Improved tumor targeting of anti–epidermal growth factor receptor Nanobodies through albumin binding: taking advantage of modular Nanobody technology

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Abstract

The ~15-kDa variable domains of camelid heavy-chain-only antibodies (called Nanobodies) can easily be formatted as multivalent or multispecific single-chain proteins. Because of fast excretion, however, they are less suitable for therapy of cancer. In this study, we aimed for improved tumor targeting of a bivalent anti–epidermal growth factor receptor (EGFR) Nanobody (αEGFR-αEGFR) by fusion to a Nanobody unit binding to albumin (αAlb). Biodistributions of αEGFR-αEGFR, αEGFR-αEGFR-αAlb (~50 kDa), αTNF-αTNF-αAlb (control, binding tumor necrosis factor-α), and the ~150-kDa anti-EGFR antibody cetuximab were compared in A431 xenograft-bearing mice. The proteins were radiolabeled with 177Lu to facilitate quantification. Tumor uptake of 177Lu-αEGFR-αEGFR decreased from 5.0 ± 1.4 to 1.1 ± 0.1 %ID/g between 6 and 72 h after injection. Due to its rapid blood clearance, tumor-to-blood ratios >80 were obtained between 6 and 72 h after injection. Blood clearance became dramatically slower and tumor uptake became significantly higher by introduction of αAlb. Blood levels of αEGFR-αEGFR-αAlb were 21.2 ± 2.5, 11.9 ± 0.6, and 4.0 ± 1.4 and tumor levels were 19.4 ± 5.5, 35.2 ± 7.5, and 28.0 ± 6.8 %ID/g at 6, 24, and 72 h after injection, respectively. Tumor uptake was at least as high as for cetuximab (15.5 ± 3.9, 27.1 ± 7.9, and 25.6 ± 6.1 %ID/g) and significantly higher than for αTNF-αTNF-αAlb. αEGFR-αEGFR-αAlb showed faster and deeper tumor penetration than cetuximab. These data show that simple fusion of αEGFR and αAlb building blocks results in a bifunctional Nanobody format, which seems more favorable for therapy as far as pharmacokinetics and tumor deposition are concerned. [Mol Cancer Ther 2008;7(8):2288–97]

Introduction

During the last decade, monoclonal antibodies (mAb) are gaining momentum in disease-specific therapy. Presently, the U.S. Food and Drug Administration has approved 21 mAbs for therapy, mostly for the systemic treatment of cancer (1). Particularly attractive for treatment of solid tumors are cetuximab [Erbitux directed to epidermal growth factor receptor (EGFR), HER1], trastuzumab (Herceptin to HER2), and bevacizumab (Avastin to vascular endothelial growth factor; refs. 2–4). These mAbs are aimed to completely neutralize/block growth factors or growth factor receptors, the key drivers of tumor growth and survival, for a prolonged period. Despite clinical enthusiasm, however, it is fair to state that efficacy of current mAbs is still quite limited, with benefit for just a portion of patients, whereas costs of mAb therapy are excessive (5). This conclusion comes with very recent insights that selective targeting of just one single tumor target might be insufficient for fully effective cancer treatment, a reason why also mixtures of mAbs are exploited in multitarget anticancer therapy (6, 7). The rationale for this approach is that cancer cells have the inherent ability to use several growth factor (receptor) systems for growth advantage and survival (8).

All mAbs except one that have been approved until now are intact mAbs. This is remarkable, because these large-sized molecules (~150 kDa) are considered to have limited capacity for tumor penetration (9). For this reason, much effort has been put in the evaluation of mAb fragments but without convincing therapeutic success to date (10). Although production of such fragments is not always straightforward and can result in diminished affinity, the main in vivo limitation of antibody fragments is their rapid blood clearance and the associated low tumor uptake. Especially molecules smaller than 60 to 70 kDa are generally filtered out by the kidneys very rapidly and are therefore not well qualified for long-term blockage of the growth factor receptor.

In the present study, we considered whether Nanobodies could deal with the aforementioned limitations of conventional mAbs. Nanobodies are derived from a unique antibody format that is present in species from the family of Camelidae, including llama, camel, and dromedary. These
animals contain, next to their conventional antibody repertory, an antibody class consisting of heavy chains only (11). Consequently, the variable region of the heavy chain from these “heavy-chain-only” antibodies (VHH) represents the complete binding unit of the antibody. Considering the small size of the VHH fragments (~15 kDa), this binding unit is also called Nanobody. A Nanobody is smaller than a single-chain Fv fragment, because it lacks both the light chain and the linker, whereas it has a similar affinity as variable regions of conventional mAbs. In addition, it does not show aggregations problems like single-chain Fv because of hydrophilic instead of hydrophobic patches in the VH and VH domains. Due to their single domain character, standard molecular biology techniques such as PCR allow for easy selection of appropriate Nanobody candidates from the full antibody repertory of immunized animals (12). Nanobodies show a high solubility and a good capacity to refold after denaturation while retaining their binding capacity (13, 14). Particularly attractive is the flexibility of drug formatting whereby it is straightforward to generate multivalent and/or multispecific single-molecule formats and to produce these formats in bacteria and yeast (13, 15). The relatively small size of the gene (~360 nucleotides) permits the fusion and expression as separate building blocks into proteins as dimers (~35 kDa), trimers (~50 kDa), or multimers. Initial biodistribution studies with monospecific bivalent antitumor Nanobodies in tumor-bearing mice showed, as expected, rapid blood clearance and low tumor uptake (16). Therefore, monospecific Nanobodies do not seem qualified for long-term blockade of growth factor receptors.

Very recently, Dennis et al. showed the possibility of using noncovalent albumin association as an elegant means to improve the pharmacokinetic properties of otherwise short-lived molecules (17, 18). For their studies, they used a F(ab) fragment of Herceptin, to which an albumin-binding peptide was fused, for imaging (18). We hypothesized that noncovalent interaction with albumin might be particularly attractive in Nanobody applications to improve their pharmacokinetics and tumor-targeting properties for therapy. To test this possibility, a single-chain Nanobody was constructed consisting of two EGFR-binding units (εEGFR) and one unit binding to mouse (and human) serum albumin (αAlb). In case of full functionality, this bispecific ~50-kDa Nanobody should show a prolonged half-life in blood as well as efficient tumor targeting. For this purpose, the εEGFR-αEGFR-αAlb Nanobody was evaluated for biodistribution as well as for tumor uptake and penetration along with the intact 150-kDa anti-EGFR mAb cetuximab in A431 xenograft-bearing nude mice. This Nanobody, as well as cetuximab, was radiolabeled to facilitate accurate quantitative analysis.

Materials and Methods

Nanobodies, Antibody, and Radioactivity

Nanobodies were obtained from Ablynx. Procedures for immunization of Llama glama and production, selection, and characterization of antagonistic anti-EGFR Nanobodies with or without αAlb unit have been described in great detail by Roovers et al. (12).

Nanobody εEGFR-αEGFR-αAlb consists of two units for specific binding to human EGFR and one unit for specific binding to murine and human serum albumin (αAlb, designated previously MSA21; see ref. 12). Nanobody εEGFR-αEGFR only consists of the EGFR-binding units. Nanobody αTNF-αTNF-αAlb comprises two units for specific binding to human (and not mouse) tumor necrosis factor-α (TNF-α; ref. 19). All Nanobody constructs contain a COOH-terminal His6 tag and a c-Myc tag.

Surface plasmon resonance data sets for the monovalent Nanobodies were obtained on the BIACore 3000 instrument (GE Healthcare), essentially as described before (20). For this purpose, BIACore CM5 sensor chips (GE Healthcare) were coated with purified EGFR (Sigma) or in house purified (human or mouse) albumin according to the manufacturer’s instructions at densities of ~5,000 or 500 relative units, respectively. Because no appropriate regeneration conditions for the EGFR coated CM5 chip were identified, the affinity of the EGFR-specific Nanobody was estimated via kinetic titration (21) avoiding successive regeneration steps. Association (k_a) and dissociation (k_d) rate constants as well as affinities (K_d) were calculated using BIACore BIAevaluation software 4.1. Kinetics and affinity constants are shown in Table 1.

Cetuximab (Erbitux concentration 2.0 mg/mL) was purchased from Merck. Cetuximab is a mouse-human chimeric anti-EGFR mAb that binds with high affinity (K_d = 0.3 nmol/L) to EGFR (22).

177Lu (725 GBq/mg) was obtained from Perkin-Elmer. 125I (3.7 GBq/mL) was purchased from Amersham Biosciences.

Radiolabeling

Radioimmunoconjugates were prepared for three sets of experiments in which biodistribution of Nanobodies and/or cetuximab was assessed and compared in tumor-bearing mice. For radiolabeling, the radionuclides 177Lu and 125I were used. 177Lu is a residualizing radiometal that shows high retention in tumor cells on internalization and catabolism of a 177Lu-labeled mAb and therefore optimally reflects antibody accumulation in the tumor. In contrast, 125I is a nonresidualizing radiohalogen, which becomes released from the tumor after internalization and catabolism of a 125I-labeled mAb. Combined use of both radio nuclides gives an indication of the level of in vivo internalization (23). For preparation of 177Lu-labeled monoclonal antibodies, 177Lu was coupled to Nanobodies or cetuximab via the chelate p-SCN-Bz-DOTA (Macroscylics), essentially as described previously (23). For preparation of 125I-labeled conjugates, 125I was directly coupled to Nanobodies or cetuximab essentially as described by Visser et al. (24). The following conjugates were prepared: 177Lu-cetuximab, 125I-cetuximab, 177Lu-εEGFR-αEGFR-αAlb, 125I-εEGFR-αEGFR-αAlb, 177Lu-εEGFR-αEGFR, and 177Lu-αTNF-αTNF-αAlb.

Analyses of Radioimmunoconjugates

All conjugates were analyzed by instant TLC for radiochemical purity, by high-performance liquid chromatography and SDS-PAGE followed by phosphorimager analysis.
for integrity, and by a cell-binding assay for immunoreactivity as described previously (23).

No binding assay was available for the $^{177}$Lu-αTNF-αAlb conjugate.

**Biodistribution Studies**

For assessment of biodistribution and the tumor-targeting capacity of Nanobodies in comparison with cetuximab, three sets of experiments were done with nude mice bearing s.c. implanted human xenografts of the vulvar tumor line A431 at one or two lateral sides. Female mice (Hsd athymic nu/nu, 25-32 g; Harlan) were 8 to 10 weeks old at the time of the experiments. All animal experiments were done according to NIH Principles of Laboratory Animal Care and Dutch national law ("Wet op de dierproeven," Stb 1985, 336).

In a first experiment, the biodistribution of the reference mAb cetuximab was assessed using the two different radiolabels. To this end, 14 mice with 24 tumors were injected i.p. with $^{177}$Lu-cetuximab, $^{125}$I-cetuximab, and unlabeled mAb cetuximab was assessed using the two different radioactivity counting, tumors were snap frozen immediately after radioactivity counting, tumors were snap frozen at the time of the experiments. All animal experiments were performed according to NIH Principles of Laboratory Animal Care and Dutch national law ("Wet op de dierproeven," Stb 1985, 336).

In a second experiment, biodistribution of the injected Nanobodies and cetuximab throughout the xenografts was assessed. In short, 5-μm-thick sections were cut on a cryotome and mounted on glass slides, dried, and fixed in 2% paraformaldehyde in PBS for 10 min. Next, sections were washed with PBS. This and all subsequent incubations were done at room temperature. A blocking step consisted of 2% normal swine serum in PBS/1% bovine serum albumin for 30 min.

For assessment of Nanobody distribution, sections were incubated for 1 h with rabbit anti-llama VHH (Ablynx) diluted 1:100 in PBS/1% bovine serum albumin for 30 min. After incubation with secondary antibody, sections were stained with Gill-III hematoxylin (Merck). After washing with demiwater and counterstained by nuclear stain with Gill-III hematoxylin (Merck). After washing with tap water, coverslips were mounted with Kaiser’s glycerol gelatin (Merck).

Maximal binding was assessed according to the same protocol (see above), except that, after blocking, the sections were incubated for 1 h with the particular Nanobody or cetuximab (10 μg/mL in PBS/1% bovine serum albumin) that also had been used for injection. The sections were washed three times with PBS followed by the procedures.

**Table 1. Kinetics and affinity constants of monovalent Nanobodies and mAb directed against albumin and EGFR**

<table>
<thead>
<tr>
<th>Antibody-antigen</th>
<th>$k_d$ (mol/L$^{-1}$ s$^{-1}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
<th>$K_d$ (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>αAlb Nanobody-mouse albumin</td>
<td>3.7 × 10$^5$</td>
<td>2.8 × 10$^{-3}$</td>
<td>7.5</td>
</tr>
<tr>
<td>αAlb Nanobody-human albumin</td>
<td>5.8 × 10$^5$</td>
<td>9.8 × 10$^{-4}$</td>
<td>1.7</td>
</tr>
<tr>
<td>αEGFR Nanobody-human EGFR</td>
<td>1.0 × 10$^6$</td>
<td>3.6 × 10$^{-3}$</td>
<td>3.6</td>
</tr>
<tr>
<td>Erbitux F(ab) fragment-human EGFR</td>
<td>8.5 × 10$^5$</td>
<td>1.7 × 10$^{-3}$</td>
<td>2.0</td>
</tr>
</tbody>
</table>
described above. Stainings without primary or secondary (rabbit anti-human or anti llama) antibody were used as negative control. Semiquantitative visual evaluation of stainings was based on homogeneity (0-100%) and intensity (compared with maximal intensity).

Statistics

Differences in tissue uptake between conjugates were statistically analyzed for each time point with Excel software (Microsoft) using paired or unpaired Student’s t test. Two-sided significance levels were calculated, and \( P < 0.01 \) was considered statistically significant.

Results

Radiolabeling

Labeling of Nanobody-p-SCN-Bz-DOTA and cetuximab-p-SCN-Bz-DOTA with \(^{125}\)I resulted in overall labeling yields of >75%. The radiochemical purity ranged from 98% to 99.7%. Phosphorimager analysis of SDS-PAGE gels as well as high-performance liquid chromatography analysis revealed optimal integrity of the Nanobodies and cetuximab. The immunoreactivity of the conjugates was >79% at the highest cell concentration for all \(^{125}\)I conjugates. In addition, the labeling of Nanobody or cetuximab with \(^{125}\)I resulted in overall labeling yields of >75%. The radiochemical purity was >99.7%. Conjugates showed optimal integrity: single peaks on high-performance liquid chromatography and single bands on gel, as has been published previously for radiolabeled Nanobodies (26). The immunoreactivity of \(^{125}\)I-Nanobody and of \(^{125}\)I-cetuximab was 90.7% and 98.0%, respectively, at the highest cell concentration.

Biodistribution of \(^{177}\)Lu-Cetuximab and \(^{125}\)I-Cetuximab

To assess the effect of abundant conjugate internalization and radionuclide residualization on radioactivity distribution, biodistribution studies were first done with the reference anti-EGFR antibody cetuximab. To this end, \(^{177}\)Lu-cetuximab and \(^{125}\)I-cetuximab were coincjected i.p. in A431-bearing mice at a total protein dose of 1 mg. As shown by Fig. 1, radioactivity uptake levels in tumors differed markedly for both conjugates, with much higher levels for the residualizing radionuclide \(^{177}\)Lu than for nonresidualizing \(^{125}\)I. For example, at 72 h after injection, the mean tumor uptake was 21.9 %ID/g for \(^{177}\)Lu-cetuximab compared with 4.5 %ID/g for \(^{125}\)I-cetuximab (\( P = 0.002 \)). Significant higher uptake of \(^{177}\)Lu than that of \(^{125}\)I was also found in organs involved in conjugate catalysis like liver, kidney, and spleen. In contrast, uptake values in most other organs and in blood were similar for both conjugates, the latter indicating that pharmacokinetics of the antibody was not altered as a result of radiolabeling. These results made us decide to use \(^{177}\)Lu conjugates for subsequent biodistribution studies with Nanobodies.

Biodistribution of Anti-EGFR Nanobodies with or without \(\alpha\)Alb Unit

The tumor-targeting potential of anti-EGFR Nanobodies was evaluated in the same model. We anticipated that introduction of an albumin-binding unit would result in a prolonged blood half-life with accompanying higher tumor uptake levels. To test this possibility, biodistribution of the following conjugates was assessed at equimolar doses in parallel: \(^{177}\)Lu-\(\alpha\)-EGFR-\(\alpha\)-EGFR-\(\alpha\)-Alb, \(^{177}\)Lu-\(\alpha\)-TNF-\(\alpha\)-TNF-\(\alpha\)-Alb, and \(^{177}\)Lu-cetuximab (reference). Biodistribution at 6, 24, and 72 h after injection is shown by Fig. 2, whereas Fig. 3 shows tumor-to-normal tissue ratios. The \(^{177}\)Lu-\(\alpha\)-EGFR-\(\alpha\)-EGFR Nanobody showed very rapid clearance from the blood, with a level of 0.06 ± 0.02 %ID/g at 6 h after injection (Fig. 2A). Tissue uptake levels above 1 %ID/g were only found for tumor and kidney. Tumor uptake was 5.0 ± 1.4, 3.2 ± 0.6, and 1.1 ± 0.1 at 6, 24, and 72 h after injection, respectively. Extremely high kidney uptake was observed at those time points (168.4 ± 9.3, 167.8 ± 29.3, and 86.5 ± 30.7, respectively), which is typical for \(^{177}\)Lu-labeled small proteins that are rapidly cleared via the kidneys (16).

Introduction of an albumin-binding unit in the molecule \(^{177}\)Lu-\(\alpha\)-EGFR-\(\alpha\)-EGFR-\(\alpha\)-Alb resulted in a dramatic change in the biodistribution pattern (Fig. 2B). Whereas blood clearance became much slower, with levels of 21.2 ± 2.5, 11.9 ± 0.6, and 4.0 ± 1.4 %ID/g at 6, 24, and 72 h after injection, respectively, tumor uptake at these time points was significantly higher: 19.4 ± 5.5, 35.2 ± 7.5, and 28.0 ± 6.8 %ID/g. These data indicate that introduction of an \(\alpha\)-Alb unit resulted in a 25-fold higher tumor uptake of the anti-EGFR Nanobody at 72 h after injection. Due to the slower body clearance kidney uptake was strongly reduced and did not exceed 11 %ID/g.

\(^{177}\)Lu-\(\alpha\)-TNF-\(\alpha\)-TNF-\(\alpha\)-Alb was included as a control Nanobody to evaluate the possibility that interaction of \(\alpha\)-Alb with albumin might have introduced a second targeting mechanism and therefore might have contributed to the high tumor uptake of \(\alpha\)-EGFR-\(\alpha\)-EGFR-\(\alpha\)-Alb (Fig. 2C). This possibility is realistic because albumin has been pursued previously as delivery vehicle for chemotherapy (27). \(^{177}\)Lu-\(\alpha\)-TNF-\(\alpha\)-TNF-\(\alpha\)-Alb showed a similar blood clearance as \(^{177}\)Lu-\(\alpha\)-EGFR-\(\alpha\)-EGFR-\(\alpha\)-Alb (except at 6 h after injection), indicating that blood clearance of these types of constructs is dominated by the \(\alpha\)-Alb-albumin interaction. In contrast, tumor uptake of \(^{177}\)Lu-\(\alpha\)-TNF-\(\alpha\)-TNF-\(\alpha\)-Alb was significantly lower than of \(^{177}\)Lu-\(\alpha\)-EGFR-\(\alpha\)-EGFR-\(\alpha\)-Alb and did not exceed 10.2 ± 1.5 %ID/g (at 24 h after injection). Because tumor uptake was slightly higher than in normal tissues, however, some contribution of tumor targeting via albumin (or TNF-\(\alpha\)) cannot be excluded.

Finally, \(^{177}\)Lu-cetuximab was included for comparison (Fig. 2D). Blood levels as well as uptake levels in most normal tissues (except for liver and kidney) were very similar to those of \(^{177}\)Lu-\(\alpha\)-EGFR-\(\alpha\)-EGFR-\(\alpha\)-Alb. Tumor levels, however, tended to be slightly lower (not significantly) for \(^{177}\)Lu-cetuximab: 15.5 ± 3.9, 27.1 ± 7.9, and 25.6 ± 6.1 %ID/g at 6, 24, and 72 h after injection, respectively.

Tumor-to-normal tissue ratios derived from aforementioned biodistribution studies have been summarized in Fig. 3. Whereas \(^{177}\)Lu-\(\alpha\)-EGFR-\(\alpha\)-EGFR-\(\alpha\)-Alb showed superior tumor uptake levels, which is important for anti-EGFR therapy, \(^{177}\)Lu-\(\alpha\)-EGFR-\(\alpha\)-EGFR showed superior tumor-to-normal
tissue ratios (except for kidneys). For example, tumor-to-blood ratios increased for the latter construct from 86.9 ± 32.0 at 6 h after injection to 369.0 ± 139.0 at 72 h after injection, respectively. On the other hand, tumor-to-blood ratios of $^{177}$Lu-αEGFR-αEGFR-αAlb tended to be higher than those of $^{177}$Lu-cetuximab (significant at 24 and 72 h after injection): 1.0 ± 0.1 versus 0.8 ± 0.1, 3.0 ± 0.3 versus 2.2 ± 0.6, and 7.9 ± 2.5 versus 3.5 ± 0.1 at 6, 24, and 72 h after injection, respectively.

**Distribution of Anti-EGFR Nanobodies and Cetuximab throughout Tumors**

Tumors derived from aforementioned biodistribution studies were used for immunohistochemical analysis of Nanobody and cetuximab distribution throughout the tumor. Staining of αEGFR-αEGFR-αAlb showed an intense and mostly homogeneous staining pattern at all three time points, 6, 24, and 72 h after injection, with 80% to 100% of the tumor cells stained. Figure 4A shows a typical staining pattern for αEGFR-αEGFR-αAlb at 6 h after injection. Preincubation of sections with αEGFR-αEGFR-αAlb to assess maximum staining did not further increase staining intensity (Fig. 4B). Staining was confined to the cell membranes of tumor cells, whereas very weak staining was observed in the stromal compartment (Fig. 4C). In contrast, staining of cetuximab was much more heterogeneous at all time points and did not exceed 60% of the cells. Staining was intense at 6 h (see Fig. 4D) and 24 h after injection and became weaker at 72 h after injection (data not shown).

Preincubation of tumor sections with cetuximab to assess maximum staining resulted in a homogeneous staining pattern (Fig. 4E). Staining of αEGFR-αEGFR was homogeneous but weak, whereas staining of αTNF-αTNF-αAlb was very weak without clear delineation of tumor cells nests (data not shown). No staining was observed in the absence of either αEGFR-αEGFR-αAlb or cetuximab (Fig. 4F).

**Biodistribution of $^{177}$Lu-αEGFR-αEGFR-αAlb and $^{125}$I-αEGFR-αEGFR-αAlb**

More extensive studies were done with αEGFR-αEGFR-αAlb to obtain detailed information on pharmacokinetics, biodistribution, tumor targeting, and in vivo internalization under EGFR saturating conditions as described previously for cetuximab (Fig. 1). To this end, $^{177}$Lu-αEGFR-αEGFR-αAlb and $^{125}$I-αEGFR-αEGFR-αAlb at a total dose of 1 mg were i.p. administered to tumor-free and A431-bearing mice. Pharmacokinetics were assessed by repeated sampling of five tumor-free mice and showed a similar pattern for both conjugates (Fig. 5), as was previously the case for cetuximab (Fig. 1). Interindividual differences were very

![Figure 1. Biodistribution of coinjected $^{177}$Lu-cetuximab (A) and $^{125}$I-cetuximab (B) in A431 tumor-bearing nude mice up to 5 d after injection. Total administered antibody dose: 1 mg. Mean (%ID/g) ± SD at each time point after i.p. injection ($n = 3$ or $4$ animals per time point).](https://mct.aacrjournals.org/content/mct/7/8/2292/F1.large.jpg)
small, especially at later time points, and congruent for both conjugates. Nanobody blood levels at 48 h after injection were ~50% of the maximum levels observed at 4 h after injection.

$^{177}$Lu-αEGFR-αEGFR-αAlb and $^{125}$I-αEGFR-αEGFR-αAlb were also coinjected to assess biodistribution in A431 tumor-bearing mice. As shown by Fig. 6, radioactivity uptake in tumors differed markedly for both conjugates, which indicates in vivo internalization of the Nanobody (as was also observed for cetuximab; see Fig. 1). Tumor uptake of $^{177}$Lu-αEGFR-αEGFR-αAlb was almost constant at ~12 %ID/g during the period 48 to 120 h after injection. To test the possibility that in vivo EGFR saturation by Nanobody had occurred, the following calculation was made to estimate the number of Nanobody molecules bound per A431 cell in vivo: an uptake of 12 %ID/g means that 120 μg Nanobody (MW ~50 kDa) had been bound per gram of tumor, which corresponds to ~1.5 × 10$^{15}$ Nanobody molecules. Assuming that 1 g tumor contains ~10$^{9}$ tumor cells, this would mean that ~1.5 × 10$^{9}$ Nanobody molecules had bound per A431 tumor cell in vivo. The percentage tumor uptake of $^{177}$Lu-cetuximab in the same model and at the same mAb dose of 1 mg was higher than for $^{177}$Lu-αEGFR-αEGFR-αAlb: ~18 versus ~12 %ID/g. However, when performing a similar calculation as above for cetuximab (150 kDa), it appears that less molecules of cetuximab had been bound per tumor cell (~0.7 × 10$^{9}$) than of αEGFR-αEGFR-αAlb.

Blood kinetics of i.p. injected $^{177}$Lu-αEGFR-αEGFR-αAlb appeared slightly faster in tumor-bearing mice than in tumor-free mice. Whereas blood levels decreased from 21.1 to 4.6 %ID/g in tumor-free mice between 24 and 120 h after injection, in tumor-bearing mice a decrease from 16.5 to 2.9 %ID/g was observed (Figs. 5 and 6).

**Discussion**

Nanobodies can easily be produced as multivalent and/or multispecific single molecule formats consisting of ~15-kDa building blocks. In this study, we exploited this flexibility to modulate the pharmacokinetics of anti-EGFR Nanobodies by introduction of an albumin-binding unit. As a starting point, a bivalent αEGFR-αEGFR Nanobody format was chosen, because previous studies had shown that bivalent anti-EGFR Nanobodies were more effective in competing for EGF binding to EGFR than monovalent Nanobodies (12). Moreover, this choice enabled direct comparison with the bivalent anti-EGFR mAb cetuximab. The αEGFR-αEGFR Nanobody showed rapid blood clearance, very high tumor-to-normal tissue ratios (except for kidney), but moderate levels of tumor uptake (5.0 ± 1.4 %ID/g at 6 h after injection), which rapidly decreased.

**Figure 2.** Biodistribution of $^{177}$Lu-αEGFR-αEGFR (A), $^{177}$Lu-αEGFR-αEGFR-αAlb (B), $^{177}$Lu-αTNF-αTNF-αAlb (C), and $^{177}$Lu-cetuximab (D) in A431 tumor-bearing nude mice at 6, 24, and 72 h after i.v. injection. Conjugates were administered at equimolar doses of 66, 100, 100, and 300 μg, respectively. Mean (%ID/g) ± SD at each time point after injection ($n = 3$ animals per time point).
over time. Although such a Nanobody format might be ideal for imaging purposes to confirm receptor expression within a short period, it is not suitable for therapy.

An ideal Nanobody format for therapy is a format that is able to block EGFR at each individual tumor cell immediately after its administration for a prolonged period. In the present study, we show that the aforementioned anti-EGFR Nanobody obtained such characteristics by introduction of an albumin-binding unit: the αEGFR–αEGFR–αAlb showed (a) rapid, high, and selective tumor
accumulation when administered at a relatively low dose; (b) prolonged residence time in blood and tumor; (c) homogeneous distribution throughout the tumor; and (d) apparent saturation of EGFR when administered at a relatively high dose. With respect to the latter, by estimation $1.5 \times 10^6$ αEGFR-αEGFR-αAlb molecules were found to be bound per A431 tumor cell in vivo on administration of a dose of 1 mg (Fig. 6). This number corresponds very closely with the number of EGFR molecules expressed per A431 cell in high-density in vitro cultures (28). Tumor uptake was predominantly directed by the αEGFR-binding units, because the αTNF-αTNF-αAlb control Nanobody showed comparable pharmacokinetics but much lower tumor uptake. Some contribution of the αAlb unit to tumor targeting, however, cannot be excluded and needs further evaluation. Biodistribution and tumor-targeting characteristics were very similar for the 50-kDa αEGFR-αEGFR-αAlb Nanobody and the ~150-kDa intact reference mAb cetuximab. However, a more homogeneous tumor distribution was found for the Nanobody. Whether this was related to the smaller size of the Nanobody (9), to differences in affinity (20, 29), or to other factors needs further investigations. A potential disadvantage of the prolonged residence time of Nanobodies in blood is the increased chance for the development of immunoreactions. However, Nanobodies have a high homology with VH domains of human antibodies and can be further humanized to decrease their immunogenicity.

Several strategies have been described in literature to reduce blood clearance of targeting molecules. Most of

Figure 4. *In vivo* targeting of A431 tumors by $^{177}$Lu-αEGFR-αEGFR-αAlb and $^{177}$Lu-cetuximab. Following *in vivo* tumor targeting as described in Fig. 3, tumors were excised at 6 h after injection and processed for immunohistochemical detection of αEGFR-αEGFR-αAlb (A–C) or cetuximab (D and E). Sections B and E have been incubated with the same conjugate as injected *in vivo* and represent maximal binding. C, an enlargement, showing that staining is confined to the cell membranes of tumor cells, with minimal staining of the stromal compartment. F, a negative control staining in the absence of primary antibody (either αEGFR-αEGFR-αAlb or cetuximab).

Figure 5. Blood concentrations of coinjected $^{177}$Lu-αEGFR-αEGFR-αAlb and $^{125}$I-αEGFR-αEGFR-αAlb in tumor-free nude mice ($n = 5$) up to 9 d after i.p. injection. Total administered antibody dose: 1 mg.
these strategies focus on the enlargement of the targeting molecule above the critical size for renal clearance (60-70 kDa) by fusion to other proteins like albumin (30) and the Fc portion of an IgG (31) or by glycosylation (32) or pegylation (33). The use of bispecific antibodies as described herein, aiming a noncovalent association with albumin as a means to improve biodistribution and tumor deposition, was pioneered by Dennis et al. from the Genentech labs (17). In an article that was published while our article was under preparation, they described the tumor-targeting potential of a bifunctional molecule derived from trastuzumab (Herceptin) capable of simultaneous binding of albumin and the tumor antigen HER2 (18). This molecule designated AB.Fab4D5 was constructed from the F(ab) fragment of trastuzumab by recombinant fusion of a linker and an albumin-binding peptide to either the COOH terminus of the heavy chain or light chain. Before use, AB.Fab4D5 was purified by an albumin affinity column to ensure a properly folded albumin-binding peptide. Although AB.Fab4D5 showed a much more rapid blood clearance than reference mAb trastuzumab, resulting in ~5 times lower blood levels at 2 days after injection, similar tumor deposition was obtained for both as revealed from quantitative imaging studies. As a result, tumor-to-blood ratios were superior for AB.Fab4D5, making this agent ideal for tumor imaging. No quantitative data were obtained for uptake in other organs.

In contrast to AB.Fab4D5, addition of the albumin-binding unit αAlb to single-chain αEGFR-αEGFR resulted in a blood clearance, which was very similar to the clearance of reference intact IgG mAb cetuximab. This might be due to a better accessibility and/or higher affinity of the αAlb unit in comparison with the albumin-binding peptide of AB.Fab4D5. αEGFR-αEGFR-αAlb showed blood levels of 21.2 ± 2.5 and 11.9 ± 0.6 %ID/g at 4 and 24 h after injection. Interestingly, Meijs et al. (34) previously assessed the pharmacokinetics of radiolabeled mouse serum albumin in mice (34). To this end, mouse serum albumin was labeled with the radionuclides 89Zr and 123I. Blood levels of both mouse serum albumin conjugates after i.v. injection were ~24 and ~10 %ID/g at 4 and 24 h after injection, respectively. The very similar pharmacokinetics of αEGFR-αEGFR-αAlb and mouse serum albumin indicates that the αAlb-binding unit ensured a very strong association between these molecules in vivo. Because the αAlb-binding unit also shows high affinity for human serum albumin,
and human serum albumin was shown to have a blood half-life of ~20 days (35), it can be anticipated that αEGFR-αEGFR-αAlb will show a much longer half-life in humans. 

Aforementioned data indicate that simple recombinant fusion of αEGFR and αAlb building blocks results in a single molecule format with bifunctional properties, which seem more favorable for therapy as far as pharmacokinetics, target binding, and tumor penetration are concerned. In addition, preliminary tumor xenograft studies indicated that first-generation αEGFR-αEGFR-αAlb type Nanobodies indeed are effective in delaying the outgrowth of A431-derived solid tumors (12), although to a lesser extent than cetuximab.4 These findings encourage further exploration of the Nanobody toolbox, especially with respect to the generation of multivalent and/or multispecific therapeutic formats.

4 Personal communication with Dr. R. Roovers.

Disclosure of Potential Conflicts of Interest
T. Laeremans, T. Dreier, and H.J. de Haard, Ablynx, NV employees. The other authors disclosed no potential conflicts of interest.

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