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Title: ~~Pharmacokinetic and pharmacodynamic advantages of a liganded protein nanocarrier targeting~~Double targeting to tumor and stromal cancer cells in an aggressive CXCR4+ ~~cancer~~ colorectal cancer mice models~~cells~~

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Running title:

"PK/PD advantages for a liganded nanocarrier targeting CXCR4"

Competing interests

No potential conflicts of interest were disclosed by the authors.

ABSTRACT

Unliganded drug-nanoconjugates accumulate passively in the tumor while liganded nanoconjugates, accumulate actively, promoting drug internalization in tumor cells via endocytosis and increasing the antitumor efficacy. However, the mechanism underlying this effect remains poorly studied. We compared tumor uptake of T22-GFP-H6, a liganded protein carrier targeting the CXCR4 receptor, and the unliganded GFP-H6 carrier in subcutaneous and metastatic colorectal cancer models. The liganded carrier had a higher tumor uptake and a longer residence time than the unliganded carrier. T22-GFP-H6 was detected in the cytosol of the $CXCR4^+$ tumor cell subset while GFP-H6 was detected in tumor stroma. SDF1-α co-administration switched T22-GFP-H6 internalization from the $CXCR4^+$ epithelia to the stroma. Our results suggest that a targeting ligand confers pharmacokinetic advantages such as enhanced uptake in the tumor and pharmacodynamic advantages such as high and selective accumulation in the cytosol of the tumor cell. These results validate T22-GFP-H6 as a CXCR4-targeted drug carrier.

Keywords: drug delivery, liganded protein nanocarrier, PK/PD advantages, target cell internalization, tumor uptake, CXCR4 receptor, colorectal cancer models

Background

Nanomedicine promises to improve cancer therapy. The use of nanoparticles as vehicles for drug delivery in cancer therapy significantly changes the pharmacokinetics and the delivery efficiency of the drug payload to tumor tissue.[1] FDA-approved nanomedicines such as pegylated-liposomal-doxorubicin (used to treat gynecological and hematological neoplasias) and nab-paclitaxel (used to treat advanced stage breast, lung or pancreatic cancer) use a passive targeting strategy. Both nanoparticle drugs improve tumor response as compared to the corresponding free drug.[2] The nanometric size of these drug carriers increases tumor accumulation because of the enhanced permeability and retention (EPR) effect and alters their biodistribution to normal tissues.[3] However, only a modest 5-33% of the administered dose of pegylated-liposomal-doxorubicin nanoparticles reaches the tumor because of their uptake by the mononuclear phagocytic system (MPS) in normal tissues.[4] The drug concentration of nab-paclitaxel that reaches the tumor is 30% higher than the concentration of paclitaxel when administered as a free drug.[5] In addition to liposomes, protein-based carriers, such as albumin and monoclonal antibodies (i.e. antibody-drug conjugates) are increasingly used for drug delivery. [6,7]

A strategy that exploits the use of receptor-mediated endocytosis could theroretically improve drug uptake in tumor tissue and consequently enhance its antitumor effect while reducing its accumulation in normal tissues and its systemic toxicity.[8] This could be accomplished by incorporating a ligand able to bind a receptor overexpressed in the tumor cell membrane. Such a liganded carrier will undergo endocytosis and deliver the payload drug into the cytosol. [8,9] It has been observed that liganded conjugates increase antitumor efficacy when compared to unliganged conjugates. This effect is most likely induced by an increase in the conjugated payload drug in the tumor cell cytosol[10]. The mechanism underlying this effect, however, remains poorly studied.[11]

We aimed to develop liganded protein nanoparticles as carriers for targeted drug delivery to tumor cells that overexpress CXCR4. Such protein carriers can transport drugs that have been easily conjugated through tioethers or amide bonds[12]. Expression of the chemokine membrane receptor CXCR4 is associated with metastatic spread and aggressiveness in many tumors, including colorectal cancer (CRC) [13,14] an expression level that in tumors is 10-20 fold higher than in normal tissues[15,16]. We previously designed and produced the liganded T22-GFP-H6 carrier that incorporates the CXCR4 receptor specific ligand T22 and assembles as a nanoparticle.[17] In cell

culture experiments, this T22-GFP-H6 targets CXCR4$^+$ cells through specific CXCR4 receptor-mediated binding and internalization.[17]

Here, we test the capacity of the T22-GFP-H6 protein carrier to internalize into CXCR4$^+$ tumor cells associates with enhanced tumor uptake. To that purpose, we compare the differences in tumor biodistribution between liganded T22-GFP-H6 and its unliganded GFP-H6 version (lacking T22), using the CXCR4-overexpressing SW1417 CRC cell line-derived subcutaneous and orthotopic mouse models. ~~We also assess whether the T22-GFP-H6 internalizes into the cytosol of tumor and metastatic cells and if this happens in a CXCR4-dependent manner. Finally, we also studied the subcellular localization and biodistribution of the liganded and unliganded carriers in normal tissues.~~ By taking this approach, we have demonstrated for the first time, a double and simultaneous targeting *in vivo*, overlooked ***in vitro***, that enables the nanoconjugate reaching intracellularly both tumor and stroma cells. This fact not only improves pharmacology and biodistribution of the nanoconjugate but it also opens a new road to explore combined tumor/stroma therapies that have been recently suggested as highly promising in innovative cancer chemotherapies (REF).

Methods

T22-GFP-H6 and GFP-H6 protein production

Production and characterization of the liganded and the unliganded proteins, and the in vitro evaluation of T22-GFP-H6 internalization were previously described.[17,18] Both proteins only differ in the presence or absence of the T22 peptide, a ligand binding the CXCR4 receptor.

SW1417 cell culture

The SW1417 CRC cell line (ATCC; Manassas, USA) was cultured in DMEM (Invitrogen, UK) supplemented with 10% FBS (Sigma-Aldrich, St.Louis, USA), 50 units/ml penicillin and 50 mg/ml streptomycin (Invitrogen, UK).

SW1417 CRC models and protein administration

The CRC models were generated using the human CXCR4-overexpressing SW1417 cell line. We used 5week-old female Swiss Nu/Nu mice, weighing 18-20 g (Charles River, France), maintained

in SPF conditions. The study was approved by the institutional animal ethics Committee. Subcutaneous (SC) and orthotopic (ORT) models were generated in separated experiments. In the SC model we implanted 10mg tumor tissue obtained from donor animals. In the ORT model we injected 2 million SW1417 cells directly in the cecal wall, as described.[19]

Fluorescent monitoring of T22-GFP-H6 and GFP-H6 biodistribution in mice

Four weeks after SC tumor implantation or eight weeks after orthotopic microinjection (once tumors or metastases became visible), tumor-bearing mice received 100µg single intravenous boluses of GFP-H6 (n=5) or T22-GFP-H6 (n=5) in 20 mM Tris, 500 mM NaCl, pH 7.4 buffer. Control animals received vehicle buffer (n=5). Biodistribution to tumor and normal tissues was measured 15min, 30min, 2h, 5h and 24h after administration. At the end of the experiment, mice were euthanized, and primary tumor, organs bearing metastatic foci and non-tumor organs that express (spleen, bone marrow) or do not express (brain, lung, liver, kidney and heart) CXCR4 were obtained. Tissue samples were cut into slices and placed in separate wells to detect fluorescence emission using IVIS® Spectrum (Perkin Elmer, CA).

Biodistribution of GFP-H6 and T22-GFP-H6 proteins in tumors, metastases and normal tissues in SC and ORT models was determined measuring fluorescence intensity in ex-vivo tissue sections, emitted by their GFP domains. The fluorescent signal (FLI) was first digitalized, displayed as a pseudocolor overlay, and expressed as radiant efficiency. The FLI ratio was calculated dividing FLI signal from experimental mice by FLI auto-fluorescent signal of control mice. FLI intensity correlates with the amount administered protein accumulated in each tissue. Organ samples were fixed with 4% formaldehyde in PBS for 24 h and embedded in paraffin for histological and immunohistochemical evaluation.

Assessment of protein internalization in tumors and histopathology

40m sections were H&E stained with and analyzed histopathologically analyze by two independent observers. The number of apoptotic bodies in tumors in 10 microscopic fields (400X) were counted. Presence and localization of CXCR4 receptor and the GFP-H6 and T22-GFP-H6

proteins in tumor or metastatic tissues were immuno-histochemically assessed. 4µm paraffin-embedded tissue sections were deparaffinized, rehydrated before antigen retrieval and quenching of peroxidase activity, as described[17]. Slides were then incubated with anti-CXCR4 antibody (1:300, Abcam, UK) for 25min to detect CXCR4 expression before protein injection. Following, they were washed in PBS-T, incubated with HRP-conjugated secondary antibody for 30min at RT before chromogenic detection with DAB substrate (DAKO, Denmark). Sections were counterstained with hematoxylin, dehydrated ethanol (100/95/70/50%) and mounted using DPX medium.

To study the kinetics of nanoparticle internalization after injection, we incubated tumor sections for 30 min with an anti-GFP antibody (1:300; Santa Cruz Biotechnology, CA). Representative pictures were taken using CellB software (Olympus Soft Imaging v3.3, Japan) at 400x. In addition, histopathological analysis of CXCR4-expressing and non-CXCR4 expressing normal tissues after injection were performed.

Competition of T22-GFP-H6 cell internalization by SDF1-α co-administration

To assess whether the liganded T22-GFP-H6 carrier entered $CXCR4^+$ tumor cells specifically through the CXCR4 receptor, we used the CXCR4 natural ligand SDF1-α in a competition assay in the SW1417 $CXCR4^+$ SC model. Mice bearing 200-300 mm^3 tumors were randomized into three experimental and a control group (n=5). The T22-GFP-H6 and GFP-H6 groups received 100µg i.v. bolus of the corresponding protein. The SDF1-α+T22-GFP-H6 group received SDF1-α at a 0.15mg/kg subcutaneous dose, one hour before injection of a 100µg T22-GFP-H6 i.v. bolus. The control group received vehicle. Two hours after injection, animals were euthanized, tumors extracted, cut into slices, and placed in separate wells to detect GFP signal emission using the IVIS® Spectrum, to estimate accumulated protein as radiant efficiency. Tumors were fixed and paraffin-embedded for histological and IHC evaluation as above. Protein internalization in CXCR4-expressing tumor cells were performed combining histological and IHC analyses using anti-CXCR4 or anti-GFP antibodies.

Evaluation of T22-GFP effect in tumor progression and body weight at a repeated dose schedule

To test whether the T22-GFP-H6 carrier any effect in tumor growth, SW1417 mice bearing about 150 mm^3 subcutaneous SW1417 SC were randomized into two groups (n=9). One received a 10µg T22-GFP-H6 i.v. dose every three days per nine doses, the other received vehicle with the injection regime. Body weight and tumor volume were measured twice a week, the last calculated using the ellipsoid formula, as described [17].

Statistical analysis

The Mann-Whitney test was used to compare differences in tumor tissue fluorescence emission, body weight, tumor volume and final tumor weight between groups using the SPSS version 11.0 package (IBM, New York, USA). All quantitative values were expressed as mean±SEM. Differences between groups were considered significant at $p<0.05$.

Results

Effect of T22-mediated targeting on tumor tissue uptake

After administering a single 100µg intravenous bolus of GFP-H6, T22-GFP-H6 or buffer in the $CXCR4^+$ SW147 SC CCR model, the intensity and kinetics of tumor uptake for the unliganded and liganded proteins differed significantly, as measured by *ex vivo* fluorescence emission (Figure 1A-B). GFP-H6 fluorescence was detected earlier in tumor tissue and reached a peak signal 15min after injection. In contrast, the onset of detection of T22-GFP-H6 in tumor was 2h, and its uptake continued to increase for 24h (Figure 1A). T22-GFP-H6 peak intensity was approximately 4 times higher than for GFP-H6 (Figure 1B). In addition, tumor residency time (period during which the administered protein remained detectable) was significantly longer for the liganded (>22h) than for the unliganded (about 2h) carrier (Figure 1A-B). Similarly to the differences in tumor uptake, as measured by fluorescent emission, using IHC with and anti-GFP antibody, we found that the amount of protein accumulating in tumor tissue was significantly higher in the T22-GFP-H6 than in the GFP-H6 group (Figure 1C). Also by IHC, the peak intensity for GFP-H6 was registered at 15

min, whereas the T22-GFP-H6 peak occurred at 24h (Figure 1C). The three groups were comparable because their tumors had a similar cell percentage with similar CXCR4 expression in their membrane. All tumors displayed similar histology and number of apoptotic figures per microscopic field (Figure 1D).

CXCR4-dependence of T22-GFP-H6 uptake in tumor tissue

To evaluate whether tumor uptake was dependent on the CXCR4 receptor, we administered the CXCR4 natural ligand SDF1-α to $CXCR4^+$ SW1417 SC CRC mice before T22-GFP-H6 nanoparticle injection. We included four mouse groups (n=5) in the study: control (buffer-injected), T22-GFP-H6 (liganded carrier targeting CXCR4), GFP-H6 (unliganded carrier), and SDF1-α+T22-GFP-H6. T22-GFP-H6 or GFP-H6 was administered as 100µg i.v. bolus, whereas SDF1-α was administered as a 0.15 mg/Kg SC dose, one hour before T22-GFP-H6 injection.

Two hours after carrier administration, we recorded *ex vivo* the fluorescent emission in tumor sections (Figure 2). T22-GFP-H6 accumulation in tumor tissue ($3.9\pm0.5xE^9$, Radiant efficiency) was approximately 40% higher than GFP accumulation ($2.5\pm0.6x\ E^9$) (Figure 2A-B). Moreover, SDF1-α administration prior to T22-GFP-H6 carrier administration dramatically reduced its tumor uptake ($1.6\pm0.2x\ E^9$) (Figure 2A). No fluorescence was detected in tumor tissue of control mice. (Figure 2A).

Effect of T22-mediated targeting on cell type internalization in tumors and its inhibition by SDF1-α

We used the SW1417 SC CRC model to compare the internalization of the GFP-H6 and T22-GFP-H6 proteins in tumor tissue in the four groups (buffer, GFP-H6, T22-GFP-H6 or SDF1-α+T22-GFP-H6-treated) (Figure 3E-H). Tumors displayed in all groups a similar percentage of cells expressing the CXCR4 receptor with a similar intensity (Figure 3A-D). We also found differences in the topographical biodistribution within tumor tissue between both proteins, as assessed by IHC using an anti-GFP antibody. Thus, the liganded and the unliganded carrier

internalized in different tumor cell types. We detected T22-GFP-H6 in the cytosol of $CXCR4^+$ epithelial tumor cells but not in tumor stromal cells (Figure 3G, black asterisks). GFP-H6 was not detected in $CXCR4^+$ tumor epithelial cells, but was unexpectedly detected in the cytosol of tumor stromal cells (Figure 3F, black arrows). Thus, the presence or absence of the T22 ligand in these protein carrier induced differences, not only in their tumor uptake but also, in cell type internalization.

IHC analysis of tumor tissue in the four groups was also used to determine whether the T22-GFP-H6 protein entered tumor epithelial cells specifically through the CXCR4 receptor; that is, whether the administration of SDF1-α before T22-GFP-H6 injection inhibited the internalization of this protein carrier in $CXCR4^+$ tumor cells. T22-GFP-H6-treated tumors showed, as expected, carrier internalization only in $CXCR4^+$ epithelial tumor cells (Figure 3G, black asterisks). In contrast, the administration of SDF1-α before T22-GFP-H6 (SDF1-α+T22-GFP-H6 group) blocked the uptake of this nanoparticle in the cytosol of $CXCR4^+$ tumor epithelial cells (Figure 3H, black asterisks). Moreover, in this group, we unexpectedly found the T22-GFP-H6 carrier internalizing inside tumor stroma cells (Figure 3, H, black arrow), a pattern identical to that shown after direct administration of GFP-H6 protein (Figure 3F). No staining was detected in tumors from buffer-treated animals (Figure 3E, black asterisk). Therefore, the liganded T22-GFP-H6 carrier underwent CXCR4-dependent internalization in CXCR4+ tumor epithelial cells.

Comparison of T22-GFP-H6 and GFP-H6 biodistribution in non-tumor tissues

We also assessed whether the GFP-H6 or T22-GFP-H6 carriers accumulated in CXCR4-expressing and CXCR4 negative normal tissues, and if this caused toxicity. 5h or 24h after 100µg i.v. bolus administration, we did not detect *ex vivo* fluorescence emitted by either protein in CXCR4 negative non-tumor tissues (kidney, liver, lung, or heart) (Figure 4). The fluorescence signal in these organs was undistinguishable from the background fluorescence observed in control mice.

Differently from the observed T22-GFP-H6 accumulation in CXCR4$^+$ tumor epithelial cells, the fluorescent signal emitted by this carrier in non-tumor tissues expressing CXCR4 such as the spleen or the bone marrow was, unexpectedly, undetectable as it happened with GFP-H6 (Figure 4). Thus, most of the administered T22-GFP-H6 carrier appears to be selectively accumulated in tumor tissue. This is consistent with tumor tissue expressing significantly higher levels of CXCR4 than normal tissues. [15,16]

Target and off-target protein toxicity

The administration of a 100µg i.v. dose of GFP-H6 or T22-GFP-H6 did not induce any sign of target-dependent, off-target systemic toxicity or weight loss. In addition, we did not find any histological alteration in CXCR4 negative normal tissues (brain, lung, kidney and liver) or in CXCR4+ non-tumor tissues (bone marrow and spleen) (Figure 5). Thus, the histology of carrier-treated tissues had the same normal appearance as that displayed by buffer-treated mice (Figure 5). In summary, both protein carriers showed undetectable distribution in normal tissues and displayed absence of toxicity in all normal organs.

Biodistribution of T22-GFP-H6 to primary tumor and metastases in an orthotopic CRC model

We assessed whether the selective accumulation of the T22-GFP-H6 carrier and CXCR4$^+$ epithelial cell internalization detected in subcutaneous tumor tissue also occurred in primary tumors and metastatic foci in the CXCR4$^+$ SW1417 orthotopic model. This is relevant when T22-GFP-H6 is used as a drug-nanocarrier for the treatment of metastatic disease. Besides the local tumor, this model develops metastatic foci in lymph nodes and the peritoneum that expresses CXCR4 (Figure 6A). We assessed T22-GFP-H6 biodistribution to tumor-bearing organs and whether it was selectively internalized in the cytosol of CXCR4 overexpressing tumor cells in this model.

We observed a selective biodistribution of this nanoparticle to the primary tumor, lymph node metastasis and peritoneal carcinomatotic foci (Figure 6B) 2h after a 20g T22-GFP-H6 bolus injection, as measured by *ex vivo* fluorescence emission. The intensity of CXCR4 expression in the

membrane of the primary tumor and peritoneal metastases was similarly high, a level more intense than CXCR4 expression in lymph node foci (Figure 6B). We observed a similarly high T22-GFP-H6 uptake in primary tumor and peritoneal metastasis, as assessed by *ex vivo* fluorescent emission, reaching a peak of about 2.5 units. However, in lymph node metastatic foci, and in consonance with their lower level of CXCR4 expression, there was less T22-GFP-H6 uptake, being its peak of 1.2 units (Figure 6B). Importantly, the level of T22-GFP-H6 uptake in $CXCR4^+$ tumor epithelial cells, as assessed by IHC appears to correlate with the level of CXCR4 receptor expression in the membrane of tumor cells when localized at different sites (Figure 6A). Thus, internalization of the nanoparticle in the cytosol of $CXCR4^+$ tumor cells was similarly intense in primary tumor and peritoneal metastases, and higher than that detected in lymph node metastatic foci (Figure 6, C), correlating with the values of fluorescence emission (Figure 6B). Thus, using two different methodologies for carrier detection, we confirmed the existence of an association between the level of CXCR4 expression in the membrane and T22-GFP-H6 uptake in tumor cells.

Lack of T22-GFP-H6 effect on tumor growth or body weight after a repeated dose schedule

We evaluated the effect of a repeated dose schedule of T22-GFP-H6 nanoparticle administration (10µg, q3d x 9 doses) in the SC SW1417 model. We measured tumor volume over the treatment period in the experimental group and compared it to tumor volume measurements registered in buffer-treated animals following the same schedule. No statistically significant differences in tumor volume were observed between groups at any time point (Figure 7A). In addition, no statistically significant differences in body weight were observed between T22-GFP-H6 and vehicle-treated animals (Figure 7B).

Discussion

The addition of a ligand to a nanoparticle-drug conjugate increases antitumor efficacy[10]; however, the mechanism underlying this effect remains unknown. There is no agreement on whether a ligand triggering tumor cell internalization could enhance tumor biodistribution[11]. PEGylated nanoparticles did not help in solving this controversy since PEGylation inhibits MPS clearance and indirectly increases tumor uptake[11].

Our approach allowed addressing this issue directly since the two compared protein carriers (liganded and unliganded) are non-PEGylated and achieve higher than 90% fluorescent emission in tumor tissue, while being undetectable in normal tissues. In contrast, for most nanoparticles, only 1-10% of the injected dose reaches the tumor. We observed that a liganded carrier that selectively undergoes internalization in the subset of CXCR4+ target tumor cells enhances carrier uptake by the tumor.

A link between CXCR4-mediated internalization of T22-GFP-H6 and enhanced tumor uptake

The liganded carrier, T22-GFP-H6, displayed an enhanced uptake in tumor tissue in the SC CRC model, reaching a 4 fold higher peak and longer residency time than the unliganded GFP-H6 carrier, as measured by both fluorescence emission and IHC protein detection. In addition, whereas T22-GFP-H6 achieved a highly selective internalization into the cytosol of epithelial $CXCR4^+$ tumor cells, the unliganded GFP-H6 carrier did not internalize in these cells. These findings were replicated in the o7rthotopic CRC model, since most of the T22-GFP-H6 emitted fluorescence was located in the primary tumor, lymphatic and peritoneal metastases, and this protein was exclusively detected in $CXCR4^+$ tumor and metastatic cell cytosol, as determined by IHC.

The demonstration that SDF1-α inhibited both T22-GFP-H6 internalization in the CXCR4+ tumor cell cytosol and tumor uptake *in vivo* established the CXCR4 receptor-dependent internalization for this liganded carrier, validating our previous observation in $CXCR4^+$ CRC cells *in vitro.*[17]. Interestingly, in the orthotopic model, the intensity of T22-GFP-H6 internalization was

dependent on the level of CXCR4 receptor expression at each particular site. Thus, the T22-GFP-H6 carrier displayed a higher uptake in primary tumors and peritoneal metastases (which expressed high level of CXCR4) than in lymph node metastases (which expressed low CXCR4 levels). All these results support a contribution for T22-induced tumor cell internalization in enhancing tumor uptake.

Pharmacokinetic and pharmacodynamic advantages for the liganded carrier T22-GFP-H6

The incorporation of a targeting ligand to a carrier may have both pharmacokinetic (PK) and pharmacodynamic (PD) advantages. Regarding PK, the liganded carrier may enhance retention and residency time in tumor tissue, having a peak at a later time (24h) because of its capacity to bind and internalize in target cells through specific receptor (in our case, CXCR4) mediated endocytosis. In contrast, the unliganded carrier reaches an earlier peak (15 min) and may return to the circulation from the extracellular space because the lack of a ligand leaves the carrier subjected to the convective current going from the tumor interior (with higher interstitial fluid pressure) toward the periphery[20]. An increase in tumor uptake has also been reported for lipoplexes or liposomes targeting the Transferrin receptor as compared to untargeted nanoparticles[21-24].

The less intense uptake of the unliganded GFP-H6 and its accumulation in the tumor stroma (instead of in tumor epithelial cells) was replicated when we compited the CXCR4-dependent internalization of T22-GFP-H6 by administering SDF1-α. These results suggest a dominant effect for the T22 liganded carrier on internalization in CXCR4+ cells over its biodistribution to tumor stroma. These findings are consistent with the report of a 6-fold higher tumor uptake for a liganded lipidic nanoparticle targeting Her2+ cells as compared to the corresponding unliganded nanoparticle; this last displaying a lower uptake in tumor stroma, without internalization in tumor epithelial cells.[25]

Most importantly, on top of its enhanced tumor distribution, the liganded T22-GFP-H6 carrier displays an important pharmacodynamic advantage, that is, its exclusive internalization in the

cytosol of the small subset of tumor cells that express the target receptor -in our case the 10-15% CXCR4+ cells within the whole tumor tissue- whereas the unliganded nanoparticle does not target any particular tumor cell subset. This means that the T22-GFP-H6 carrier reaches a dramatically high concentration inside the CXCR4+ target cell cytosol, since it is exclusively detected in the cytosol of these cells by IHC, and therefore, most of the fluorescence emitted by the tumor should come from the carrier molecules internalized in these cells.

The PK/PD advantages of T22-GFP-H6 over GFP-H6 occur without detectable toxicity on normal tissues. Thus, there is a lack of distribution for both the liganded and the unliganded carriers to CXCR4- (brain, lung, heart, kidney or liver) or CXCR4 + (spleen and bone marrow) normal tissues since their fluorescence emission signal was undistinguishable from background. This is probably related to the 10-20 fold lower level of CXCR4 expression in normal as compared to tumor tissues,[15,16] a finding consistent with the lack of histopathological alterations registered in all studied normal organs and with the lack of mouse body-weight loss. These results differ for instance from the described uptake of liposomal or polymeric particles, both PEGylated and non-PEGylated, by the MPS system in the normal liver, which represents an important problem. [11,26]

T22-GFP-H6 as a nanocarrier for antitumor drug delivery to CXCR4-dependent tumors

We have already demonstrated the carrier capacity for T22-GFP-H6, since we could complex this protein to nucleic acid constructs, which could afterwards undergo endocytosis in CXCR4+ cells, triggering construct expression in target cells.[1] We are currently using the T22-GFP-H6 carrier to generate drug nanconjugates with the aim of achieving targeted drug delivery to CXCR4+ tumor cells. To that purpose, we are using established protocols, for site-specific bioconjugation of proteins (e.g. thioethers or amide bonds linkages)[12], to achieve the covalent conjugation of T22-GFP-H6 to antitumor drugs and toxins without interfering with their activity.

We expect that these novel drug-conjugates, obtained by bioconjugation, maintain the cell-specific targeting properties, and display the same PK/PD advantages here described for the unconjugated carrier. Consequently, we expect that these liganded T22-GFP-H6-drug conjugates achieve a higher

tumor uptake and an even higher accumulation in the cytosol of CXCR4+ cells, as compared to the corresponding unligaded GFP-H6 conjugate. This will, in turn, achieve a dramatic increase in the delivery of the conjugated drug and of the free-drug concentration in the target tumor cell cytosol, which could explain the enhanced antitumor effect previously described for non-protein-based liganded nanoconjugates[10] and for the antibody-drug conjugates[27], without significant accumulation or toxicity on normal tissues.

In agreement with this rationale, the $Her2^{+}$ targeted antibody-drug conjugate (ADC) trastuzumab emtansine, used to treat refractory $Her2^{+}$ breast cancer patients,[28] shows receptor-mediated internalization[29,30] and achieves a 300-500% increase in tumor drug concentration compared to the untargeted drug conjugate.[31] In contrast, the tumor uptake of the unliganded nanoconjugates is significantlty lower. Thus, the unliganded albumin-based nanoparticle nab-paclitaxel achieves only a 30% increase in tumor drug concentration as compared to the free drug,[5] a finding consistent with only 5% of the injected intravenous dose of 89Zr-albumin reaching the tumor. [32]

Our results support the notion that a targeting ligand confers pharmacokinetic (enhanced tumor uptake) and pharmacodynamic (high and selective accumulation in the cytosol in a small subset of target tumor cells) advantages. These findings validate T22-GFP-H6 as a highly efficient nanocarrier for selective drug delivery to the cytosol of CXCR4 tumor cells, and support the development of novel antitumor-drug nanoconjugates based on the this carrier. This novel approach could significantly improve current therapy for the wide variety of tumors whose growth and dissemination depends on CXCR4 receptor overexpression, which include all neoplasias in which CXCR4 is a poor prognosis marker.[13,33]

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Authors´contributions

Conception and design: RM, AV, EV, MVC, UU ; Development of Methodology: MVC, UU, PA, RS, MT, MAM; Acquisition of data: MVC, UU, EV, RS, PA, IC, MAP; Analysis and interpretation of data: RM, AV, MVC, EV, UU, MAM, MT, ALP, MAP; Writing, review, and/or revision of manuscript: RM, AV, MVC, EV, UU, MAM, MT, ALP, MAP; Study supervision: RM, AV, EV, MVC, UU

Legend to figures

Figure 1. Differences in intensity and kinetics of tumor accumulation between the T22-GFP-H6 and GFP-H6 protein carriers. The liganded T22-GFP-H6 carrier differed from the unliganded GFP-H6 carrier in tumor uptake after the administration of 100ug i.v. dose each, in the SW1417 $CXCR4^+$ subcutaneous CRC model, as assessed by fluorescent emission (panel A). The differences were observed in the onset of uptake detection (2 h vs. 15 min), in maximal intensity (10.5 vs. 2.5 units) and in time at the peak (24 h vs. 15 min). No fluorescence was detected in buffer-treated controls (panels A & B). Similarly to the differences in tumor uptake, as measured by fluorescent emission, we found that the protein carrier accumulation, detected by IHC using an anti-GFP antibody, at its peak inside CXCR4+ tumor cells was significantly higher in T22-GFP-H6 (peak at 24h) than in the GFP-H6 (peak at 15 min) (panel C). No differences in histology (H&E staining), percentage or expression intensity of CXCR4 positive cells (IHC staining), or apoptotic figures (nuclear staining by Hoescht) in tumor tissue were detected between the compared groups (panel D). Abbreviatures: FLI: Fluorescent imaging; GFP: green fluorescent protein; T22: CXCR4 receptor ligand

Figure 2. CXCR4-dependent uptake of T22-GFP-H6 in tumor tissue. (A) Fluorescence emission in representative SW1417 $CXCR4^+$ subcutaneous tumors from buffer, GFP-H6, T22-GFP-H6 or SDF1-α+T22-GFP-H6 treated animals, recorded *ex vivo* 2h after 100µg oiv administration. (B) Mean fluorescent signal, expressed as ratio of radiant efficiency in the 4 compared groups. Note the inhibition of T22-GFP-H6 uptake by the tumor when co-administering SDF1-α (** $p< 0.001$). The administration of SDF1-α (0.15 mg/kg) was performed as a SC 1h before a 100µg T22-GFP-H6 i.v. bolus injection.

Figure 3. Differences in tumor cell type internalization between T22-GFP-H6 and GFP-H6 carriers. CXCR4 expression (A,B,C,D) and GFP-H6 or T22-GFP-H6 carrier detection with an anti-GFP antibody (E,F,G,H) in representative tumors, in Buffer, GFP-H6, T22-GFP-H6 or SDF1-

α+T22-GFP-H6-treated groups. Two hours after administration, the unliganded GFP-H6 carrier was internalized into tumor stromal cells (F, black arrows) whereas the liganded T22-GFP-H6 carrier selectively internalized into $CXCR4^+$ tumor epithelial cells (G, black asterisks). Note that CXCR4 receptor downregulation by the co-administration of SDF1-α, completely blocked T22-GFP-H6 internalization into the cytosol of $CXCR4^+$ tumor epithelial cells (H, black asterisks), and promoted instead T22-GFP-H6 internalization into tumor stromal cells (H, black arrows). Lack of anti-GFP IHC staining was observed in epithelial (black asterisks) or stromal (black arrows) cells in buffer-treated tumors (E).

Figure 4. Lack of accumulation of the GFP-H6 or T22-GFP-H6 carriers in normal tissues. No fluorescence was detected *ex vivo* 5h or 24h after the administration of 100μg of GFP-H6 or T22-GFP-H6 in non-tumor tissues that do not express CXCR4 (A, brain, lung heart, kidney and liver) or in those that do express CXCR4 (B, spleen and bone marrow)

Figure 5. Lack of histological alterations in non-tumor tissues. No histological alterations were detected in H&E stained tissue sections of CXCR4 negative organs (brain. lung, kidney, liver) or CXCR4 expressing organs (spleen, bone marrow) 24 hours after the administration of 100 μg of T22-GFP-H6 or GFP-H6

Figure 6. Liganded T22-GFP-H6 carrier uptake in primary tumor and metastases and selective internalization in CXCR4 overexpressing tumor cells in an orthotopic CRC model. Macrophotographs (A, upper panel), histology (H&E staining, A, middle panel) and CXCR4 expression (A, bottom panel) in primary tumor, and in lymph node or peritoneal metastases in the SW1417 orthotopic CRC model. Selective biodistribution of the liganded T22-GFP-H6 carrier to primary tumor, lymph node and peritoneal metastases measured by *ex vivo* fluorescence emission (B) and its selective internalization into the $CXCR4^+$ tumor cell cytosol in primary tumor and lymphatic or peritoneal metastasis (C) 2h after the administration of a 20μg T22-GFP-H6 i.v. bolus. Fluorescence emission or anti-CXCR4 staining was undetectable in tumors from buffer-treated

animals (B). Note the lower intensity of CXCR4 expression in the membrane of lymphatic metastases (A, lower panel), as compared to the tumor in other locations, and its association with lower T22-GFP-H6 in lymphatic metastases (B, 1.2 versus 2.5 fluorescence intensity peak) or carrier uptake in tumor cells, as measured by anti-GFP antibody (C).

Figure 7. Lack of effect of T22-GFP-H6 on tumor growth or mouse body weight after repeated dose administration. The liganded T22-GFP-H6 carrier administration at a repeated dose regime (10µg, q3d, x9 doses) did not alter tumor growth (A) or reduced mouse body weight (B).

References

1. **Unzueta U et al.** Sheltering DNA in self-organizing, protein-only nano-shells as artificial viruses for gene delivery. *Nanomedicine 2014;***10(3)**: 535-41.

2. Dawidczyk CM et al. State-of-the-art in design rules for drug delivery platforms: lessons learned from FDA-approved nanomedicines. *J Control Release* 2014; **10(187)**:133-44.

3. Prabhakar U et al. Challenges and key considerations of the enhanced permeability and retention effect for nanomedicine drug delivery in oncology. *Cancer Res* 2013; **15:73 (8)**:2412-17.

4. Harrington KJ et al. Effective targeting of solid tumors in patients with locally advanced cancers by radiolabeled pegylated liposomes. *Clin Cancer Res* 2001; **7(2)**: 243-54.

5. Desai N, Trieu V, Damascelli B, Soon-Shiong P. SPARC Expression Correlates with Tumor Response to Albumin-Bound Paclitaxel in Head and Neck Cancer Patients. *Transl Oncol* 2009; **2(2):** 59-64.

6. Duncan R, Gaspar R. Nanomedicine(s) under the microscope. *Mol Pharm 2011*; **5:8 (6)**: 2101-41.

7. Wang R, Billone PS, Mullett WM. Nanomedicine in Action: An Overview of Cancer Nanomedicine on the Market and in Clinical Trials. *J Nanomaterials* 2013; Article ID 629681. doi:10.1155/2013/629681

8. Dawidczyk CM, Russell LM, Searson PC. Nanomedicines for cancer therapy: state-of-the-art and limitations to pre-clinical studies that hinder future developments. *Front Chem* 2014; **25:2**:69.

9. Byrne JD, Betancourt T, Brannon-Peppas L. Active targeting schemes for nanoparticle systems in cancer therapeutics. *Adv Drug Deliv Rev* 2008; **14;60(15)**: 1615-26.

10. Li SD, Huang L. Gene therapy progress and prospects: non-viral gene therapy by systemic delivery. Gene Ther 2006; **13(18)**:1313-9.

11. Pirollo KF, Chang EH. Does a targeting ligand influence nanoparticle tumor localization or uptake? *Trends Biotechnol* 2008; **26(10)**:552-8.

12. Kalia J, Raines RT. Advances in Bioconjugation. *Curr Org Chem* 2010;**14(2)**: 138-47.

13. Balkwill F. The significance of cancer cell expression of the chemokine receptor CXCR4. *Sem Cancer Biol* 2004; **14(3)**: 171-9.

[14.] Kim J et al. Chemokine receptor CXCR4 expression in colorectal cancer patients increases the risk for recurrence and for poor survival. *J Clin Oncol* 2005; **20;23(12)**: 2744-53.

[15.] Fischer T, Nagel F, Jacobs S, Stumm R, Schulz S. Reassessment of CXCR4 chemokine receptor expression in human normal and neoplastic tissues using the novel rabbit monoclonal antibody UMB-2. *PLoS One* 2008; **3(12)**: e4069.

[16.] Nimmagadda S, Pullambhatla M, Pomper MG. Immunoimaging of CXCR4 expression in brain tumor xenografts using SPECT/CT. *J Nucl Med* 2009; **50(7)**: 1124-30.

[17.] Unzueta U et al. Intracellular CXCR4 cell targeting with T22-empowered protein-only nanoparticles. Int J Nanomedicine 2012; **7**:4533-44.

[18.] Céspedes MV et al. In vivo architectonic stability of fully de novo designed protein-only nanoparticles. *ACS Nano* 2014; **27:8(5)**: 4166-76.

[19.] Céspedes MV et al. Orthotopic microinjection of human colon cancer cells in nude mice induces tumor foci in all clinically relevant metastatic sites. *Am J Pathol* 2007; **170(3)**:1077-85.

[20.] Flessner MF, Choi J, Credit K, Deverkadra R, Henderson K. Resistance of tumor interstitial pressure to the penetration of intraperitoneally delivered antibodies into metastatic ovarian tumors. *Clin Cancer Res* 2005; **15;11(8)**:3117-25.

[21.] Xu L et al. Systemic p53 gene therapy of cancer with immunolipoplexes targeted by anti-transferrin receptor scFv. *Mol Med* 2001; **7(10):**723-34.

[22.] Kircheis R et al. Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application. *Gene Ther* 2001; **8(1):**28-40.

[23.] Xu L et al. Systemic tumor-targeted gene delivery by anti-transferrin receptor scFv-immunoliposomes. *Mol Cancer Ther* 2002; **1(5)**:337-46.

[24.] Yu W et al. Enhanced transfection efficiency of a systemically delivered tumor-targeting immunolipoplex by inclusion of a pH-sensitive histidylated oligolysine peptide. *Nucleic Acids Res* 2004; **16;32(5)**:e48.

[25.] Kirpotin DB, Drummond DC, Shao Y, Shalaby MR, Hong K, Nielsen UB, Marks JD, Benz CC, Park JW. Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models. *Cancer Res* 2006; **66(13)**: 6732-40.

[26.] Ruoslahti EL, Bhatia SN, Sailor MJ. Targeting of drugs and nanoparticles to tumors. *J Cell Biol* 2010; 22:188(6): 759-68.

[27.] Scott AM, Wolchok JD, Old LJ. Antibody therapy of cancer. *Nat Rev Cancer* 2012; **22:12(4)**: 278-87.

[28.] Verma S et al. EMILIA Study Group. Trastuzumab emtansine for HER2-positive advanced breast cancer. *N Engl J Med* 2012; **8;367(19)**: 1783-91.

[29.] Austin CD et al. Endocytosis and sorting of ErbB2 and the site of action of cancer therapeutics trastuzumab and geldanamycin. *Mol Biol Cell* 2004; **15(12)**: 5268-82.

[30.] Lewis Phillips GD et al. Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. *Cancer Res* 2008; 9280-90.

[31.] Erickson HK et al. The effect of different linkers on target cell catabolism and pharmacokinetics/ pharmacodynamics of trastuzumab maytansinoid conjugates. *Mol Cancer Ther* 2012; **11(5)**: 1133-42.

[32.] Heneweer CL et al. Magnitude of enhanced permeability and retention effect in tumors with different phenotypes: 89Zr-albumin as a model system. *J Nucl Med* 2011; **52(4)**: 625-33.

[33.] Sun X et al. CXCL12/CXCR4/CXCR7 chemokine axis. *Cancer Metastasis Rev* 2010; **29**:709-22.