Nanobody-albumin nanoparticles for the delivery of the multikinase inhibitor 17864 to EGFR overexpressing tumor cells

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ABSTRACT SUMMARY
A novel, epidermal growth factor receptor (EGFR)-targeted nanomedicine has been developed based on albumin nanoparticles loaded with a kinase inhibitor and surface functionalized with the single variable domain of an antibody (nanobody) leading to the internalization of the nanoparticles via clathrin mediated endocytosis and inhibition of cell proliferation.

INTRODUCTION
Nanomedicine helps to improve the low therapeutic index of highly potent drugs that limit their use in the clinic. Albumin nanoparticles have gained attention due to their biocompatibility and ease of surface functionalization. We report the preparation of a novel type of glutaraldehyde crosslinked albumin nanoparticles (NP) loaded with a kinase inhibitor, 17864 via a platinum based linker (Lx) and further functionalized with cameloid nanobodies against EGFR (EGa1) that allows for binding and internalization to EGFR overexpressing tumor cells. We examined cellular uptake, intracellular routing and antiproliferative activity on human head and neck squamous cell carcinoma (HNSCC) UM-SCC-14C (14C).

EXPERIMENTAL METHODS
Albumin nanoparticles were prepared according to Langer et al. wherein human serum albumin was dissolved in water and the pH adjusted to 8.3. To this solution 92% ethanol was added dropwise to obtain an albumin nanosuspension.1 These nanoparticles were stabilized with glutaraldehyde and the particles were purified by centrifugation at 35,000 g. 17864-Lx was conjugated by overnight incubation (10:1 mol/mol albumin) at 37°C in 20 mM tricine buffer pH 8.5. The 17864 content of the nanoparticles was determined after cleavage of the 17864-Lx coordinative bond by an overnight incubation with KSCN at 80°C, followed by extraction with ACN.2

Nanoparticles were surface functionalized with NHS-PEG3500-Maleimide (10:1 PEG:NP, mol/mol) and later reacted with EGa1-SATA (N-Succinimidyl-S-Acetyl-Thioacetate) (1:8 mol/mol). Alexa-488-conjugated NP were allowed to internalize for 30 min in complete culture medium. After incubation, cells were washed with PBS to stop the internalization. Surface bound nanoparticles were removed by an acid wash and cells were fixed with 4% paraformaldehyde.

Antiproliferative effects of 17864-Lx NP were determined by a BrdU assay (Roche Diagnostics). 14C cells were incubated with NP diluted in medium to concentrations ranging from 25 to 800 μg NP/ml corresponding to 3–100 μM 17864 for 48h.

RESULTS AND DISCUSSION
Albumin nanoparticles of 100 nm size and narrow polydispersity index were obtained (Table 1).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Loading capacity</th>
<th>Size (nm)</th>
<th>Polydispersity index</th>
<th>Zetapotential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17864-Lx</td>
<td>4.0 ±</td>
<td>100</td>
<td>0.05</td>
<td>-26</td>
</tr>
<tr>
<td>NP (D-NP)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGa1-PEG-D-NP</td>
<td>0.3</td>
<td>138</td>
<td>0.14</td>
<td>-21</td>
</tr>
</tbody>
</table>

Table 1. Properties of nanoparticles after drug loading and surface functionalization

Drug release from the NP was tailored by using a platinum linker that can release the drug in reducing environment like the cytoplasm and stay stable in PBS or 10% serum (Figure 1). Hence, this requires the NP to be internalized into the cell in order to release the drug. Activated EGF receptors are internalized into cells by clathrin-mediated endocytosis (CME) (at physiological plasma concentration of EGF; 1 ng/ml). Phospholipids play an important role in CME, especially during the formation of the clathrin coated vesicles that bud off from the cell membrane. While the vesicles are forming, adaptor proteins involved in clathrin-mediated endocytosis interact.
with the phospholipid, phosphoinositide-4,5-biphosphate (PIP2) in the membrane. 1-Butanol, inhibits the formation of PIP2, consequently blocking CME.3-5

Figure 1. The release of 17864 from NANAPs in PBS, PBS with 10% serum and 5 mM DTT at pH 7.4, 37ºC

EGa1-PEG-NPs were internalized in the presence of 2-propanol (which does not interfere with CME) as shown in Fig.2A. In contrast, EGa1-PEG-NPs were not internalized in the presence of 1-butanol (Figure 2B). From this result, it is concluded that EGa1-PEG-NPs are taken up specifically via CME and non-PEGylated NPs without EGa1 were internalized via clathrin-independent mechanisms, (Figure 2A–B, arrows 2a and 2b).

17864 inhibits multiple tyrosine kinases involved in tumor cell proliferation. Only EGa1-PEG-D-NP efficiently inhibited tumor cell proliferation with an IC50 of 40 μM whereas the drug loaded PEGylated and non-PEGylated NP did not show any effect (Figure 3).

CONCLUSION

In conclusion, we have developed a tumor-directed novel drug carrier system loaded with the multikinase inhibitor 17864 conjugated to the albumin backbone via a platinum based linker (Lx). The pathway of uptake was crucial for the intracellular release of 17864 from Lx. The tunable characteristics of the NANAPs make them promising new materials favorable for in vivo use as targeted nanomedicines in the treatment EGFR positive cancers.

REFERENCES