SIKVAV peptide functionalized ultra-small gold nanoparticles for selective targeting of $\alpha_6\beta_1$ integrin in hepatocellular carcinoma

M Roskamp$^1$, T Coulter$^1$, Y Ding$^1$, R Perrins$^1$, C Espinosa Garcia$^1$, A Pace$^1$, S Hale$^1$, A Robinson$^1$, P Williams$^1$, U Aguilera Peral$^1$, K Patel$^1$, D Palmer$^1$

Midatech Pharma PLC, Abingdon, UK

dan@proficitpartners.com

Abstract. Ultra-small glycan-passivated gold nanoparticles of <2nm diameter were functionalised with a short HS-EG(8)-COOH ligand. The nanoparticles were subsequently labelled, in a stoichiometrically controllable manner, with integrin-binding peptide SIKVAV and the maytansinoid cytotoxin DM4. In vitro assays showed significantly increased integrin-mediated uptake of SIKVAV labelled nanoparticles in HepG2 cells. SIKVAV targeted nanoparticle binding was shown to be outcompeted with free SIKVAV peptide, indicating target specific uptake. DM4 was passively attached to nanoparticles via sulphydryl ligand exchange at the gold nanoparticle surface, which rendered them highly cytotoxic (IC50 ~1 x 10^3M). In a rat model, pharmacokinetic studies showed that nanoparticle biodistribution was strongly altered by labelling with either peptide and DM4 moieties.

1. Introduction

Ultra-small gold nanoparticles (GNPs) have broad applicability in the field of drug delivery and tissue-specific drug delivery$^{1,3}$. The term “ultra-small” is used to define nanoparticles with a core size of <2nm$^4$. Numerous conflicting reports describe the toxicity of ultra-small GNPs$^{2,5-11}$. Our own in vivo studies show across various species, that GNPs of this size with thiolated ligand shells can have negligible toxicity and immunogenicity. Differences in the ligand shell composition of GNP’s may be a likely cause for differences in observed toxicity$^7$. Ultra-small nanoparticles offer particularly high surface / volume ratio, which has important consequences for drug-loading and dose-efficiency. GNPs are also useful as drug-delivery vehicles, as their physicochemical properties may be fine-tuned to support various classes of therapeutic compounds$^{12,14}$.

Much of our research concerns the synthesis and functionalisation of glycan-passivated GNPs, which are afforded long-term stability and water-solubility by sulphydryl-modified monosaccharide ligands such as 2’-thioethyl-α-D galactopyranoside$^{15,16}$. Active targeting to specific tissues or cell types can be achieved by incorporating high-affinity species (e.g. peptides) within the ligand shell of a GNP construct$^{17,19}$. For cancer therapy, such nanoparticle constructs are intended to enable the selective delivery of a pharmaceutical ingredient (often a cytotoxin) to tumour tissues, thus sparing healthy tissues and minimising off-target toxicities.

In this study, the peptide sequence (SIKVAV) derived from the basement membrane glycoprotein laminin α1 chain$^{9,21}$, was covalently attached via the N-terminus to carboxylated ultra-small GNPs. The sequence is known to bind to cell-surface integrins α3β1 and α6β1$^{22}$. Importantly, the α6 integrin is known to be...
highly expressed by some cancer stem cells, notably those residing within the glioblastoma perivascular niche\textsuperscript{23}. Similarly, hepatocytes are known to undergo unusual changes in expression of integrins upon carcinogenesis, including increased expression of laminin receptor α6β1\textsuperscript{24}. Other extracellular matrix-derived peptide sequences have been used to confer active-targeting functionality to a range of therapeutic nanoparticles. In a recent study, the widely studied integrin-binding sequence c(RGDfC) was covalently attached to ~5nm gold nanoparticles for the purposes of vascular targeting\textsuperscript{18}. Additionally, the functionalization of larger (~300nm) carboxylate-functionalised nanoparticles with another laminin-derived peptide sequence (YIGSR) has previously been described\textsuperscript{25} and shown to enable successful in vivo targeting to lung metastases.

The potent tubulin inhibitor tansine, which is derived from a naturally occurring ansamycin antibiotic, is an extremely potent cytotoxin that inhibits microtubule assembly and disrupts mitosis\textsuperscript{26}. The structurally homologous mertansinoid DM4 has a terminal sulfhydryl function that can be used for conjugation as it is not related to activity. Despite high potency, DM4 has significant systemic side effects leading to poor tolerance and narrow therapeutic window. Here, we explore a straightforward approach to the synthesis of DM4-loaded GNPs via ligand exchange. This method was used to synthesise a novel GNP construct comprising SIKVAV peptide targeting ligands and DM4 cytotoxic ligands.

2. Results & Discussion

A previously characterised GNP construct was synthesised with a target ligand shell of 50\% galactose-C2 thiol and 50\% HS-EQ(8)-COOH. The product (A) was characterised by DLS, \textsuperscript{1}H-NMR, TEM and HPLC and was found to have, an average gold core size of ca. 1.8 nm and a hydrodynamic diameter of ca. 5 nm. Following purification, the GNP was reacted with an EDC/NHS mixture to convert carboxylic acid ligands to reactive NHS esters, then incubated with SIKVAV peptide. By varying the SIKVAV:GNP stoichiometry, GNPs could be produced with between 2 to 13 covalently coupled SIKVAV moieties per 200 gold atoms (Figure 1). For all further testing, a product GNP (B) with a ligand shell comprising [AuNP (α-Gal-C2)/(S-EQ(8)COOH)/(S-EQ(8)CONHSIKVAV)(11)] was chosen. This AuNP has an average of 8 SIKVAV peptides per 200 gold atoms.

![Figure 1](image1.png)

Figure 1 (A). DLS analysis. All samples were measured in PBS and showed an average hydrodynamic diameter of approximately 5 nm. For each nanoparticle, the average of 3 measurements is shown. (B). HPLC analysis showed a shift of retention time for GNPs bearing varying equivalents of SIKVAV peptide.

To compare the effect of targeting ligand incorporation into our ultra-small GNPs, the (α6β1) expressing cell line HEPG2\textsuperscript{24} was exposed to non-targeted (GNP-A) and SIKVAV-targeted (B) particles. Silver enhancement staining revealed cell uptake to be ~2.5 fold higher in the SIKVAV-targeted GNPs (Figure 2a). To test the specificity of the observed increased GNP uptake, HEPG2 cells were pre-treated with free SIKVAV peptide before treatment with SIKVAV-labelled GNP B. Internalised gold content was measured by ICP-MS. These data revealed that pre-blocking with SIKVAV resulted in ~50\% reduction in cell uptake (Figure 2b), achieving a similar level to that of the non-targeted GNP.
2.3 \( \text{groups that are incompatible with the reducing} \)
\( \text{not greatly diminish its cytotoxicity} \)
\( \text{conclude} \)
\( \text{with IC50 values of 1.0 \times 10^{-4}} \)
\( \text{highest concentration} \)
\( \text{was determined by MTT assay. GNP constructs} \)
\( \text{between 300 and 0.1ng/mL DM4 (GNPs A & B were tested at matching gold concentrations),} \)
\( T \)
\( \text{2.2} \)
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\( \text{product} \)
\( \text{peptide labelled} \)
\( \text{were isolated} \)
\( \text{The t} \)
\( \text{2.1} \)
\( \text{experiments.} \)
\( \text{MTT assay. Results were analyzed using a Student's} \)
\( \text{were treated with GNPs functionalized with DM4 plus or minus SIKVA} \)
\( \text{acid/10.5M NaCl pH2.8: 10min at RT). Cell pellet gold content was measured by ICP} \)
\( \text{incubated either in the absence (−) or presence (+) of free SIKVAV peptide (500nM). After exposure, cells were acid washed (0.2M Acetic} \)
\( \text{Figure 2A GNP-SIKVAV uptake in human hepatocellular carcinoma cells} \)
\( \text{Attachment of DM4} \)
\( \text{toxicities of} \)
\( \text{Deacetyl N2'-} \)
\( \text{Here, we attempted to further functionalise GNPs with DM4 moieties by ligand exchange.} \)
\( \text{samples} \)
\( \text{DM4} \)
\( \text{The product nanoparticles were} \)
\( \text{HPLC analysis, the} \)
\( \text{DM4} \)
\( \text{D} \)
\( \text{were studied in} \)
\( \text{The two GNP samples} \)
\( \text{were isolated from the reaction mixture and carefully washed to remove traces of non-bound DM4. The non-peptide labelled GNP} \)
\( \text{DM4} \)
\( \text{towards DM4 plus or minus SIKVAV targeting peptide for 48h before measurement of cell viability by} \)
\( \text{results were analyzed using a Student's} \)
\( \text{Data are means ±} \)
\( \text{standard deviation of at least three independent experiments.} \)
\( \text{Attachment of DM4} \)
\( \text{By treating glycan-passivated GNPs with another sulfhydryl ligand, ligand substitution is observed. The cytotoxin N2',Deacetyl-N2'-} \)
\( \text{cytotoxin N2'} \)
\( \text{Deacetyl-N2'-} \)
\( \text{ligand substitution is observed. The} \)
\( \text{DM4} \)
\( \text{possesses a terminal sulfhydryl function. Here, we attempted to further functionalise GNPs with DM4 moieties by ligand exchange.} \)
\( \text{The two GNP samples} \)
\( \text{were treated with 12 equivalents of DM4 per 200 gold atoms. After 3h, GNPs} \)
\( \text{were isolated from the reaction mixture and carefully washed to remove traces of non-bound DM4. The non-peptide labelled GNP} \)
\( \text{DM4} \)
\( \text{The product nanoparticles were water soluble and showed no aggregation or precipitation in PBS. Similarly, a SIKVAV-labelled GNP} \)
\( \text{HPLC analysis showed the isolated product GNP} \)
\( \text{HPLC analysis showed the isolated product GNP} \)
\( \text{DM4} \)
\( \text{DM4} \)
\( \text{HNPS/cell for 1.5h} \)
\( \text{were shown to be highly cytotoxic,} \)
\( \text{From these results, we conclude that the attachment of DM4 (IC50 = 4.1 \times 10^{-8}M) to ultra-small GNPs via the sulfhydryl function does not greatly diminish its cytotoxicity in vitro. The simple approach to drug-loading via competing sulfhydryl ligand exchange is potentially useful for the post-manufacture attachment of compounds with labile functional groups that are incompatible with the reducing, basic conditions of GNP synthesis.} \)
\( \text{In vivo biodistribution and PK} \)
The GNP samples A-D were administered intravenously to male Wistar rats to elucidate the effects of peptide targeting & DM4 loading upon PK and biodistribution. Blood samples were collected at 1, 2, 4, 8 and 24h post injection. Attachment of the peptide sequence SIKVAV, which has a calculated LogP value of -0.75 is expected to introduce positive charge (from lysine residues) to the otherwise negatively charged GNP construct A. On the other hand, DM4 has a calculated LogP value of 5.72 and is expected to add significant hydrophobicity to the nanoparticle construct. Addition of SIKVAV reduced the zeta potential from -40 mV for A to -12 mV for C consistent with addition of a positively charged peptide. Incorporation of SIKVAV peptide or DM4 cytotoxin did affect both biodistribution and pharmacokinetics. Construct A showed the longest circulating half-life (Figure 3a). Construct B, labelled with an average of 7 SIKVAV peptide moieties per 200 gold atoms showed a similar elimination rate. Constructs loaded with DM4 moieties (C & D) showed a significantly increased elimination rate. From this preliminary study, it was not possible to derive meaningful PK parameters, hence further work is required to fully understand the impact of DM4 labelling on elimination rate and volume of distribution.

To more fully understand the fate of injected GNPs, tissue samples were harvested from liver, lungs, brain, eyes, faeces, femur, heart, kidney, large intestine, muscle, skin, plasma, small intestine, spleen, stomach, testes, urine and blood cells. For many of these tissues, ICP-MS analysis showed gold deposition to be extremely low and close to the limit of detection. Hence, we concentrated our analysis on the samples containing the bulk of the dose, i.e. liver, kidney, faeces, plasma and urine. Construct A showed minimal tissue deposition, with just 9% of dose recovered in the kidney and over 65% recovered in urine. Interestingly, the incorporation of SIKVAV peptide (B) caused minimal change in distribution to the liver and plasma, but a marked increase in kidney deposition (45% of dose) and a decreased elimination in urine (35%). GNPs loaded with DM4 (C) showed a different pattern of distribution, in which liver uptake was double that of A, whilst plasma levels were the lowest detected at 0.6% of dose (corresponding with pharmacokinetic profile above). Again, deposition to kidney increased to 19.5%, and urine content decreased to 22% of the given dose. Finally, construct D, bearing both SIKVAV and DM4 moieties, showed the highest uptake in kidney (60%) and a large reduction in urine content (27% of dose) (Figure 3b). Accumulation of peptide GNP’s in the kidney has been observed in previous studies (unpublished observation).

![Figure 3](image)

**Figure 3 (A)**. PK profiles of GNPs in Wistar rats: Gold-content measured in blood samples following single tail vein injection of GNPs A-D (300µg/kg dose). Concentrations measured by ICP-MS (B). Biodistribution of GNPs 24h post dosing. Gold-content found in major organs & tissues following tail vein injection of GNPs A-D. Concentrations measured by ICP-MS. GNP A = GalC₂-EG₈-COOH; B = GalC₂-EG₈-CONHSIKVAV; C = GalC₂-EG₈-COOH-DM4; D = GalC₂-EG₈-CONHSIKVAV-DM4

### 3. Materials & Methods

#### 3.1 Chemicals & materials

Dimethyl sulfoxide, Hydrogen tetrachloroaurate (III) trihydrate (HAuCl₄·3H₂O), EDC (N-(3-Dimethylaminopropyl)-N’-ethoxycarbodiimide hydrochloride); DMEM medium, Ethanol (absolute); L-Glutamine, Methylthiazolyl diphenyl-tetrazolium bromide; foetal calf serum; NHS (N-Hydroxy-succinimide); Non-essential amino acids; ProteoSilver™ Silver Stain Kit; Sodium borohydride; Sodium hydroxide and Sodium pyruvate were all obtained from Sigma-Aldrich, UK. N₂’-Deacetyl-N₂’-(4-mercapto-4-methyl-1-oxopentyl)-maytansine, referred to as DM4, was purchased from Carbosynth, UK. Di-2’-thioethyl-α-D galactopyranoside disulfide was purchased from Galchimia, A Coruña, Spain. SIKVAV peptide was purchased from Bachem, Switzerland. 1-mercaptop-3,6,9,12,15,18,21,24-octaoxaheptacosan-27-oic acid (HS-EG₁₈-COOH) was purchased from Iris
Biotechnology, Germany. Amicon Ultra-15 centrifugal filter units, MWCO=10K were purchased from Millipore Sigma, UK. Deionized water was obtained from an Elga Purelab Option-Q (DV25) (electrical resistivity 18.2MΩ).

Analytical methods

Analytical methods used for physico-chemical characterisation of GNPs are fully described in the supplementary information. Briefly, UV-Vis absorption spectra were measured using a BMG Labtech SPECTRostar Nano microwell plate spectrophotometer. Dynamic light scattering was measured in triplicate using a Zetasizer Nano ZSP (Malvern Instruments Ltd, UK), at 25°C in distilled water. Mean volume-weighted hydrodynamic diameter of the particles was used as a measure of GNP size. GNP-bound ligands were analysed by 1H-NMR after dissolution of gold cores by treatment with KCl/KOH (0.3M/0.1M, respectively in D2O). HPLC analysis was performed using an Agilent 1260 Infinity system with a Zorbax Express Peptide C-18 column (4.6 x 100 mm, 2.7μm; Sigma; 35°C column temperature; flow rate 1ml/min). Ligand shell composition and DM4 loading were analysed by releasing the ligands from GNPs via incubation in 100mM Dithiothreitol for 3h.

3.2 Nanoparticle Synthesis

Carboxyl-functionalized ultra-small GNPs were synthesised at a 1mg scale via a modified Brust-Schiffrin method78. Aqueous solutions of 1-mercaptopropane-3,6,9,12,15,18,21,24-octaoxahexacosan-27-oic acid (HS-EG17-COOH) (7.5mL, 20mM) and di-2'-thioethyl-α-D galactopyranoside disulfide (2.5mL, 20mM) were mixed in a round bottomed flask. With continuous stirring, an aqueous solution of HAuCl4 (88μL, 333mM) was added. The pH was adjusted to 11.6 by adding 2M NaOH solution. A solution of sodium borohydride (1mL, 1.0M) was then added as quickly as possible. The resulting dark purple/black solution was stirred for a further 1 hour before purification by ultra-centrifugation and repeated washing in distilled water in an Amicon Ultra-15 (MWCO 10kDa) tube. Characterisation by DLS, HPLC and 1H-NMR revealed the product GNP (A) had an average diameter of 4nm and an average ligand shell comprising 40% galactose-C2 and 60% HS-EG17-COOH, which is expressed as [AuNP(α-Gal-C2)40] / (S-EG17-COOH)60].

3.3 Conjugation of SIKVAV peptide

GNP construct A (1.0mL, 3g/L Au) was activated with a mixture of EDC (55eq. per gold) and NHS (110eq. per gold) in DMSO for 2 hours. The activated GNPs were diluted in deionised water and centrifuged in an Amicon Ultra-15 tube to remove unreacted reagents. A solution of SIKVAV in 40mM HEPES buffer (pH7.8) was prepared (2.0mL, 0.263mM), to which the activated carboxyl GNPs were added via pipette. The mixture was stirred at room temperature overnight. The product was purified by ultra-centrifugation and repeated washing in deionised water in an Amicon Ultra-15 (MWCO 10kDa) tube.

3.4 DM4 Ligand exchange reactions

DM4 in absolute ethanol (500μL, 2.36mM) was carefully pipetted into a glass vial. An aqueous solution of the GNP [(α-Gal-C2)40] / (S-EG17-COOH)60] was added (500μL, 4g/L Au). The mixture was stirred for 3h at RT. After this time, the nanoparticle solution was transferred to an Amicon Ultra-15 tube and diluted with 7mL 50% ethanol solution. The product was then purified by repeated centrifugation at 5000rpm, during which the solvent was gradually changed to water. A small, white solid pellet (unreacted DM4) was removed by careful pipetting. HPLC analysis showed the extent of ligand exchange and DM4 loading per nanoparticle. Nanoparticle-bound DM4 was quantified by comparison with a standard curve. Using an analogous method, SIKVAV-labelled nanoparticles were treated with free DM4 for 3h to allow passive ligand exchange, yielding a brown solution that was analysed for DM4 content by HPLC.

3.5 Biological Methods

HEPG2 cells (human hepatocellular carcinoma, ATTC) were cultured in DMEM supplemented with 10% (v/v) FBS, 1% (v/v), L-Glutamine, non-essential amino acid and sodium pyruvate, at 37°C & 5% CO2 atmosphere. Cells were sub-cultured regularly and used at passage +5-10. MTT cytotoxicity assays were performed using a standard procedure, outlined in supplementary information. Adherent cell cultures were prepared in 96 well-plates and incubated overnight. Growth medium was replaced with solutions of test GNPs in low FBS medium (2%) at a concentration of 1.0x10⁷ GNPs per cell (20μL volume). Cultures were incubated for 90 min at 37°C, before being washed with warm PBS. Cells were fixed in 4% PFA solution (50μL per well) and incubated at RT for 10 min. Cell cultures were permeabilised by addition...
of Triton X-100 in PBS (0.1%, 50μL per well) for 10 min, then carefully washed with PBS. Working quickly, cell cultures were treated with the pre-mixed silver stain solutions at RT (100μL each) and imaged by light microscopy.

GNP uptake was also quantified (gold content) by ICP-MS. HEPG2 cells were seeded in 6 well-plates at a density of 1.2*10^5 cells in 2mL growth medium and allowed to grow to 95% confluence. The medium was replaced with a solution of 1.0x10^6 GNP per cell in low-serum medium (400μL, 2% FBS). Treated cultures were incubated for 3 hours, then washed with PBS (1mL). Cells were scrapped from well-plates, transferred to 2mL tubes and centrifuged at 200 x g for 5 min before being washed with a chilled solution of acetic acid (0.2M in 10.5M NaCl, pH2.8; 1mL; 4°C). After 6 min, samples were centrifuged at 200 x g for 5 min then incubated at RT for 10 min. Pellets were washed a further two times in PBS (1mL) and then frozen at -20°C. Before analysis, the pellets were digested in a mixture of nitric acid, hydrochloric acid and hydrogen peroxide (1.6mL, 0.5mL, 0.04mL, respectively) and diluted to 10mL volume in deionized water. The ensuing solutions were analysed using an Agilent 7700X ICP mass spectrometer alongside appropriate calibration standards.

For PK and biodistribution studies, male Wistar rats (7-8 weeks old, n = 4) were injected via the tail vein with a single dose of GNP sample at 0.3 mg(Au)/kg gold. Blood plasma samples (250μL) were collected at 0, 1, 2, 4, 8 and 24h post injection. All major organs & tissues were harvested and wet-weighed. Plasma & tissue samples were digested in Teflon microwave vessels using a CEM Discover Microwave digestion system. Gold concentrations were then measured by ICP-MS with appropriate calibration standards and expressed as a percentage of injected dose per gram of tissue.

4. Conclusion

Over-expression of integrins such as α6β1 is reported to be a characteristic of some cancer stem cells, glioblastomas and hepatocellular carcinomas. Hence, increased specific cytotoxicity and diminished systemic toxicity could be expected of a therapeutic GNP bearing SIKVAV peptides that bind with high affinity to α6β1 integrins. SIKVAV was covalently attached to glycan/EG_16-COOH ultra-small GNP via EDC/NHS coupling to the carboxylated ligand HS-EG_16-COOH. The product GNP were further derivatised with the microtubule inhibitor DM4, yielding stable, water-soluble constructs. In vitro experiments showed SIKVAV labelling produced a significant increase in nanoparticle uptake in HEPG2 cells. Similarly, in vitro toxicity studies showed DM4-loaded GNP to be highly toxic to this cell line. We observed a marked increase in nanoparticle elimination rate upon attachment of DM4 moieties, which is worthy of further study. We propose further development of the GNP construct, to extend plasma half-life and to improve target binding efficacy by modifying the targeting peptide / drug/ligand ratio.

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