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A Small Molecule Drug Conjugate (SMDC) of DUPA and a Duocarmycin Built on the Solid Phase

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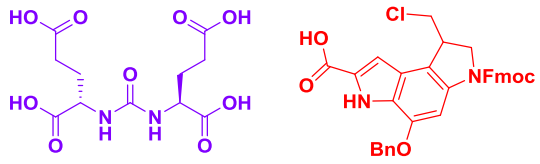
In a proof-of-concept study, solid phase synthesis allowed the rapid generation of a small molecule drug conjugate in which the glutamate carboxypeptidase II (GCPII) targeting small molecule DUPA was conjugated to the alkylating subunit of the potent cytotoxin duocarmycin SA. The doubly targeted SMDC contained a cathepsin B cleavable linker, which was shown to be active and selective against cathepsin B over-expressing and GCPII-expressing tumour cell lines.

Introduction

Targeted cancer therapy is among the most promising strategies for the realisation of side effect free oncology treatment.[1] The development of precision medicine will allow promising cytotoxic moieties that are already known to be delivered to specific sites of action.[2]

Small molecule drug conjugates (SMDCs) present an exciting technique to harness the potential of cytotoxic compounds that demonstrate therapeutic windows too small for clinical use.[3,4] Small molecules that target receptors or antigens overexpressed in cancers may not be useful as treatments for these disease states in themselves, but, in conjunction with ultrapotent cytotoxins, a symbiotic partnership is generated to act as a viable solution. SMDCs offer advantages over antibody drug conjugates (ADCs) and peptide drug conjugates (PDCs). SMDCs are inexpensive, completely characterised, readily tuneable and non-immunogenic.[3] Additionally, as the name suggests, SMDCs are small, low molecular weight conjugates, benefitting cell permeability in solid tumours and stability.[4] This concept has been championed by the use of folate, with the vast majority of SMDCs exploiting this efficient directing group.[2-4] However, several other examples exist which target the fusion protein BCR/ABL, aminopeptidase N and glutamate carboxypeptidase II (GCPII), among others.

GCPII, also known as prostate specific membrane antigen (PSMA), *N*-acetyl-α-linked acidic dipeptidase I (Naaladase I) or folate hydrolase (FOLH1), is over expressed in almost all prostate cancers, as well as several other cancer lines.[5] GCPII expression in cancerous cells is approximately 1000-fold more than healthy tissue.[6] Once a ligand is bound, GCPII undergoes endocytosis, unloading the ligand, and recycling back to the cell surface.[7] These factors highlight GCPII as an excellent candidate for tumour-targeted drug delivery.[8-10] Its importance as a diagnostic and therapeutic target have resulted in extensive study into inhibitors of GCPII, with several potent and selective compounds identified.[11] 2-[3-(1,3- dicarboxypropyl)ureido]-pentanedioic acid (DUPA, **1**, Figure 1) has a high affinity for GCPII (K_i = 8 nM),[12-14] and has been widely exploited for diagnostics. Studies demonstrated that DUPA-linked cytotoxic drugs such as tubulysin B hydrazide and desacetyl vinblastine hydrazide are able to eradicate tumours in vivo and eliminate nonspecific toxicity associated with the unconjugated drugs.[9]



1 **2**

Figure 1. Structures of GCPII ligand DUPA, **1** (2-[3-(1,3-dicarboxypropyl)ureido]pentanedioic acid) and duocarmycin synthetic unit Fmoc-DSA(OBn)-OH, **2**.

The duocarmycins are a family of DNA-alkylating natural products that demonstrate ultrapotent cytotoxic activity.[15,16] Despite several clinical trials and implementation in a number of antibody drug conjugates, the duocarmycins are yet to reach the market. Work by Neri and co-workers demonstrated the applicability of duocarmycin payloads to SMDCs, attaching a modified CBI unit to a carbonic anhydrase IX (CAIX) targeting ligand.[17] However, the use of the duocarmycins in SMDCs has been limited to this proof-of-concept work. With our recent development of a duocarmycin payload ready for solid phase peptide synthesis (SPPS),[18] Fmoc-DSA(OBn)-OH (**2**, Figure 1), this family of cytotoxins is poised for implementation in all

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directing strategies. SMDCs have resulted in a number of compounds entering clinical trials, most of which exploit folate as a directing small molecule.[3,4] We disclose here a proof-of-concept study demonstrating a duocarmycin alkylating unit directed by the GCPII targeting ligand DUPA, built on the solid phase. Additionally, the implementation of a cathepsin B enzyme cleavable sequence provides a secondary directing method, demonstrating the duocarmycins have high potential for SMDCs.

Results and Discussion

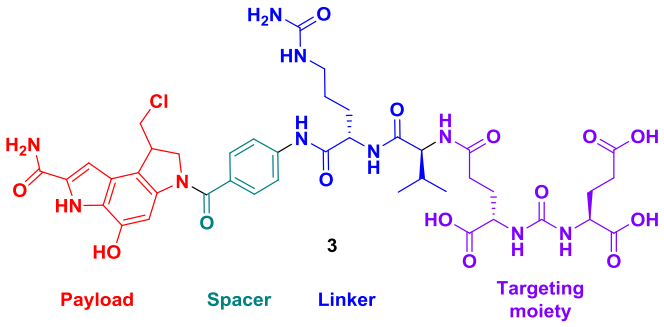
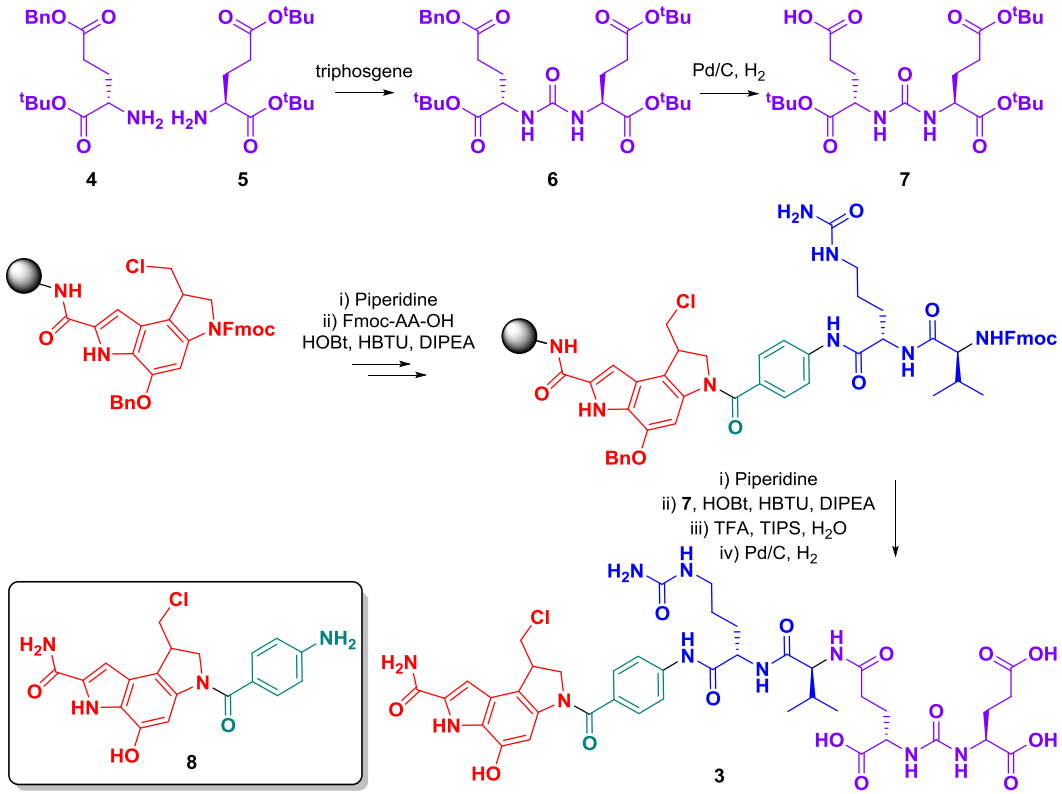


Figure 2. Duocarmycin DUPA small molecule drug conjugate structure, **3**.

SMDCs are typically made up of the targeting small molecule, a spacer, a linker – often cleavable – and finally the warhead. In an exhibition of the utility of the Fmoc-DSA(OBn)-OH unit the design of our SMDC (**3**, Figure 2) focussed on the ability to be synthesised on the solid phase. We have demonstrated previously that a neutral C-terminus is required for the activity of the Fmoc-DSA-OH unit,[18] and as such synthesis on Rink amide resin was selected to provide a terminal amide. The racemic duocarmycin was employed to allow the proof-of-concept to be demonstrated with synthetic ease, balancing the likely sacrifice of activity.[19] The routinely employed PABA-Cit-Val cathepsin B enzyme cleavable sequence was chosen as both the spacer and linker for two reasons. Firstly, the secondary targeting effect, with cathepsin B being overexpressed in cancer cell lines.[20,21] Secondly, the para-aminobenzoic acid (PABA) unit can also act as a DNA binding unit.[22] The synthetic ease and availability of Fmoc-PABA-OH made it more attractive for this proof-of-concept study compared to, say, an indole unit – more reminiscent of the natural products – despite the known reduction in efficacy. Finally, the GCPII binding ligand 2-[3-(1,3-dicarboxypropyl)ureido]pentanedioic acid (DUPA, **1**) was chosen as a directing group because of its high affinity for GCPII (K_i = 8 nM) and the carboxylic acid handle readily obtained via the synthesis described by Cushman and co-workers.[14] The tri-^{t}butyl protected DUPA was prepared as previously described (Scheme 1).[14] Briefly, α,γ-di-tert-butyl glutamate **4** was treated with triphosgene and Et_3N, followed by addition of glutamate **5**, with benzyl protection on the γ-carboxylate and a ^{t}butyl protecting group on the α-carboxylate, provided protected DUPA **6**. Hydrogenation with Pd/C under a hydrogen atmosphere yielded tri-^{t}butyl protected DUPA **7**.[10,13]



Scheme 1. Synthesis of tri-^{t}butyl protected DUPA as reported by Cushman and co-workers.[14] Solid phase synthesis of the DSA DUPA small molecule drug conjugate. Payload duocarmycin **8.**

Synthesis of the SMDC began with the loading of Fmoc-DSA(OBn)-OH, **1** onto rink amide resin (Scheme 1). Coupling was achieved with HOBt and HBTU in the presence of DIPEA over 24 hours. These conditions allow for complete coupling, with only 1.2 equivalents of the Fmoc-DSA(OBn)-OH unit. Subsequently, Fmoc solid phase peptide synthesis techniques were routinely employed to add first Fmoc-PABA-OH, followed by Fmoc-Cit-OH and Fmoc-Val-OH. Removal of the final Fmoc protecting group provided a terminal amine which was then coupled using the same amide coupling conditions to the appropriately protected DUPA unit **7**. Cleavage from the resin and deprotection was achieved with a cleavage cocktail of 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIPS) and 2.5% water. Following evaporation and trituration with diethyl ether the crude peptide was debenzylated using H_2/Pd-C to provide the liberated DSA phenolic OH. Purification was achieved with reverse phase preparative HPLC, providing the desired SMDC **3**. The analogous process was employed, terminating at the addition of Fmoc-PABA-OH, with subsequent deprotection, cleavage and benzyl deprotection to generate the payload **8** (Scheme 1).

With the desired construct obtained, evaluation of the ability of DUPA to direct and harness the potent activity of the duocarmycins could be evaluated. Commonly, when GCPII is being examined in prostate cancer, the cell lines LNCaP (GCPII positive) and PC3 (GCPII negative) are employed.[23,24] To extend the applicability of this proof-of-concept further we examined the SMDC in lung cancer cell lines H292 and A549, breast cancer cell lines MCF-7 and SKBR3 and melanoma cell line SKMEL28. The non-cancerous cell line 16HBE14o was used as a control. Each of the cell lines was analysed using flow cytometry to evaluate the expression of GCPII. 16HBE14o and PC3 showed no expression of GCPII and LNCaP demonstrated high levels of GCPII. Additionally, H292, A549, MCF-7, and SKMEL28 also demonstrated elevated expression of GCPII. SKBR3 demonstrated a slightly reduced expression of GCPII (See SI). All cell lines were examined for their cathepsin B activity using a Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (Z-RR-AMC) assay. The ability to cleave the Arg-Arg sequence and release the fluorescent amino-4-methylcoumarin was compared to the noncancerous cell line 16HBE14o. All cancer cells lines demonstrated increased cathepsin B activity, while 16HBE14o demonstrated no appreciable cathepsin B activity (See SI). Each of the cell lines were then evaluated in an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cell viability assay (Table 1). The SMDC (**3**), the benzyl-protected DSA version (**3-OBn**), as well as the enzyme cleaved payload (**8**) were incubated with cell cultures for 72 hours, followed by treatment with MTS. All cell lines demonstrated sensitivity to the precleaved PABA-DSA payload **8** in the nanomolar range. Cell lines which demonstrated high GCPII expression and cathepsin B activity had IC_{50} values at high nanomolar concentrations when treated with **3**. Excitingly, SKBR3 cells, which showed reduced GCPII expression, demonstrated correspondingly weaker compound **3** efficacy. Additionally, PC3 cells, which are GCPII negative but showed high cathepsin B activity, demonstrated an IC_{50} greater than 100 μM, indicating GCPII plays a key role in the activity of the SMDC. The non-cancerous cell line 16HBE14o which demonstrated no GCPII expression, and no appreciable cathepsin B activity also showed no sensitivity to the SMDC. The benzyl-protected DSA SMDC (**3-OBn**) demonstrated no toxicity to cell lines at 100 μM, suggesting DNA alkylation is responsible for the SMDC's efficacy.

Table 1: IC_{50} results obtained for compounds **3**, **3-OBn** and **8** to selected cancerous and non-cancerous cell lines. Errors are 95% confidence intervals.

Cell Line	3 (nM)	3-OBn	8 (nM)
LNCaP	201.4 [142.5; 284.6]	>100 μM	15.7 [10.8; 23.0]
PC3	>100 μM	>100 μM	4.7 [4.020; 5.464]
H292	476.2 [312.8; 724.9]	>100 μM	7.3 [6.873; 7.701]
A549	308.7 [208.8; 456.4]	>100 μM	36.6 [25.7; 52.3]
MCF-7	190.4 [43.60; 831.3]	>100 μM	181.8 [132.1; 250.0]
SKBR3	3564 [1704; 7456]	>100 μM	2.3 [1.9; 2.8]
SKMEL28	290.4 [174.1; 484.2]	>100 μM	22.5 [17.0; 29.7]
16HBE14o	>100 μM	>100 μM	85.4 [73.1; 99.8]

To provide evidence that GCPII is responsible for the activity of the SMDC a competition assay was employed. LNCaP and PC3 cells were incubated with the known GCPII inhibitor 2-(phosphonomethyl)-pentandioic acid (2-PMPA)[25] at 10 μM before treatment with the DUPA-DSA SMDC and the payload PABA-DSA. Cell viability was determined with the MTS assay (Figure 3). As expected treatment with **8** in the presence of 2-PMPA demonstrated no significant change (PC3 cells IC_{50} = 6.1 nM [5.2; 7.1], LNCaP cells IC_{50} = 25.5 nM [17.7; 36.5]), nor **3** in the presence of 2-PMPA to PC3 cells (IC_{50} > 100 μM). However, the efficacy of **3** to the GCPII overexpressing LNCaP was greatly diminished by the presence of 2-PMPA (IC_{50} > 100 μM), suggesting GCPII is not available for binding, weakening the ability of the SMDC to enter cells.

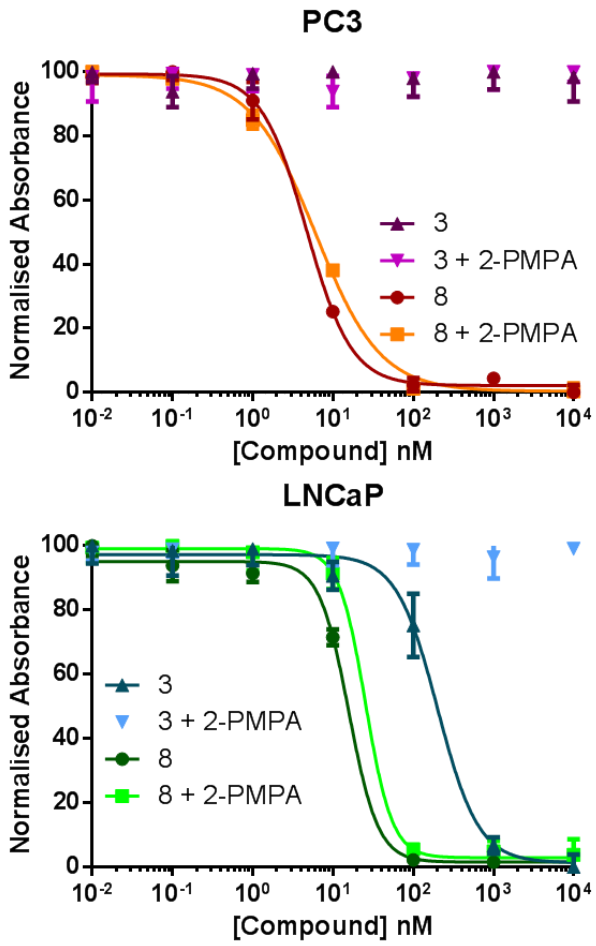


Figure 3. Competitive binding antiproliferation assay of SMDC **3** and payload **8**, in the absence of GCPII binder 2-PMPA, or in the presence of 2-PMPA (10 μM). Y-axis is the normalised absorbance at 490 nm after treatment with MTS for 3 hr. Results are representative of three independent experiments.

Conclusions

This proof-of-concept study has provided new insight into harnessing the ultrapotent duocarmycin family. Employing the low nanomolar GCPII binding DUPA molecule has allowed for the targeting of an important oncological target. The use of a cathepsin B enzyme cleavable sequence has imparted a secondary targeting effect, further directing the payload to cancerous cells. The SMDC employing a duocarmycin alkylating unit had demonstrated nanomolar IC_{50} values to cell lines which express GCPII and overexpress cathepsin B, but show no appreciable toxicity to cell lines which do not express GCPII. Additionally, levels of expression correlate to the toxicity of the SMDC. Excitingly, cell lines which do not express GCPII and do not demonstrate overexpression of cathepsin B demonstrated no sensitivity to the SMDC, despite low nanomolar IC_{50} for the DSA-PABA warhead. The SMDCs efficacy appears to be reliant on GCPII, with a competition assay greatly reducing the efficacy of the SMDC to overexpressing LNCaP cells. This work suggests that the duocarmycins are suitable payloads for small molecule drug conjugates, and that they can be readily and rapidly produced on the solid phase. Work continues to improve the toxicity of the payload while maintaining the excellent directing nature of the conjugate.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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