Characterization of Payload Release From an Improved Camptothecin Drug-Linker

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Background

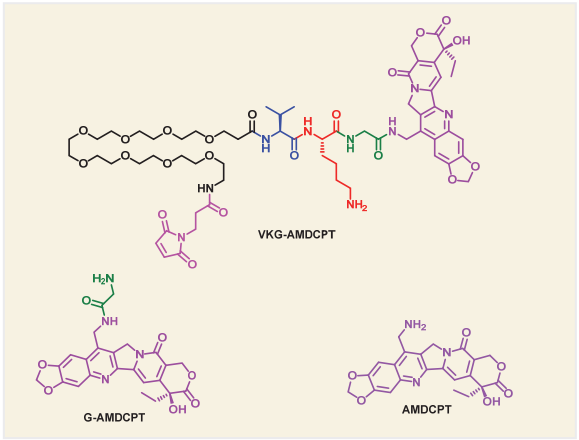
- Camptothecins (CPT), an important class of molecules that have been employed in cancer therapy for the past 20 years, interact with the DNA-topoisomerase I complex, leading to DNA damage and cell death
- This class of small molecules is also known to induce toxicities including diarrhea and myelosuppression
- Antibody-drug conjugates (ADCs) have emerged as a method to help mitigate small molecule toxicities by targeting them to tumors via conjugation to tumor-specific antibodies; the linker that is used to conjugate a small molecule payload to an antibody strongly influences the release rate and efficacy
- Two ADCs employing camptothecin analogues were recently approved
- We sought to study the small molecule release from an improved CPT drug-linker, maleimidopropionyl-PEG8-valine-lysine-glycine-7-aminomethyl-10,11-methylenedioxycamptothecin (VKG-AMDCPT), building on our previous experience with highly cytotoxic methylenedioxy camptothecin analogues (DOI: 10.1021/bc9001097)

Camptothecin structure and linkage to antibody

A Drug-Linker with Multiple Cleavage Possibilities

- When employed as an ADC, maleimidopropionyl-PEG7-valine-lysine-glycine-7-aminomethyl-10,11-methylenedioxycamptothecin (VKG-AMDCPT), an improved, highly active and enzyme-cleavable tripeptide CPT drug-linker, has the ability to release multiple species upon internalization and degradation
- The two most likely released molecules include 7-amino methyl 10,11-methylenedioxy CPT (AMDCPT) and the corresponding glycine adduct of AMDCPT (G-AMDCPT)
- When prepared as an ADC with a ratio 8 drugs per antibody, potency is observed across a diverse panel of cancer cell lines *in vitro*

Figure 1. VKG-AMDCPT and its potential released drugs[1]

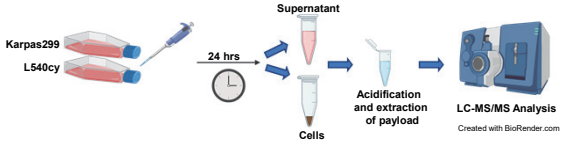


Method for evaluation of payloads in cell culture

Quantitative Mass Spectrometry

Figure 2. Workflow for detection of released payloads

- L540cy and Karpas299 cells were dosed with 100 ng/mL of cAC10-VKG-AMDCPT ADC and incubated at 37°C and 5% CO_2. After 24 hours, cells were harvested, washed and pellets and cell culture media were frozen down until small molecule quantitation was performed



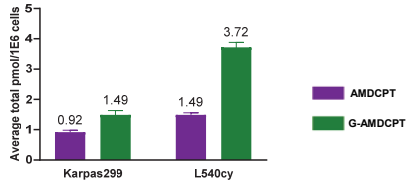
- Prior to extraction of small molecule, treated samples were acidified with formic acid to convert any molecules present in the carboxylate form to their respective lactones, providing consistent and robust extraction efficiencies
- Following acidification, released payloads were then isolated by organic extraction and protein precipitation and quantitated by LC-MS/MS utilizing an internal standard on a SCIEX 6500

VKG-AMDCPT ADC releases two drug species

Total payload released from ADC

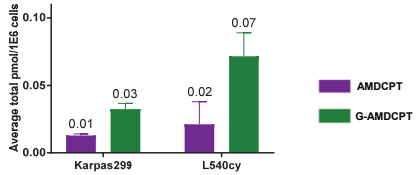
- cAC10-VKG-AMDCPT ADC releases detectable amounts of AMDCPT and G-AMDCPT (Figure 3)
- In total, more G-AMDCPT is released than AMDCPT (~60-70% of the total payload detected both intra- and extra-cellularly for both cell lines)

Figure 3. Quantification of AMDCPT and G-AMDCPT drug release from cAC10-VKG-AMDCPT ADC in CD30-positive Karpas299 and L540cy cancer cell lines



Intracellular payload quantitation

Figure 4. Identity and quantity of payloads in cell pellets



- AMDCPT and G-AMDCPT were detected intracellularly and extracellularly (Figure 4 and 5 respectively)
- More G-AMDCPT than AMDCPT is detected in both locations
- The cellular concentration of each drug detected inside cells varied between 5-80 nM

Extracellular payload quantitation and comparison to intracellular payload

- Substantially higher quantities of both species were found extracellularly, with 40-50X more total drug found outside of the cell (Figure 5)
- Less than 2.5% of each payload released from ADC was retained inside cells after 24 hours

Figure 5. Identity and quantity of released payloads in culture media

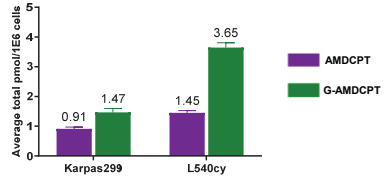
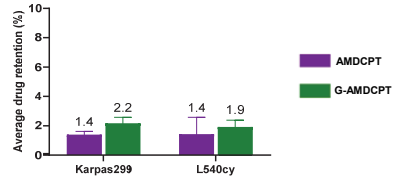


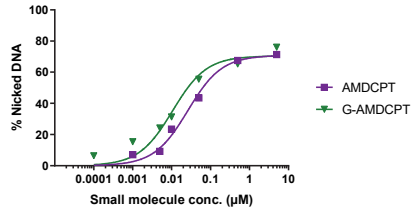
Figure 6. Retention of released payloads in cells



Biochemical potency of released payloads

- Both AMDCPT and G-AMDCPT have similar concentrations required for cleavable complex (CC) formation (10-25 nM, Figure 7)

Figure 7. Cleavable complex formation assay (CC50)



- Cleavage by human topoisomerase I was determined by measuring nicked/open circular DNA with a titration of test article via gel electrophoresis. The CC50 was calculated as a percentage of the maximum cleavage obtained for each compound.
- Reversibility of CC formation was measured via gel electrophoresis after the addition of exogenous linear DNA to the formed CC. Rate constants were 0.01 and 0.02 min^{-1} for AMDCPT and G-AMDCPT, respectively.

In vitro potency of released payloads

- AMDCPT and G-AMDCPT both display potent *in vitro* on-cell activity
- AMDCPT exhibits increased potency compared to G-AMDCPT
- The ability of the small molecules to cross the cellular membrane is often a factor in their in vitro IC50 values, with more charged molecules often having reduced potency due to reduced membrane penetration of more polar molecules, which may account for the reduced cellular potency of G-AMDCPT compared with AMDCPT

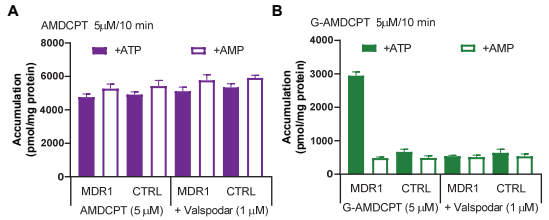
Table 1 Cytotoxicity of CPT payloads

Cytotoxicity (96 hr CellTiterGlo)	IC50 (nM)						
	L540cy	786-O	BxPC-3	MM.1R	MOLM13	SK-MEL-5	U-266
AMDCPT	1	1	1	1	4	1	1
G-AMDCPT	24	71	62	18	27	59	39

MDR1 pump susceptibility of released payloads

- In vitro drug transport assays indicate that G-AMGCPT, but not AMDCPT, is a substrate for MDR1
- cAC10-VKG-AMDCPT is active on Del:BVR, a cell line overexpressing MDR1, in the presence or absence of MDR1 inhibition by Zosquidar suggesting that the mixture of released drugs provides activity that can overcome MDR1 activity

Figure 8. Accumulation of small molecules in MDR1 and control vesicles in a vesicular transport substrate assay with or without a reference inhibitor



Conclusions

- LC-MS/MS assays confirm that cAC10-VKG-AMDCPT releases both AMDCPT and G-AMDCPT in Karpas299 and L540cy cells
- G-AMDCPT accounts for approximately 60-70% of the released payload, with AMDCPT making up the remainder
- Retention of AMDCPT and G-AMDCPT in cells is less than 2.5% of the total released drug
- The observed intracellular concentrations of drug positively corresponds with the CC50 values of these payloads
- AMDCPT and G-AMDCPT have similar biochemical potencies and are active as small molecules *in vitro*
- G-AMDCPT has reduced in vitro potency compared to AMDCPT, possibly due to reduced cellular uptake of the glycine adduct
- G-AMGCPT, but not AMDCPT, is a substrate for MDR1
- cAC10-VKG-AMDCPT has activity in an MDR1-expressing cell line, likely due to the ability of AMGCPT to evade MDR1 pumps
- VKG-AMDCPT is the drug linker in SGN-CD30C

References:
1. Lyski, R. Abstract 2885. AACR Virtual Meeting II, June 22-24, 2020
2. Li, F. Cancer Res 2016; 76(9); 2710-9

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