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A Highly Potent Maytansinoid Analogue and its Use as a Cytotoxic Therapeutic Agent in Gold Nanoparticles for the Treatment of Hepatocellular Carcinoma

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Abstract: Gold nanoparticles are promising drug delivery agents with the potential to deliver chemotherapeutic agents to tumour sites. The highly cytotoxic maytansinoid tubulin inhibitor DM1 has been attached to gold nanoparticles and shows tumour growth inhibition in mouse models of hepatocellular carcinoma. Attempting to improve the stability of the gold-cytotoxin bond led to the design and synthesis of novel maytansinoids with improved potency in cell viability assays and improved *in vivo* tolerability compared to the DM1 analogues. These novel maytansines may also have applications in other methods of drug delivery, for example as the cytotoxic component of antibody drug conjugates.

Gold nanoparticles (GNPs) have shown promise as drug delivery agents as they are non-toxic, easy to prepare with controlled size distributions and functionalisable with a range of ligands. The ability to construct tuneable, multi-modal GNP entities allows the precise control of surface properties for targeting, stability and, importantly, the release of therapeutic payloads,[1,2] and GNPs have been reported as potential cancer therapies.[3-5] One advantage of GNPs is that it is possible to load relatively large amounts of hydrophobic drugs yet retain good aqueous solubility by the appropriate use of hydrophilic ligands attached to the gold, effectively changing the *in vivo* distribution and pharmacokinetics of the cytotoxic agent. The key requirement is that the active component remains

attached to the gold core until the nanoparticle reaches the target tissue whereupon it is selectively released in response to the altered physiological state of the diseased (tumour-) microenvironment compared to healthy tissue. A variety of functional groups will bind to gold, but the gold-sulfur bond is one of the strongest and is readily formed by the interaction of organic thiols with nanoscale gold.[6] Consequently, a thiol based cytotoxic agent would be a valuable tool in the design of a GNP based delivery system for cancer therapy.

Hepatocellular carcinoma (HCC) is the sixth commonest cancer worldwide with a very poor prognosis,[7] life expectancy after diagnosis is typically 6-9 months.[8,9] Although new emerging technologies such as immuno-oncology are under increasingly active development,[10] the current standard of care for HCC remains surgical resection and/or treatment with sorafenib, a broad-spectrum kinase inhibitor with a poor tolerability profile and only limited benefit in prolonging patient survival.[11] We have reported our studies on MTC-100038,[12] a GNP conjugated to a cytotoxin in mouse models of HCC, but it should be noted that without any targeting agents attached to the gold core, this type of GNP moiety would not be expected to show any tumour selectivity.

Maytansine **1** figure 1, an ansa macrolide isolated from *Maytenus ovatus*,[13] is a highly potent antimitotic agent that exerts an antiproliferative effect by inhibiting microtubule assembly on binding to tubulin with a K_D of around 1 μmol/L.[14] Despite a promising *in vitro* profile,[15] clinical trials with maytansine in cancer patients failed because of poor efficacy and unacceptable systemic toxicity.[16] Although the narrow therapeutic window precluded further clinical development work, maytansines continued to excite interest because of their high cytotoxicity (100 to 1000-fold more cytotoxic than the tubulin binders vincristine and vinblastine[17]). Antibody drug conjugates (ADCs) utilise monoclonal antibodies that bind to tumour specific receptors with high affinity to provide both a therapeutic entity, as well as a vehicle for the targeted delivery of chemotherapeutic agents to cancer cells.[18,19] As the antibody concentration at the tumour site is typically low, a highly potent chemotherapeutic agent is required. Maytansine, with an *in vitro* IC_{50} potency below that of typical

antibody concentrations at the tumour, has proved an attractive candidate for ADCs. Much effort has been expended in optimising suitable linkers to conjugate cytotoxins to antibodies,[20] for maytansines this has been achieved by the addition of thiol groups to give DM1, **2**, and DM4, **3**, figure 1, with little loss in potency.[21a,b] The success of this approach may be judged by the FDA approved Kadcyla®, an ADC consisting of an anti-HER2 antibody conjugated to DM1,[22] for the treatment of breast cancer, other maytansine based ADCs are progressing through clinical trials.[23] In some respects cytotoxin conjugated GNPs may be viewed as having some similarities to ADCs, and potentially have some advantages such as their relative ease of synthesis. However, there remain significant challenges in the therapeutic development of payload conjugated GNPs, though promising results for a TNF conjugated GNP in early clinical trials in patients with solid tumours have been reported.[24]

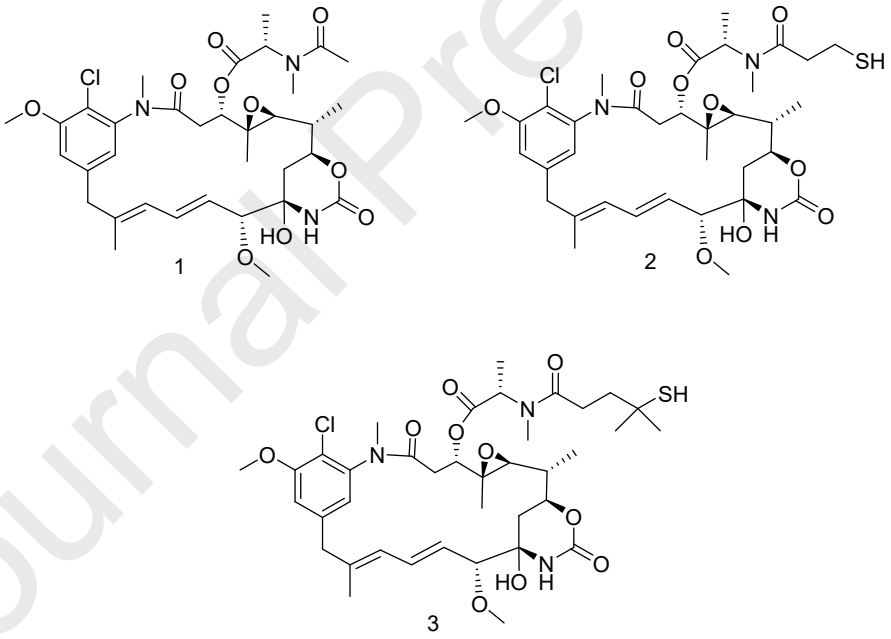


Figure 1. Maytansine analogues

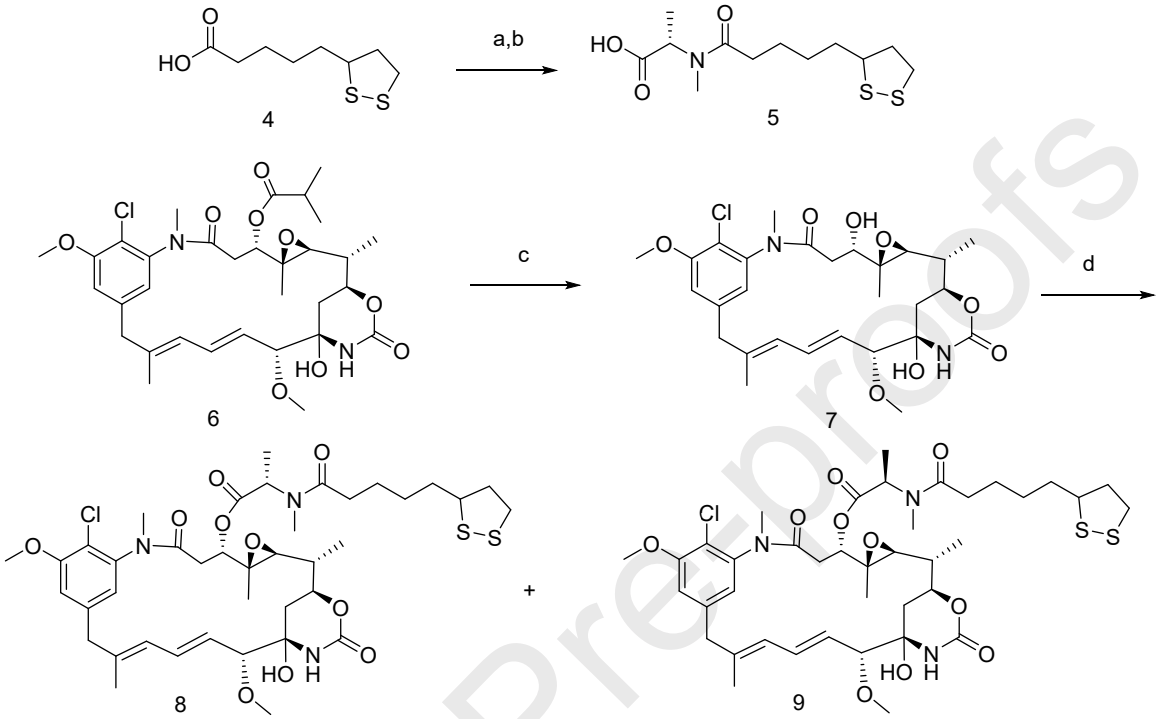
DM1 appeared to be an obvious candidate for conjugation to a GNP based on the relative ease of access, its cytotoxic potency and clinical validation together with the well-known ability of sulfur to form strong bonds with gold on the nanoscale.[25] MTC-100038,[12] a 2nm diameter gold core nanoparticle with galactose and carboxylate oligomeric ethylene glycol passivating and solubilising

ligands, with up to 6 molecules of DM1 per GNP. MTC-100038 was assessed for tolerability in NOD/SCID mice giving a 3-fold improvement in tolerability compared to DM1 alone, and significant tumour growth inhibition in an HCC HepB3 murine model bearing subcutaneous xenografts. However, measuring the rate of cleavage of DM1 from MTC-100038 in biological fluids and consequent correlation with the observed effects proved challenging (the small quantities, metabolism[26] and disulphide bond formation between DM1 and plasma proteins contributing to the challenges). We therefore sought an alternative method of linking the maytansine moiety to the gold to simplify analysis and provide improved stability.

Dihydrolipoic acid (DHLA), a dithiol obtained by the reduction of readily available thioctic acid, is an attractive ligand for addition to GNPs because it adopts a conformation that allows binding by both sulfur atoms rendering the ligand to be more resistant to displacement by a variety of factors including pH, ionic strength, heat, light and chemical attack by thiol containing moieties. This approach has been shown to improve the colloidal stability of GNPs[27] and resistance to intracellular glutathione mediated ligand cleavage.[28] The comparative stability and binding of monothiol, dithiols or disulphide ligand binding to GNPs has been reported,[29] however we are not aware of any reports of the use of this method to link complex cytotoxic molecules to nanoparticles. Once released from the GNP the disulphide bond would be expected to reform under oxidising conditions, making covalent binding to plasma proteins less likely, as well as being less prone to metabolism than DM1, thereby making analysis easier.

The 1,2-dithiolane analogue of DM1 was prepared as shown in Scheme 1. For the initial studies we used racemic lipoic acid **4** which was coupled to methyl N-methyl-L-alaninate using standard peptide coupling conditions and the ester hydrolysed with base to give compound **5**. Ansamitocin P3 **6** was reduced[21a] to maytansinol **7** and coupled with compound **5** using DCC and $ZnCl_2$ to give the desired product **8**. The latter step caused the racemisation of the alanine centre, an occurrence that is well-precedented.[21a] Although the yields for this step were disappointing, literature reports suggest that a

range of other reagents and conditions have been studied with DCC/$ZnCl_2$ giving the optimal results. The two products **8** and **9** were separated by preparative tlc, their purity being confirmed by SFC and HPLC (see supplementary data).



Scheme 1: (a) EDCI, HOBT, DIPEA, methyl N-methyl-L-alaninate, DCM, rt; (b) NaOH, H2O, MeOH, rt 16h; (c) $LiAl(OMe)_3H$, THF, -40°C; (d) DCC, $ZnCl_2$, compound **5**, DCM, rt, 24h

The cytotoxicity of **8** and **9** was measured by an MTT assay[30] against U87MG (glioblastoma) and Hep3B (hepatocellular carcinoma) cell lines and we were pleasantly surprised to observe that one of the isomers was significantly more potent than DM1, table 1. This is consistent with the reports for DM1 analogues where the eutomer was determined to be have the *S* configuration at the alanine centre,[21a] we consequently assigned **8** with *S*-alanine stereochemistry, though were unable to confirm this experimentally.

#	Alanine stereo-chem	1,2-dithiolane stereo-chem	Hep3B cells				U87MG cells			
			IC_{50} (nM)	n	SEM	IC_{50} fold over DM1	IC_{50} (nM)	n	SEM	IC50 fold over DM1
DM1	*S*	-	5.1	219	0.41	-	3.6	171	0.25	-

8	*S*	racemic	0.56	27	0.31	9	1.2	21	0.69	3
9	*R*	racemic	5.8	12	2.02	0.9	9.6	9	1.37	0.4
10	*S*	*R*	0.29	9	0.01	18	0.33	9	0.03	11
11	*R*	*R*	76	9	61	0.1	66	9	55.4	0.05
12	*S*	*S*	0.27	3	0.6	19	0.31	3	0.3	12
13	*R*	*S*	12.3	3	2.5	0.4	10.6	3	1.2	0.3

Table 1: Activity of 1,2-dithiolane analogues in an MTT cytotoxicity assay against Hep3B and U87MG cells

The potency of **8, 9** and DM1 led us to model their binding to β-tubulin to rationalise the observed activity and elucidate a possible mechanism of action. Three crystal structures of β-tubulin were obtained from the PDB[31] and were used for the construction of a homology model (PDB IDs: 4TUY, 4TV8, 4TV9). A data set of 25 literature compounds [21a,31] were used as references to establish the binding site for the docking. Analysis of the results from these docking studies identified several significant protein-ligand interactions, allowing discrimination between active (pIC_{50} values > 9) and inactive (pIC_{50} values < 9) compounds and the generation of a pharmacophore model to rationalise the activities, Figure 1. The sulfur moiety was observed to extend to the GDP binding site, the size of this feature represents the flexibility in the linker from the amide to the sulfur containing group. Amino acid residues Phe404 and Trp407 form van der Waals interactions to the alkyl chain of the ligands. Hydrogen bonds are formed to residues Asn101, Asn102, Lys105, Val181 and Val182. A feature observed across the chemical series was a pi-pi face-edge interactions with the aryl group of the ligands and Phe404. The alcohol moiety on the ligands was observed to form either a hydrogen bond to Ala99 mediated through a water molecule as for ligand **8**, or directly to the backbone of Gly100 as seen with ligand **9**. The water-Ala99 mediated hydrogen bond for ligand **8** is in a more preferential conformation to that formed by either **9** or DM1, this could account for the observed increase in potency for **8** over these other ligands. An analysis of the contribution to binding energies showed no significant differences between ligands **8** and **9** to account for the differences in potency, suggesting that there are other forces driving the protein-ligand interactions. A calculation of ligand

strain energy on both **8** and **9** of the docked poses showed that **8** had a ligand strain energy 12 kcal/mol lower than **9**. The energy of solvation (the energy associated with dissolving a ligand in water, such that a positive number means that dissolution is endothermic whilst a negative figure is a favourable exothermic process) for **8** and **9** was also calculated. The solvation energy of **8** was -12 kcal/mol lower than that of **9**, implying that **9** must undergo a larger conformational change to become desolvated and bind to β-tubulin than **8**. Together these results may explain the observed differences in potency between **8** and **9**.

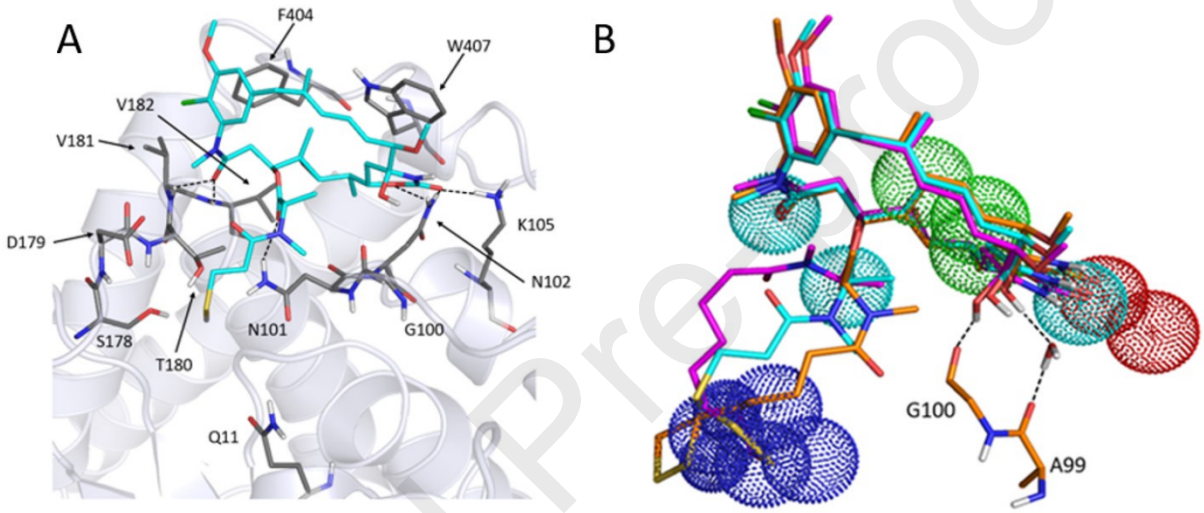
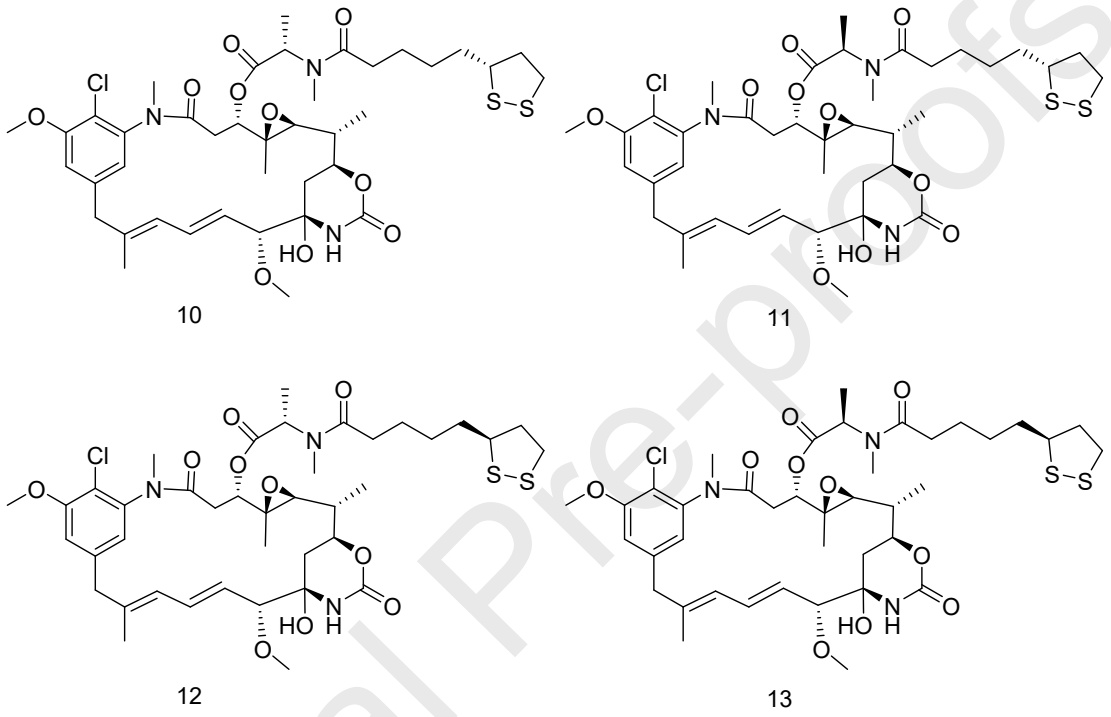


Figure 2: (A) Structure of the tubulin–DM1 complex. Left; DM1 (cyan) docked to β-tubulin (grey ribbon), showing significant interactions observed between DM1 and residues of β-tubulin. Hydrogen bonding interactions are shown as dashed lines. (B) The important pharmacophoric features for ligand binding to the maytansine site for the docked compounds **8**, **9**, DM1 (orange, magenta, cyan sticks respectively). The features are coloured dark blue; included volume required for the sulphur binding, cyan: HBA group, red; HBA-projection vectors to neighbouring side chains, green; hydrophobe. Hydrogen bonds shown as dashed lines

These unexpected results prompted us to investigate the effect of resolving the racemic 1,2-dithiolane centre. Consequently, the *S*-1,2-dithiolanes **10** and **11,** and *R*-1,2-dithiolanes **12** and **13**,

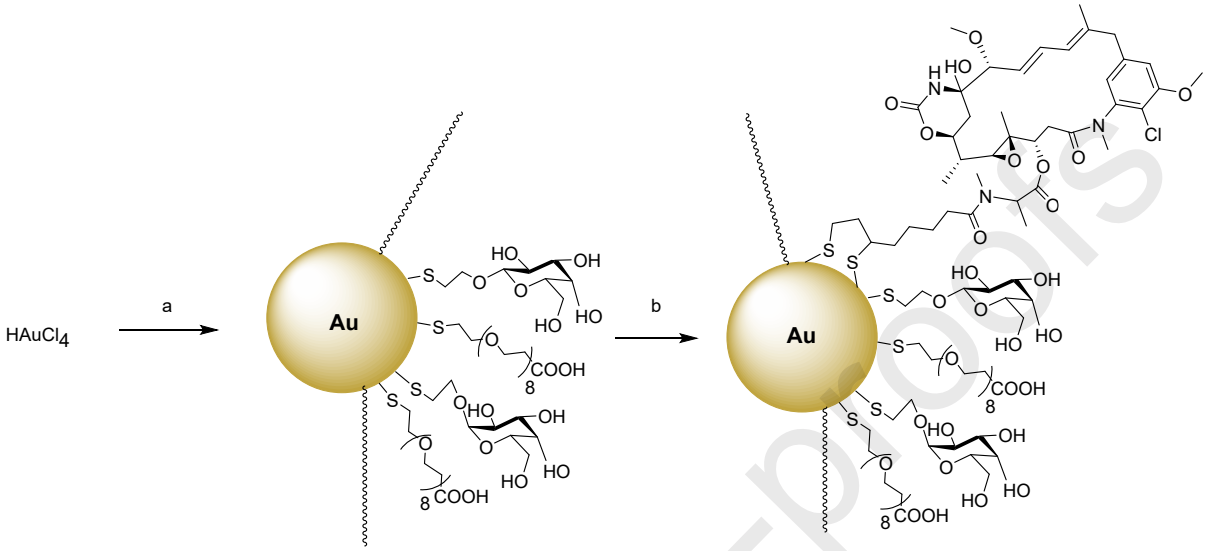
scheme 2, analogues were made in the same way using the homochiral lipoic acid starting material. However, the eutomer, putative *S*-alanine, analogues **10** and **12** were only about 2-fold more active than **8,** though interestingly, the *R*-alanine analogues, **11** and **13**, were markedly less potent than **9** in the MTT cytotoxicity assay, Table 1. These results show that binding to the GDP pocket in β-tubulin is not totally dependent on the 1,2-dithiolane stereochemistry.



Scheme 2: Structures of the chiral dithiolane analogues

We next investigated the conjugation of the 1,2-dithiolane cytotoxins to the GNP, scheme 3. The galactose-carboxylate oligomeric ethylene glycol base nanoparticle was prepared as described for MTC-100038,[12] scheme 3. Here, a Murray ligand exchange methodology[32] was used to couple the thiol group of maytansine to the gold core, thereby displacing a galactose or ethylene glycol ligand. Oh and co-workers were able to displace citrate ligands from GNPs with a disulphide[29] but our attempts were unsuccessful, presumably because of the stronger binding of the galactose- or ethylene glycol-thiol ligands to the gold core. Consequently, we used more forcing conditions (addition of TCEP and stirring for 2 hr prior to the addition of the base nanoparticle) to ensure

complete reaction in coupling **8** or **9** to the GNP. This method typically conjugated around 5 molecules of the cytotoxin to each nanoparticle (as determined by HPLC analysis), the resulting dark brown aqueous solutions were stable when stored at 4°C.



Scheme 3: Synthesis of the GNPs (a) (HOOC-PEG(8)-S-)$_2$, alpha-GalC$_2$, water, NaOH, NaBH$_4$; (b) Maytansine analogue, TCEP, DMSO, GNP, water

Screening of the *S*-alanine GNP **14** and the R-alanine GNP **15** in the MTT cytotoxicity assay against Hep3B and U87MG cell lines showed that **14** retained the relative potency of its non-conjugated precursor, being 10-fold more potent than its DM1 counterpart (MTC-100038), though the *R*-alanine analogue was substantially less potent, table 2. Whereas the stereochemistry of the homochiral 1,2-dithiolane analogues did not appear to play a significant role, when conjugated to the GNP the *S*-alanine, *R*-1,2-dithiolane **16** was significantly more active, about 30-fold more potent than MTC-100038 and about 3-fold more potent than the *S*-1,2-dithiolane analogue **18**. The reasons for this are not clear but may involve a more rapid cleavage of the *R*-1,2-dithiolane from the gold core under the conditions of the assay.

#	Alanine stereo-chem	1,2-dithiolane stereo-chem	Drug eq /GNP	Hep3B				U87MG			
				IC_{50} (nM)	n	SEM	IC_{50} fold over MTC-100038	IC_{50} (nM)	n	SEM	IC_{50} fold over MTC-100038
MTC-100038	*S*	-	5.5	19.4	9	8.8	-	14.6	18	2.82	-
14	*S*	racemic	5.5	1.5	12	0.23	13	1.8	12	0.22	8
15	*R*	racemic	5.0	88	3	20	0.2	68	3	13	0.2
16	*S*	*R*	4.3	0.58	9	0.38	33	0.46	9	0.32	32
17	*R*	*R*	3.4	49	9	14.4	0.4	30	9	9.2	0.5
18	*S*	*S*	5.9	2.06	3	0.51	6.4	1.75	3	0.6	5.6
19	*R*	*S*	4.8	113	3	32.6	0.6	83	3	2.9	0.15

Table 2: Activity of GNP analogues in an MTT cytotoxicity assay against Hep3B and U87MG cells

The effect of the cytotoxins and cytotoxin conjugated GNPs against a panel of human tumour cancer cell lines including 786-O (renal adenocarcinoma), A2780 (ovarian cancer), A375 (melanoma), A431(epidermoid carcinoma), A549 (adenocarcinoma), ACHN (renal adenocarcinoma), BXPC-3 (pancreas adenocarcinoma), as well as Hep3B and U87MG used in the above study. The cell lines showed varying susceptibility to the unconjugated cytotoxins **8** and **10**, but these molecules were at least an order of magnitude more potent than DM1. The difference between the conjugated cytotoxin GNPs **14** and **16** and the DM1 conjugated GNP, MTC-100038, were perhaps less profound but were still significant (See Supplementary Tables 3 and 4). Note that, as yet, we have been unable to determine the rate of cleavage of the cytotoxin from the GNP and that the predicted slower rate of cleavage for the 1,2-dithiolane maytansines may well have an effect on the observed cytotoxicity.

Finally, we conducted a tolerability study of maytansine **8** and GNP **14** in mice to compare to DM1 and MTC-100038. In the previous study,[12] DM1 and MTC-100038 had been exposed to a 2 x QD5 iv regime, but for this study the exposure was reduced to a weekly iv dose, which was felt to be more in keeping with a clinical dosing schedule. Tolerability was assessed by change in body weight. Under these conditions a DM1 dose of 350 ug/kg QW x 3 or 450 ug/kg QW x 2 was well tolerated but increasing to 1350 ug/kg QW x 1 saw a rapid loss in body weight, Figure 3A. In contrast, the

maximum feasible dose of **8** that could be administered, because of solubility issues, was 1350 ug/kg, this dose was well tolerated, Figure 3B. For the GNP conjugates, MTC-100038 was tolerated to 1250 ug/kg (drug equivalent dose), Figure 3C, whereas GNP **14** was tolerated to a dose of 2700 ug/kg (drug equivalent dose), Figure 3D, over twice that of MTC-100038, demonstrating that the strategy of stabilising the linkage of the cytotoxic moiety to the gold core appeared to have a significant effect in improved tolerability.

In conclusion, we have attempted to improve the properties of a cytotoxic maytansine conjugated to a GNP by improving the stability of the drug-gold linkage. These modifications had the unexpected benefit of improving the potency as determined by a cytotoxicity assay against a number of cancer cell lines. The improvements in potency could be accounted for by computational modelling. The resulting cytotoxic GNP was significantly better tolerated in a mouse study. These results are sufficiently promising to warrant further *in vivo* pharmacokinetic and biodistribution studies as well as efficacy studies in mouse models of HCC. Additionally, we need to understand the rate of cleavage of the cytotoxin to help interpret these data. We have focused on the use of these novel cytotoxic maytansines in combination with gold nanoparticles, but it is apparent to the authors that these agents may have applications in other areas such as antibody drug conjugate systems where their potency and the unusual method of attachment through the dithiolane group may offer advantages.

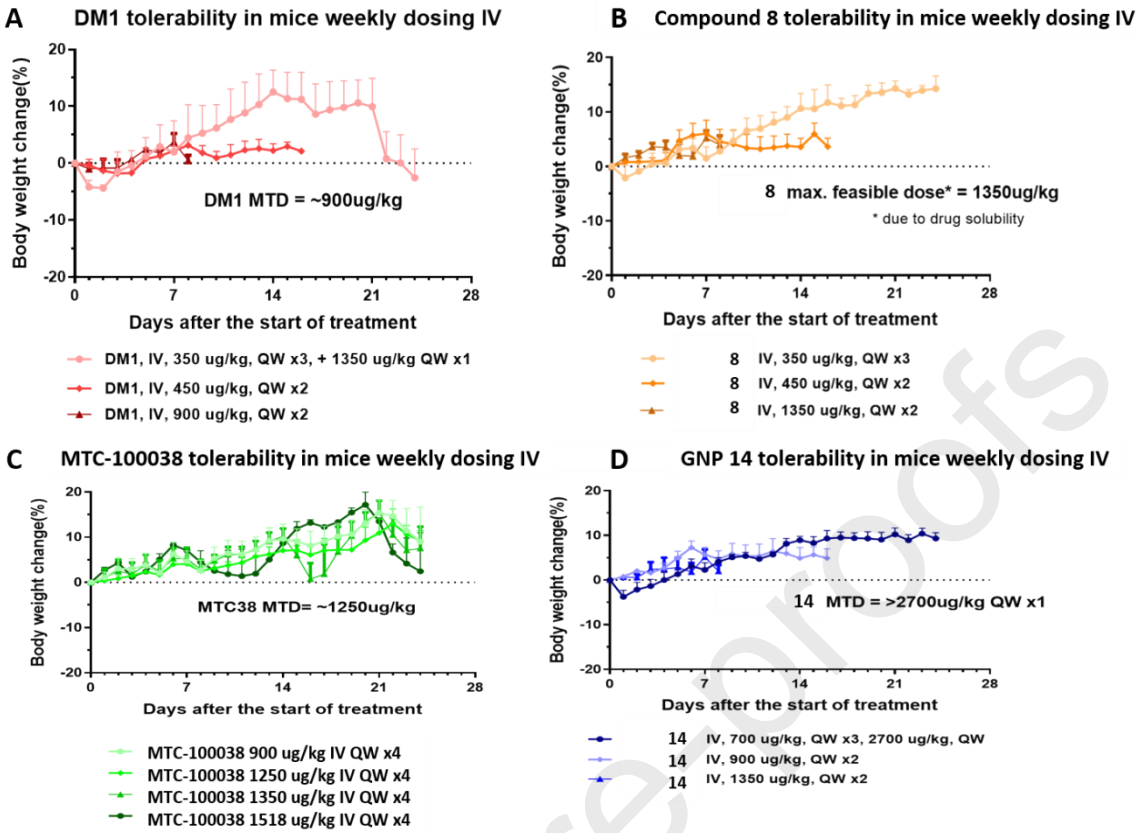


Figure 3. Tolerability in mice dosing QW. A. DM1 tolerability, B. Maytansinoid **8** tolerability, C. GNP MTC-100038 tolerability, D. GNP **14** tolerability

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Figure and scheme captions

Figure 1. Maytansine Analogues

Figure 2: (A) Structure of the tubulin–DM1 complex. Left; DM1 (cyan) docked to β-tubulin (grey ribbon), showing significant interactions observed between DM1 and residues of β-tubulin. Hydrogen bonding interactions are shown as dashed lines. (B) The important pharmacophoric features for ligand binding to the maytansine site for the docked compounds 8, 9, DM1 (orange, magenta, cyan sticks respectively). The features are coloured dark blue; included volume required for the sulphur binding, cyan: HBA group, red; HBA-projection vectors to neighbouring side chains, green; hydrophobe. Hydrogen bonds shown as dashed lines

Figure 3. Tolerability in mice dosing QW. (A) DM1 tolerability, (B) Maytansinoid 8 tolerability, (C) GNP MTC-100038 tolerability, (D) GNP 14 tolerability

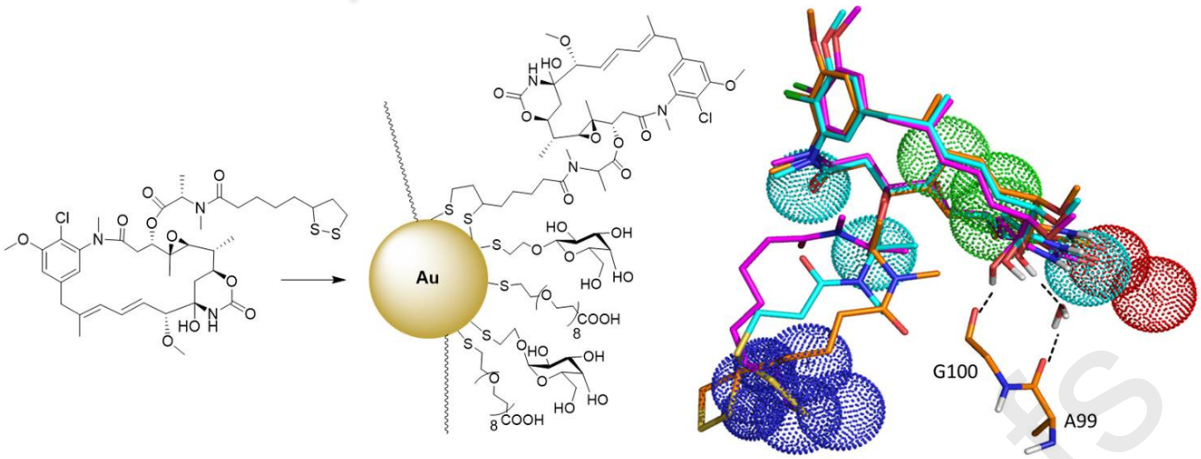
Table 1: Activity of 1,2-dithiolane analogues in an MTT cytotoxicity assay against Hep3B and U87MG cells

Table 2: Activity of GNP analogues in an MTT cytotoxicity assay against Hep3B and U87MG cells

Scheme 1: (a) EDCI, HOBT, DIPEA, methyl N-methyl-L-alaninate, DCM, rt; (b) NaOH, H2O, MeOH, rt 16h; (c) LiAl(OMe)3H, THF, -40oC; (d) DCC, ZnCl2, compound 5, DCM, rt, 24h

Scheme 2: Structures of the chiral dithiolane analogues

Scheme 3: Synthesis of the GNPs (a) (HOOC-PEG(8)-S-)2, alpha-GalC2, water, NaOH, NaBH4; (b) Maytansine analogue, TCEP, DMSO, GNP, water



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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