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Improved Physical Stability of an Antibody–Drug Conjugate Using Host–Guest Chemistry

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Improved physical stability of an Antibody Drug Conjugate using Host-Guest chemistry

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KEYWORDS: Supramolecular Chemistry, Antibody Drug Conjugates, Polyethylene Glycol, Pyrrolo Benzo Diazepine, Cucurbituril, Antibody Aggregation, Formulation.

ABSTRACT: Antibody Drug Conjugates (ADCs) are an emerging class of biopharmaceutical products for oncology, with the cytotoxic PyrroloBenzoDiazepine (PBD) family of 'warheads' well established in the clinic. While PBDs offer high potency, they are also characterized by their hydrophobicity which can make formulation of the ADC challenging. Several approaches have been investigated to improve the physico-chemical properties of PBD-containing ADCs and herein a supramolecular approach was explored using cucurbit[8]uril (CB[8]). The ability of CB[8] to simultaneously encapsulate two guests was exploited to incorporate a 12-mer polyethylene glycol harboring a methyl-viologen moiety at one terminus (MV-PEG_{12}), together with a PBD harboring an indole moiety at the C'$_2$ position (SG3811). This formulation approach successfully introduced a hydrophilic PEG to mask the hydrophobicity of SG3811, improving the physical stability of the ADC while avoiding any loss of potency related to chemical modification.

Introduction

The increase of tumor diagnoses and heterogenicity of their genomic profile requires the development of a different approach from conventional cancer therapies[1,2]. A key objective in addressing this need is the requirement to administer potent cytotoxics in a targeted manner for selective release in cancerous cells, so limiting the adverse effects of these small molecules[3].

A relatively recent class of biopharmaceutical products that fulfill those requirements are antibody drug conjugates (ADCs), wherein a potent small cytotoxic molecule is covalently bound to a monoclonal antibody (mAb) via a linker[4,5]. The cytotoxic 'warhead', has specific requisites including high potency, limited hydrophobicity, avoidance of multidrug resistance protein 1 (MDR1)-mediated efflux and suitable chemistry for attachment to the mAb[6]. A potent class of cytotoxics was first discovered from the fermentation broth of a thermophilic actinomycete[7] with the active compound later isolated, characterized and named as Anthramycin[8].

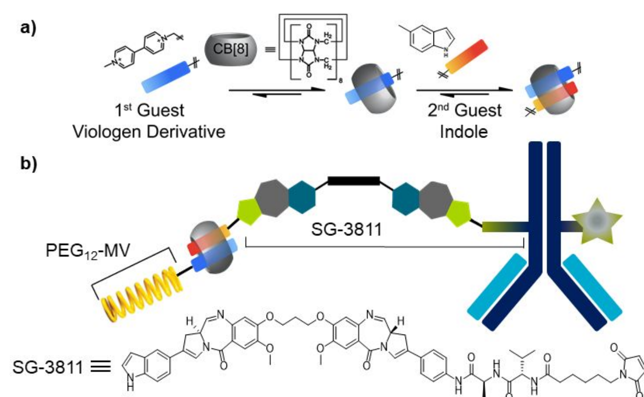


Figure 1. Schematic representation of the host-guest system: a) MV-CB[8] binary complex and heteroternary host-guest formation

with a suitable second guest b) Cartoon depicting the antibody with the MV-PEG$_{12}$ CB[8] SG-3811 heteroternary complex (represented for simplicity by a star on the right-side of the antibody cartoon).

From a structure-activity relationship (SAR) study, the anthramycin pharmacophore was identified as pyrrolo benzodiazepine (PBD), which is amongst one of the most selective DNA alkylating agents. From the dimerization of the sequence selective PBD pharmacophore, non-distorting and DNA interstrand cross-links in the minor groove of DNA cytotoxins were also obtained[9]. Several types of linker systems have been explored[10–12] classified as cleavable and non-cleavable according to the mechanism of drug release. Mutual properties in all the linkers include their stability during circulation in vivo and efficient release upon reaching the target cells and internalization. Antigen target selection is crucial in directing the cell specificity of the ADC: the antigen needs to be highly expressed on the tumor cells and very poorly or not expressed on normal cells[13]. One of the most pursued anticancer targets is the human epidermal growth factor receptor 2 (HER2), which is overexpressed in 20-25% of breast cancer and linked to oncogenic behaviour[14–17]. Currently, among the 60 antibody-based products present on the market, two are based on Trastuzumab: Herceptin and Kadcyla, the latter being an ADC[18,19].

The conjugation of hydrophobic molecules such as PBDs to mAbs increases protein-protein and inter-ADC attraction on account of the polydispersity of drug:antibody ratios[20]. The conjugation of PBDs to mAbs has also been shown to decrease protein conformational stability[21]. As a result of this loss of physical stability, ADC solubility and aggregation are two challenges encountered during their formulation[22]. A short polyethylene glycol (PEG) chain is sometimes incorporated in the PBD linker to increase the solubility of the molecule and so improve the conjugation efficiency and ADC yield[23]. However, the PEG chain in this position does not 'mask' the hydrophobic PBD once conjugated to the antibody. This study therefore aims to improve the solution stability of the ADC during storage by placing a PEG chain in a position that masks the PBD; however, the known structure activity relationship of the PBD dimer family precludes covalent conjugation of the PEG chain[24]. Therefore, to achieve that aim, a hydrophilic molecule (a short 12-mer PEG modified at one terminus with methyl-viologen, MV-PEG$_{12}$) was introduced in the ADC not *via* a covalent linker, but through cucurbit[8]uril (CB[8]) host-guest chemistry. CB[n]s are a family of synthetic macrocyclic hosts which can bind tightly and selectively to a wide range of organic compounds in aqueous solution[25,26]. CB[n]s can be described as barrel shaped molecule comprising a hydrophobic cavity, made by methylene-bridged glycoluril units, and two polar rims presenting ureido carbonyl oxygens[27–29]. According to the number of glycoluril units in the macrocycles several CB[n]s have been isolated[30]; in this series, the eight-units member, CB[8], occupies a unique spot as it can bind two aromatic molecules at the same time forming both homoternary (2:1) and heteroternary (1:1:1) complexes[31,32].

As depicted in Figure 1.a, CB[8] can bind 1 equivalent of the electro-deficient methyl-viologen (MV) with an equilibrium dissociation constant (K_d) of ~1μM, and 1 equivalent of an electron-rich aromatic molecule or moiety such as indole or phenyl, respectively, as the second guest; this property has enabled novel protein conjugation strategies. For example, CB[8] has been utilized to: i) dimerize an enzyme engineered with a N-terminal FGG tripeptide[33]; ii) generate a ternary complex with an antibody fragment (Fc, engineered with a N-terminal WGG tripeptide) and cyclic peptide harboring MV[34]. Cucurbit[7]uril (CB[7]), which binds one aromatic guest, has

been demonstrated using ^{1}H NMR to be selective for exposed phenylalanine residues in the complementary determining region of an antibody [34,35,36].

Exploiting the ability of CB[8] to form heteroternary complexes with MV-modified molecules, enables a hydrophilic molecule ($MV\text{-}PEG_{12}$) to be hosted alongside a PBD derivatized with an indole moiety at the C'$_2$ position. The intention here being to mask the hydrophobic PBD warhead and improve the solubility of the ADC. In using this approach, the $MV\text{-}PEG_{12}$ and CB[8] are non-covalently bound to the PBD and so dissociate from the ADC upon administration-dilution *in vivo*, thus avoiding potential loss in potency associated with direct PEGylation of the PBD. Previous work has demonstrated that non-covalent PEGylation can improve the physical stability of insulin, glucagon and an antibody in aqueous solution, utilizing the supramolecular interaction between CB[7]-PEG and phenylalanine at the N-terminus (insulin) or mid-chain (glucagon and antibody)[37].

Results and Discussion

PBD selection and PBD-dimer synthesis

Over the last twenty years, a library of PBD monomers has been produced and tested *in vitro,* identifying the most active molecules to progress into dimers for mAb conjugation[9,24]. Within this library, several promising PBD monomers have been discarded not for low activity but for their hydrophobic nature. Therefore, a supramolecular approach was employed to reduce the hydrophobic nature of these promising PBDs and enable their use as warheads.

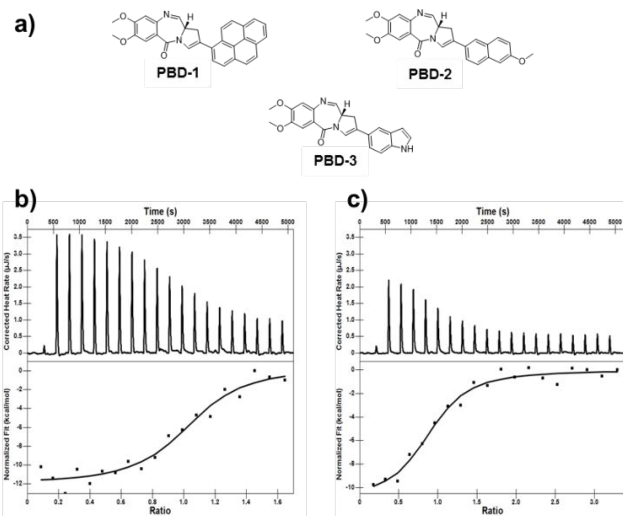
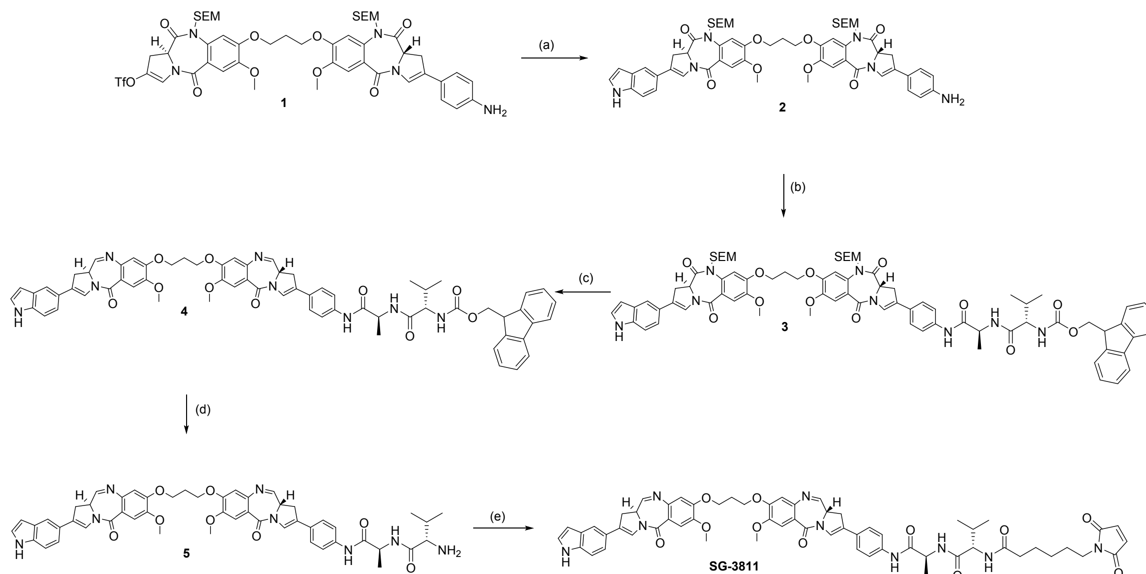


Figure 2. a) PBD monomers evaluated for host-guest interaction with MV-CB[8]. ITC binding curves of b) MV-CB[8] (1 mM) and c) $MV\text{-}PEG_{12}\subset CB[8]$ (0.42 mM) with PBD-3 (100 and 50 µM, respectively) in phosphate buffer saline (PBS).

Three PBDs were selected from our library on the basis that they harbored at the C'$_2$ position indole, 2-napthol or pyrene moieties (Figure 2.a), which are known second guests for CB[8] with micromolar affinities[28,38]. The affinity of these three PBDs for the binary complex MV-CB[8] and $MV\text{-}PEG_{12}\subset CB[8]$ was determined by isothermal titration calorimetry (ITC). One PBD monomer, termed PBD-3, showed reproducible binding to both



Scheme 1: (a) Suzuki, Y: 85%; (b) Peptide coupling, Y: 69%; (c) Reduction/SEM deprotection, Y: 64%; (d) Fmoc deprotection; (e) Maleimide caproic acid coupling, Y: 22% (over 2 steps). Full details are reported in the Supporting Information.

binary complexes with a K_d of ~3 μm (Figure 2 b,c and Table S1). PBD-1 and -2 showed weak binding in the presence of MV-CB[8] and no discernable binding in the presence of MV-PEG12⊂CB[8] (Figure S11 and Table S1). Therefore, PBD-3 was selected as best candidate to take forward in this study. Application of the PBD pharmacophore as a dimer rather than monomer is proven to result in a synergistic effect on account of sequence-specific interstrand cross-linking with DNA[39]. Thus, the unsymmetrical PBD dimer termed SG3811, harboring an indole at the C2' position and a Val-Ala-C_6-maleimide linker at the C2 position, was synthesized following a 7-step synthetic procedure (Scheme 1). The synthesis was challenging from the 2(trimethylsilyl)ethoxymethyl (SEM) deprotection step onwards, due to the high hydrophobicity of the molecule and formation of salts, but a final product with ~80% purity was obtained after chromatographic purification (Figure S7-8). Liquid chromatography–mass spectrometry (LC-MS) analysis confirmed that most of the impurities were probably aggregates stable in solution (Figure S8).

SG3811 antibody conjugation

Site specific conjugation of SG3811 to a cysteine engineered anti-HER2 mAb (C239i) which harbors a reactive cysteine on each constant heavy domain 2 (C_H2), thus setting the maximum drug to antibody ratio (DAR) to 2[40]. In brief, the maleimide on SG3811 was reacted in 10-fold molar excess with deblocked, reoxidized, Trastuzumab-C239i (T-C239i, full details are reported in the Supporting Information). On account of the poor aqueous solubility of SG3811, a small scale conjugation screening comprising several different conditions was carried out to improve the DAR, as reported in Table 1. The parameters evaluated were pH, ionic strength, temperature and co-solvent, whilst time and SG3811 equivalents were kept constant. The data obtained suggested the highest DAR values were obtained at pH 8 and 50% propylene glycol (PG) co-solvent, independently from ionic strength and temperature. Therefore, condition **7** was selected for the larger scale production which successfully yielded T-C239i-SG3811 (the ADC) with an improved DAR of 1.60. The crude ADC was then purified by ceramic hydroxy apatite Type II chromatography (CHT II)40 and characterized by reverse phase liquid chromatography (RP-LC)

Table 1 – Site specific conjugation protocols

Protocol	DAR	Concentration (mg/mL)	Buffer	pH	Temperature (°C)	Co-solvent
1	0.93	0.4	PBS	7.4	RT	33% PG
2	0.94	1.4	PBS	7.4	RT	33% PG
3	1.03	1.7	Tris 50 mM	8.1	RT	40% PG
4	1.10	1.7	Tris 50 mM	8.1	RT	40% PG 10% DMSO
5	1.09	1.7	Tris 50 mM	8.1	RT	40% PG 10% DMA
6	1.25	1.1	Tris 50 mM	8.1	RT	50% PG
7	1.26	1.5	PBS + Arg 100 mM	8.0	RT	50% PG
8	1.17	1.1	Tris 50 mM	8.1	37 °C	50% PG
9	1.24	1.5	PBS + Arg 100 mM	8.0	37 °C	50% PG

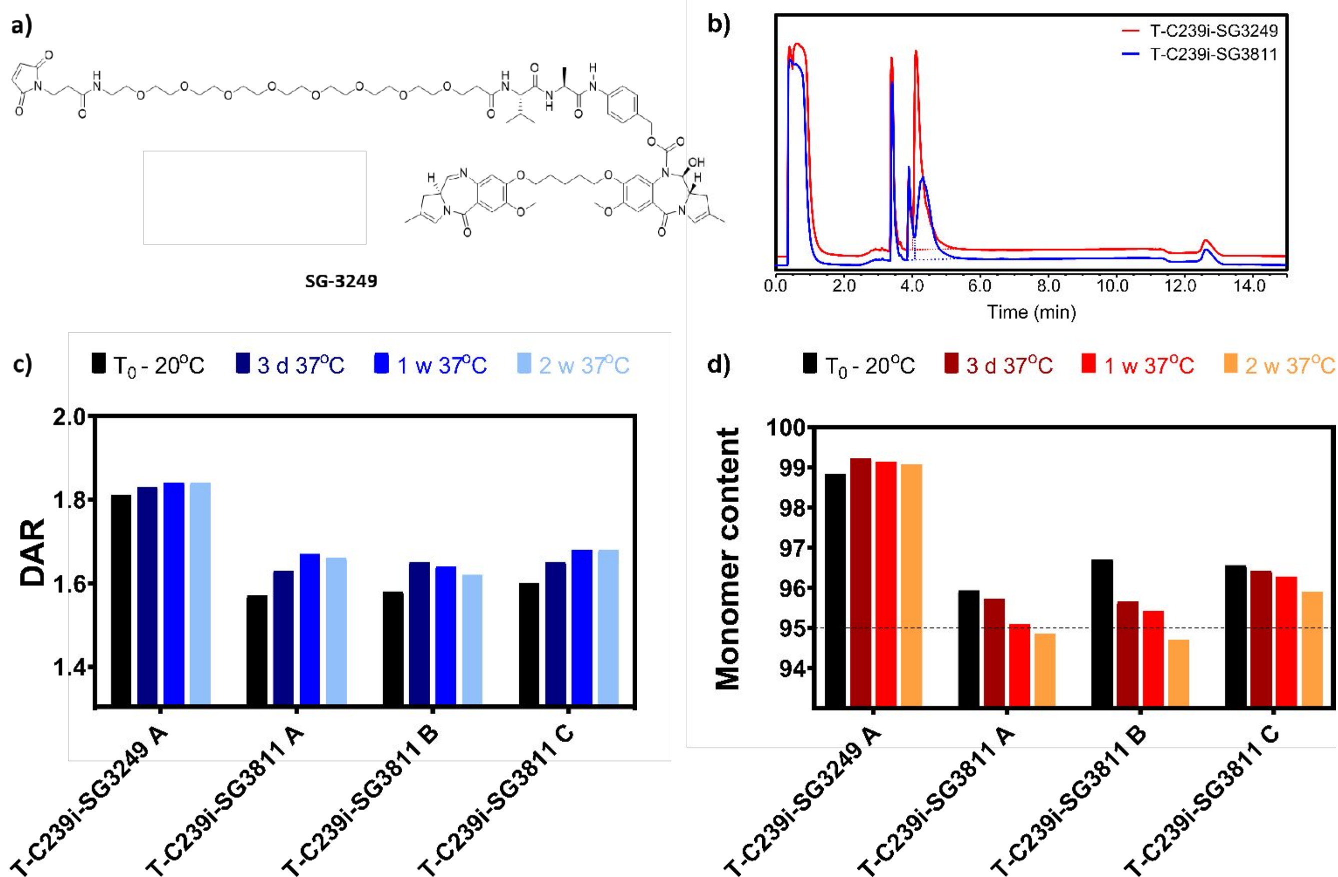


Figure 3. a) Chemical structure of SG3249; b) comparison of RP-LC data at 214 nm for the two ADCs studied in buffer A at time zero, -20 °C, for calculation of the DAR: L0 (ADC light chain), H0 and H1 peaks elute at ~ 3.5, 4.0 and 4.5 min, respectively; c) calculated DAR and d) monomer content obtained by RP-LC and SE-LC, respectively, for the two ADCs in buffers A (PBS), B (+MV-PEG_{12}) and C (+MV-$PEG_{12} \subset CB[8]$), data are reported at time 0 and at three different time points (d = day, w = week) under thermal stress (37 °C). The reproducibility of the SEC method is such that the standard deviation for each sample is < 0.1% monomer (Table S3).

and size exclusion liquid chromatography (SE-LC) to determine protein concentration, DAR and yield (Figure S9-10).

Supramolecular Formulation Approach and Stability Study

The binding affinity of SG3811 and the ADC, T-C239i-SG3811, for MV-CB[8] and MV-$PEG_{12} \subset CB[8]$ was reevaluated by ITC, since the additional complexity of the PBD dimer as well as the addition of the bulky antibody may sterically hinder the accessibility of the indole moiety for the binary host-guest complex. A K_d of 29 and 32 μM, for the SG3811and T-C239i-SG3811 respectively, was obtained (Figure S12 and Table S1), around 10-fold higher than that for the free PBD monomer (Figure 2c). These results suggest that moving from a monomer to a dimer PBD indeed hindered the host-guest binding interaction, but not the conjugation of the warhead to the specifically engineered position on the antibody. Using a 10 fold excess of the host system, this affinity is suitably positioned to maintain ~85% molar equivalent of the heteroternary complex in an ADC formulation of ~10 μM, while ensuring complete dissociation of the MV-PEG_{12} upon dilution in the bloodstream following intravenous administration.

To demonstrate proof of concept of this supramolecular approach for improving the stability of the formulated ADC, an accelerated stability study was carried out. The study included a 'control' Trastuzumab-C239i-based ADC, T-C239i-SG3249, the linker and warhead of which is shown in Figure 3a. T-C239i-SG3249 has been reported as a very potent and stable ADC, enforced by having a short PEG chain within the linker[23]. Here, both chemical and physical stability of the two ADCs were tested at five different time points over one-month, under different thermal conditions (-20, 4-8 and 37 °C). T-C239i-SG3811 was tested in three different buffers: buffer A, phosphate buffered saline (PBS); buffer B, PBS containing 160 μM MV-PEG_{12}; and buffer C, PBS containing 160 μM MV-$PEG_{12} \subset CB[8]$. Buffer B was included to test the effect of free MV-PEG_{12} on the stability of T-C239i-SG3811; note that no specific interaction between MV-CB[8] and (non-conjugated) T-C239i was observed by ITC (Figure S12c). T-C239i-SG3249, as the positive control, was only evaluated in buffer A. Based on the K_d measured by ITC and working at an ADC concentration of 1.4 mg/mL (~10 μM), a 10-fold greater concentration of MV-$PEG_{12} \subset CB[8]$ over the warhead in the formulation would equate to ~85% of heteroternary host-guest complex present in solution.

The chemical and physical stability of the two ADCs was followed by measuring DAR, monomer/aggregate ratios and warhead stability (full data are in the Supporting Information). The DAR values, measured by RP-LC, did not shown any notable change for neither of the ADCs at any point the study (Figure 3c). The DAR results demonstrate a good chemical stability of T-C239i-SG3249 in buffer A and of T-C239i-SG3811 in either buffer A, B, or C (Figure S13). This is consistent with the use of the same maleimide linker chemistry for the two ADCs.

Since SG3811 is a completely new PBD payload, the chemical stability of T-C239i-SG3811 was assessed by LC-MS at each time point for the three buffer conditions. It should be noted that, on account of the higher hydrophobicity of the SG3811 payload, the reverse phase chromatography required optimization of the elution gradient conditions to improve peak resolution in the case of T-C239i-SG3811. Nevertheless, even at the best eluting conditions, the conjugated heavy chain (H1) was still 'tailing' on the chromatogram (Figure 3b), thus complicating deconvolution of the mass spectrometry data. Therefore, in the LC-MS analysis, the ratio between non-conjugated heavy-chain (H0) and H1 was always higher than expected, as reported in Figure S15. A further molecular weight species, H1-fragment 1, was also observed for T-C239i-SG3811, however, its fractional abundance at T0 and at the three time points under thermal stress was constant, and therefore H1-fragment 1 was interpreted to be a stable impurity (Figure S15).

Evaluation of the fraction monomer by SEC-LC is shown in Figure 3d and Figure S14. T-C239i-SG3249 did not show any loss in monomer content even under thermal stress conditions over two weeks, consistent with its previously reported highly stable nature. T-C239i-SG3811 showed a lower monomer content (~96% *vs.* 98.5% for T-C239i-SG3249) at time 0. The percentage of T-C239i-SG3811 monomer remaining in buffers A and B after 3 days, 1 and 2 weeks at 37 °C was almost indistinguishable (94.5% monomer at 2 weeks, 37 °C; Figure 3d). This demonstrated that the free MV-PEG$_{12}$ had no stabilizing effect on the ADC. Only in the presence of both CB[8] and MV-PEG$_{12}$ was the aggregation of HG-SG3811 seen to be minimized, presenting a more stable profile over two weeks at 37°C. This clearly demonstrated that only the ternary host-guest complex was sufficient to mask the hydrophobic warhead.

In vitro cytotoxicity

The two anti-HER2 ADCs were evaluated for their *in vitro* cytotoxicity in two breast cancer cell lines with differing levels of HER2 expression based on immunohistochemistry: MDA-MB-468 (HER2 0) and NCI N87 (HER2 3+). Neither ADC displayed cytotoxic activity against the HER2 negative MDA-MB-468 control cell line (Figure S17)[41–43]. Both ADCs showed potent cytotoxic activity in HER2 3+ NCI N87 line with IC50 values in the order of pg/mL (Figures 4a, S16 and Tables 2, S4). Comparing the two ADCs at T_0 in buffer A (PBS), the known, high potency of T-C239i-SG3249 is reflected in the left-shift of the curve and *ca.* 2-fold lower IC50 value. The presence of free MV-PEG$_{12}$ does not notably alter the potency of T-C239i-SG3811(80 pg/mL) but the additional presence of CB[8], forming the ternary complex, caused a 4-fold loss in potency (297 pg/mL). The reason for this is not clear, since the K_d of the ternary complex would suggest near complete dissociation of MV-PEG$_{12}$⊂CB[8] from T-C239i-SG3811 upon dilution; however, retention of the complex in the cell media cannot be ruled out. Following thermal stress, T-C239i-SG3811 in buffer A and B lost 2 and 1 $\log_{10}$ potency, respectively, while in buffer C only a 2-fold loss of potency was observed (Table 2 And Figure 4b). These data highlight the importance of the host-guest interaction in order to stabilize T-C239i-SG3811 and are in agreement with the data collected from the stability study, especially the monomer loss monitored by SEC-LC.

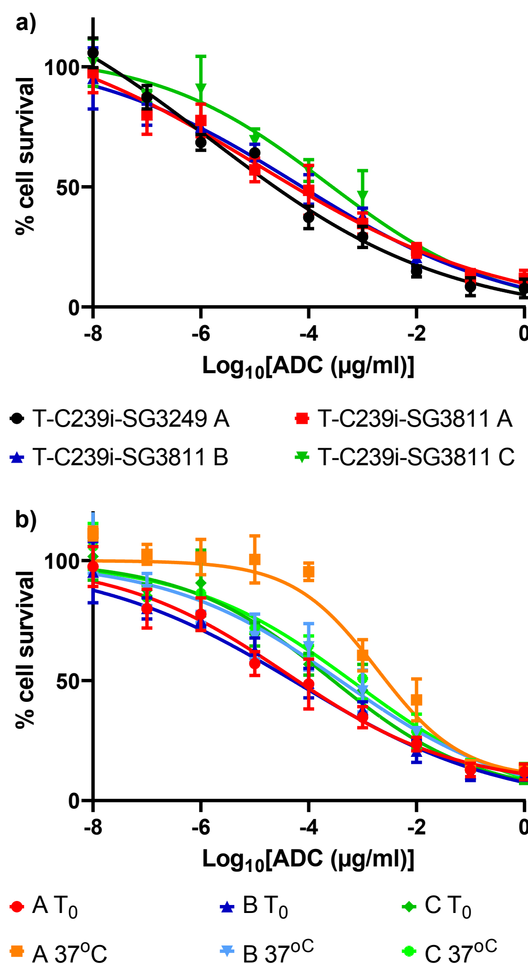


Figure 4. Dose-response curve in NCI-N87 cell line: a) showing the two ADCs under analysis, in buffers A (PBS), B (+MV-PEG$_{12}$) and C (+MV-PEG$_{12}$⊂CB[8]); b) T-C239i-SG3811 in the buffers A, B and C, at T_0 and after 7 days (T_7) at 37 °C. Data were produced in triplicates and are reported as average ± SD.

Table 2 – ADCs cytotoxicity in NCI N87 cell line

IC $_{50}$ (pg/mL ± SD)		
	T_0	T_7 37°C
T-C239i-SG 3249 (A)	38 ± 4.2	23 ± 2.2
T-C239i-SG 3811 (A)	73 ± 9.1	4502 ± 479
T-C239i-SG 3811 (B)	80 ± 8.6	351 ± 46.5
T-C239i-SG 3811 (C)	297 ± 37	575 ± 84

Conclusions

In order to explore the feasibility of a supramolecular formulation approach to stabilize a hydrophobic ADC, a potent PBD monomer was selected from the Spirogen library on account of its affinity to the macrocycle CB[8] and was then dimerized (SG3811) to obtain a more potent payload. The dimerization also led to a further increase in the hydrophobicity of the molecule; in this work, both the chemical synthesis of the warhead and site-specific conjugation of this ADC system were performed and optimized. The ability of the complex MV-PEG_{12}⊂CB[8] to improve the formulation of this ADC was then assessed through a stability study, comparing against an ADC (T-C239i-SG3249) previously reported to have high potency and stability. Both ADCs used the same linker chemistry and retained their DAR values during the course of the stability study. The presence of MV-PEG_{12} in solution did not improve the stability of T-C239i-SG3811, only the presence of the binary complex MV-PEG_{12}⊂CB[8] produced a clear improvement in physical stability under stress, suggesting that specific localization of the PEG chain is necessary to mask the hydrophobic warhead in solution. Both ADCs showed potent cytotoxic activity, in the order of pg/mL, towards the NCI N87 cell line, though the presence of the supramolecular complex reduced the potency of (non-stressed) T-C239i-SG3811 4-fold. Nevertheless, the improvement in physical stability of T-C239i-SG3811 in the presence of supramolecular complex following thermal stress was consistent with the minimal loss in cytotoxic potency. In conclusion, we show that the host-guest approach, whereby MV-PEG_{12}⊂CB[8] was used to mask a highly hydrophobic PBD warhead, improves the physical stability of the
ADC without perturbing its chemical stability and retaining potent cytotoxic activity. This novel supramolecular approach to reversibly bind hydrophilic polymers such as PEG to an ADC is generically applicable to cytotoxic warheads harboring appropriate guest moieties and enables highly hydrophobic payloads to be considered for conjugation to monoclonal antibodies.

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Author Contributions

All authors have given approval to the final version of the manuscript.

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ASSOCIATED CONTENT

Supporting Information The Supporting Information is available free of charge on the ACS Publications website at DOI: XXXX

ABBREVIATIONS

ADC, antibody drug conjugate; Arg, arginine; CB[n], cucurbit[n]uril; DAR, drug to antibody ratio; DMSO, dimethyl sulfoxide; DMA, dimethyl acetamide; Fmoc, fluorenylmethyloxycarbonyl; HER2, human epidermal growth factor receptor 2; ITC, isothermal titration calorimetry; LC-MS, liquid chromatography–mass spectrometry; MDR1, multidrug resistance protein 1; MV, methyl-viologen; PBD, pyrrolo benzodiazepine; PBS, phosphate buffered saline; PEG, polyethylene glycol; PG, propylene glycol; RP-LC, reverse phase liquid chromatography; RT; room temperature; SD, standard deviation; SEC-LC, size exclusion liquid chromatography; SAR, structure-activity relationship; SEM, 2(trimethylsilyl)ethoxymethyl; T-C239i, Trastuzumab-C239i; Tris, tris(hydroxymethyl)aminomethane.

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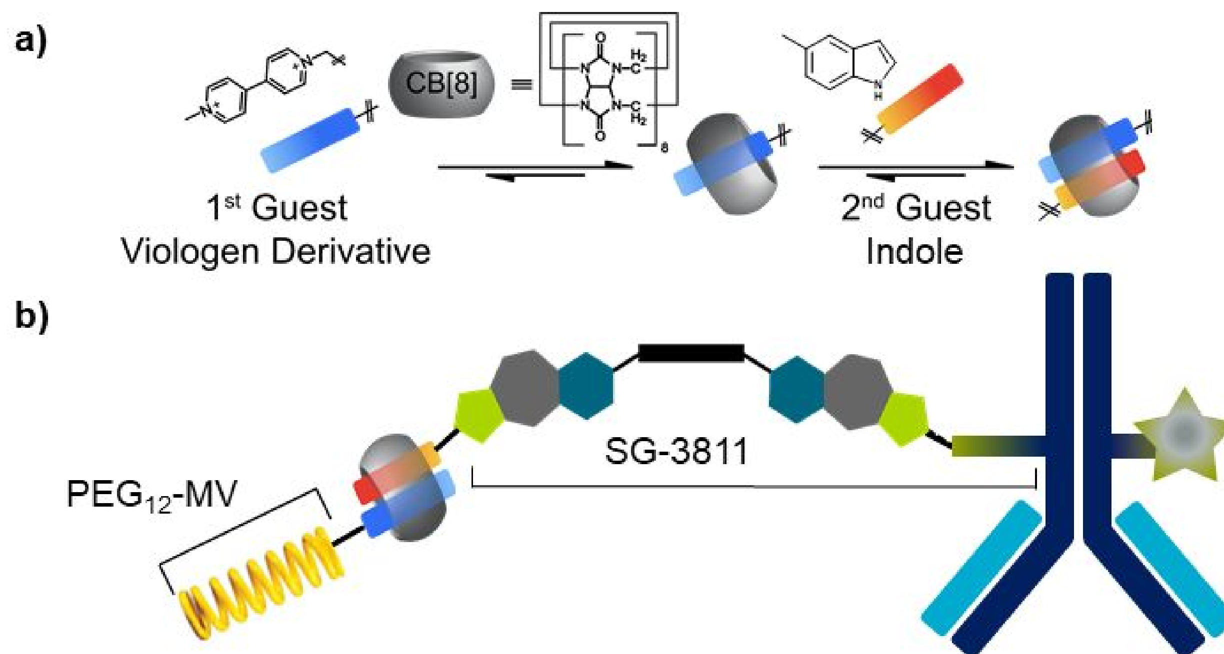

Supramolecular formulation to improve ADCs physical stability

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