating biparatopic constructs (30). Prolonged incubation at 37°C did not affect the nanobodies, and they resisted thermal denaturation, as evidenced by a melting temperature in the range of 63°C–78°C, which far exceeds the melting temperatures obtained for human VH (56.6°C; Ref. 31) or scFv (57°C; Ref. 32).

To assess the potential of nanobodies as vehicles to selectively deliver toxic principles to tumors, we fused β-lactamase from E. cloacae P99 to the high-affinity binder cAb-CEA5. This particular β-lactamase was chosen because it effectively converts many substrates into potent cytotoxic compounds (20). We intentionally used the llama γ2c hinge because the natural flexibility of the immunoglobulin hinge ensures the independent movement of the connected variable domains in immunoglobulins in the natural antibody (33). This natural linker provided high conjugate stability because protein aggregates or breakdown products were not detected after purification and storage at 4°C for >45 days. The cAb-CEA5::βL conjugate was extracted as soluble protein and was functional in all respects because the nanobody entity in the conjugate recognized its antigen with the same affinity as the monomer and the enzyme retained full enzymatic activity.

The biodistribution studies showed that cAb-CEA5::βL not only cleared rapidly from the systemic circulation but also localized pref-
ationally in tumors without the need for clearance agents (Fig. 3). In other strategies, it has been necessary to accelerate systemic conjugate clearance by administration of glycosylated antibodies before prodrug injection (34). The high specific uptake of the immunocomplex in the tumor and its rapid clearance from nontarget organs suggest that only short time periods between conjugate and prodrug administration are necessary for therapeutic efficacy. This has been confirmed experimentally; we observed cures of established tumors without toxic effects when CCM was administered 24 h after the conjugate.

In conclusion, we have shown that cAB-CEA5::H9252 has properties that are well suited for selective anticancer prodrug activation. The fusion protein is homogeneous, localizes in solid tumor masses, and clears very rapidly from the systemic circulation. Finally, the favorable biophysical and pharmacological properties of nanobodies and the ease with which they can be formatted into multifunctional protein therapeutics make them ideal as a new generation of antibody-based therapeutics.

REFERENCES


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