In vitro characterization of the mechanism of action of micvotabart pelidotin, an antibody-drug conjugate targeting extradomain-B of fibronectin

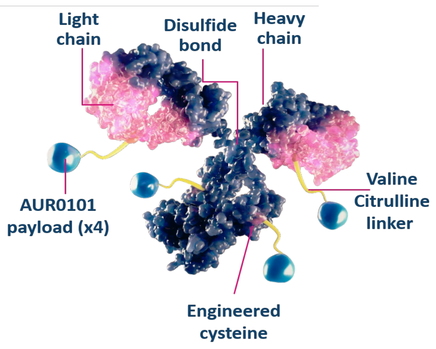
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Abstract: 54

Micvotabart pelidotin (MICVO, formerly PYX-201), a first-in-concept antibody-drug conjugate (ADC) targeting extradomain-B of fibronectin (EDB+FN)

MICVO structure



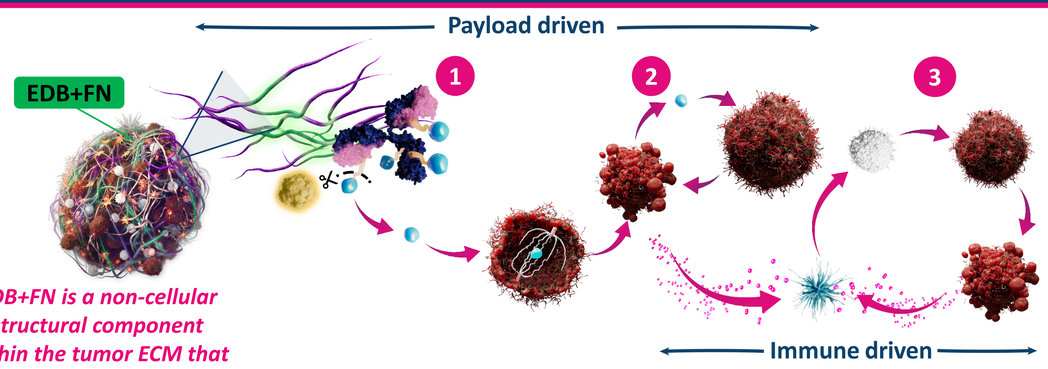
MICVO is composed of a fully human IgG1 monoclonal antibody (mAb) derived from the L19 clone. The mAb was engineered with cysteines κK183C and K290C for site-specific conjugation. The optimized auristatin payload Aur0101 was conjugated to the mAb via the PABC spacer and a mc-Val-Cit linker [1]

Key potential advantages

PURPOSE-BUILT	• Structural integrity with **high avidity-driven binding** • **Site-specific, extracellular cleavable Valine Citrulline linkers**
PREDICTABLE	• **Uniform DAR* of 4** provides improved therapeutic window • **Reduced free payload in serum,** C_{max} ~4 days post administration
POTENT	• Four **optimized auristatin 0101** microtubule directed payloads • Maximize **broad tumor-killing and biological potency**
PERMEABLE	• Optimized membrane diffusion to enable **bystander killing** • **Fast payload clearance to reduce off-target effects**

* DAR: drug-to-antibody ratio

MICVO delivers potent antitumor activity through a three-pronged mechanism of action (MOA)



EDB+FN is a non-cellular structural component within the tumor ECM that is highly expressed in tumors compared to normal adult tissues [9]. MICVO is designed for extracellular linker cleavage in the acidic TME which initiates the MOA.

1 **Direct cancer cell killing[1-4]:** MICVO designed to bind to EDB+FN and release payload within tumor ECM

2 **Bystander cancer cell killing[5]:** Cancer cell dies releasing payload for additional cycles of cancer cell killing

3 **Immunogenic cell death[6-8]:** Cancer cell death releases immune stimulatory molecules leading to T-cell activation



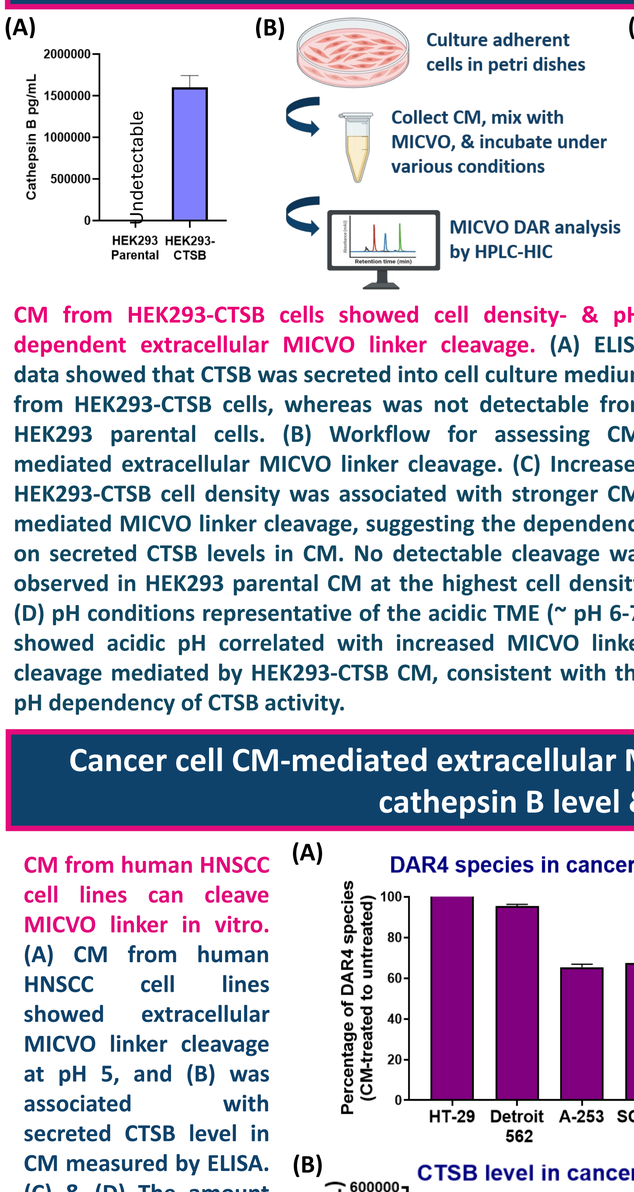
Objectives

- Preclinical studies have demonstrated broad antitumor activity of MICVO across multiple solid tumor mouse models [1, 4, 5, 6]. In patient-derived xenograft (PDX) models, EDB+FN protein expression levels did not correlate with MICVO's efficacy, but an enzyme gene signature was associated with MICVO response [4]. Consistent with these findings, several recombinant cathepsins were shown to efficiently cleave the MICVO linker in vitro in a concentration- and pH-dependent manner [3].
- Tumors commonly overexpress proteases, including cathepsins, which can be secreted into the tumor ECM [10]. The objective of this study is to show that extracellular proteases secreted in conditioned media (CM) from a cathepsin B (CTSB) overexpressing cell line and human HNSCC cancer cell lines endogenously expressing CTSB can efficiently cleave the linker of MICVO to release the payload independent of ADC internalization, demonstrating MICVO's non-cellular associated MOA. The in vitro antibody-dependent cellular cytotoxicity (ADCC) property of MICVO is also reported here.

Methods

- **CM-mediated extracellular MICVO linker cleavage.** HEK293 parental and CTSB-expressing cells were seeded at a density of 0.5 × 10^6 cells/mL (or a range of 0.031 to 0.5 × 10^6 cells/mL in the cell density assay), and human cancer cells were seeded at 1 × 10^6 cells/mL. Cells were cultured overnight in their respective media without FBS. Freshly collected CM was incubated either overnight or 24hrs with MICVO at pH 5 (or a range of pH 6–7 in the pH-dependency assay). MICVO linker cleavage was assessed by High-Performance Liquid Chromatography with Hydrophobic Interaction Chromatography (HPLC-HIC). The percentage of DAR4 species was calculated as the ratio of DAR4 species in CM-treated MICVO compared to untreated MICVO.
- **Extracellularly released payload-mediated cytotoxicity assay.** CM were collected following overnight culture of HEK293 parental & CTSB cells, and endogenous CTSB-expressing human HNSCC A-253 and SCC-9 cancer cells, each seeded at a density of 0.5 × 10^6 cells/mL. MICVO was mixed with freshly collected CM and incubated at pH 5 for 4 hours, then added to EDB+FN negative HT-29 cells at a final MICVO concentration of 1.67 µg/mL for 4 days.
- **Binding affinity analysis by Octet Bio-Layer Interferometry (BLI).** His-tagged FcγRIIIA V176 and F176 variants, which play critical roles in NK cell-mediated ADCC, were captured onto HIS1K biosensors at 50 nM and 250 nM, respectively. MICVO, L19 intermediate mAb, and L19 WT mAb were used as analytes at concentrations ranging from 10–160 nM for the V176 variant and 50–800 nM for the F176 variant.
- **In vitro ADCC assay.** EDB+FN-positive Caki-2 cells were seeded in 96-well plates at 7,500 cells/well and cultured overnight. Cells were then treated in duplicate with a 9-point, 1:3 serial dilution of MICVO, L19 intermediate mAb, or L19 WT mAb, together with effector cells from Promega's ADCC Bioreporter Assay kit at a 10:1 effector-to-target (E:T) ratio. Plates were incubated for 6 hours at 37°C, and luminescence was measured.

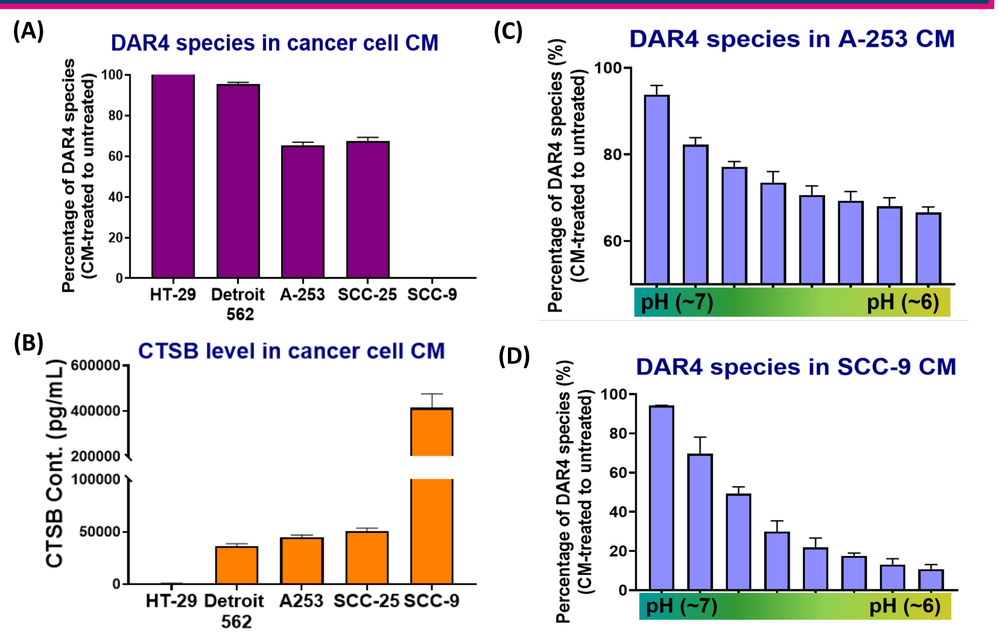
CM from HEK293-CTSB cells can efficiently cleave MICVO linker in vitro



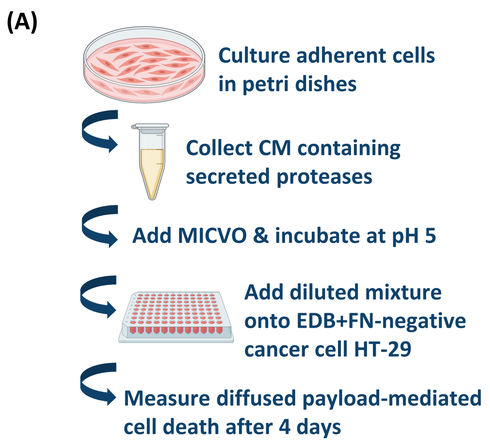
CM from HEK293-CTSB cells showed cell density- & pH-dependent extracellular MICVO linker cleavage. (A) ELISA data showed that CTSB was secreted into cell culture medium from HEK293-CTSB cells, whereas was not detectable from HEK293 parental cells. (B) Workflow for assessing CM-mediated extracellular MICVO linker cleavage. (C) Increased HEK293-CTSB cell density was associated with stronger CM-mediated MICVO linker cleavage, suggesting the dependency on secreted CTSB levels in CM. No detectable cleavage was observed in HEK293 parental CM at the highest cell density. (D) pH conditions representative of the acidic TME (~ pH 6-7) showed acidic pH correlated with increased MICVO linker cleavage mediated by HEK293-CTSB CM, consistent with the pH dependency of CTSB activity.

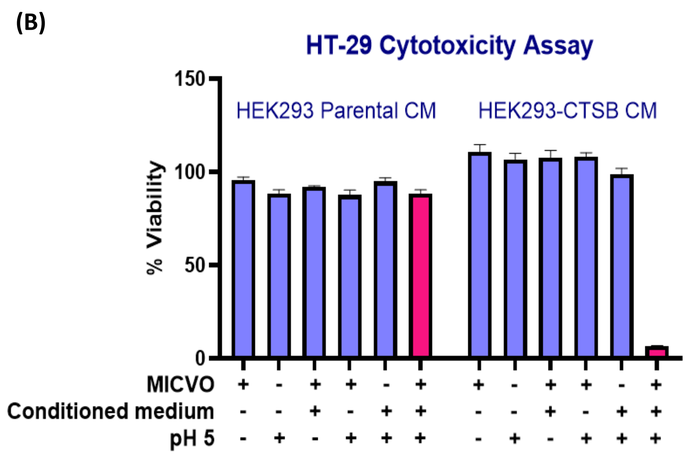
Cancer cell CM-mediated extracellular MICVO linker cleavage is associated with cathepsin B level & acidic conditions

CM from human HNSCC cell lines can cleave MICVO linker in vitro. (A) CM from human HNSCC cell lines showed extracellular MICVO linker cleavage at pH 5, and (B) was associated with secreted CTSB level in CM measured by ELISA. (C) & (D) The amount of CM-mediated extracellular MICVO linker cleavage was associated with acidic pH in CM for both HNSCC cell lines A-253 & SCC-9.

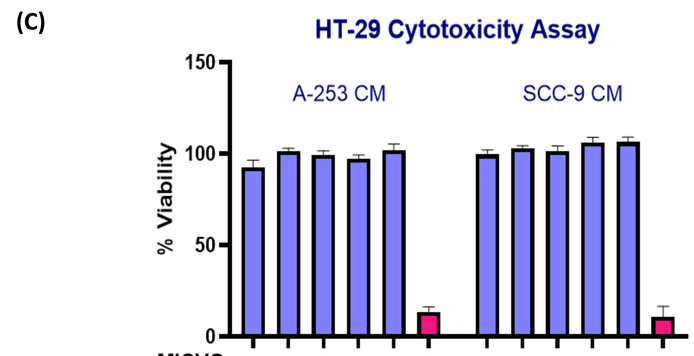


CM-mediated in vitro extracellular payload release enables the killing of EDB+FN-negative tumor cells





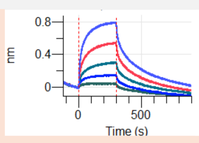
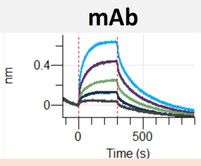
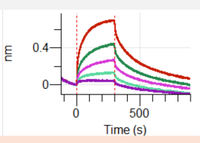
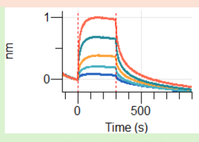
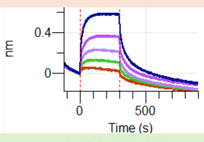
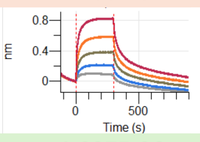
EDB+FN-negative cancer cells can be killed by in vitro extracellularly released MICVO payload in CM at pH 5. (A) Workflow for in vitro payload release mediated by CM and cytotoxicity assay. CM were collected from (B) HEK293 parental & CTSB cells, and (C) endogenous CTSB-expressing human HNSCC A-253 and SCC-9 cells. The CM+MICVO mixture under pH 5 caused significant cell death except for negative control HEK293 parental cells (pink bars). No cell killing was observed for all other control groups (violet bars), demonstrating that the killing of EDB+FN-negative HT-29 cells was specifically caused by protease-mediated payload release of MICVO in CM under acidic conditions.



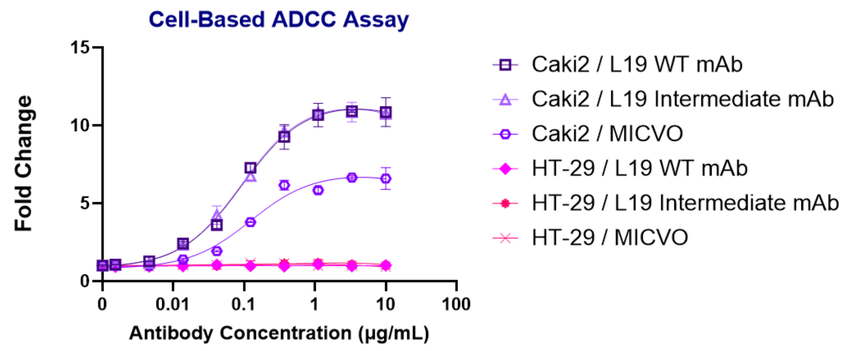
MICVO shows moderate in vitro ADCC activity in cell-based assay

MICVO retains binding affinity to FcγRIIIA receptors with moderate in vitro ADCC activity observed. (A) MICVO, L19 intermediate mAb, and L19 WT mAb showed comparable binding affinities to FcγRIIIA V176 & F176 variants by Octet BLI analysis. (B) In vitro ADCC activity data for MICVO, L19 intermediate mAb, and L19 WT mAb on EDB+FN-positive Caki2 and EDB+FN-negative HT-29 cells. MICVO, L19 intermediate mAb, and L19 WT mAb moderately induced ~ 5x, 10x and 10x ADCC activity, respectively, in Caki-2 cells, but not in HT-29 cells.

(A)

Binding Affinity	MICVO	L19 intermediate mAb	L19 WT mAb
FcγRIIIA V176			
KD (M)	1.338E-07	1.436E-07	1.406E-07
FcγRIIIA F176			
KD (M)	3.117E-06	3.831E-06	1.027E-06

(B)



Conclusions

- Conditioned media from human CTSB-overexpressing cell line and multiple human HNSCC cell lines can cleave MICVO linker extracellularly in a pH-dependent manner, which was associated with the protease (e.g. CTSB) level secreted in the conditioned media.
- Extracellularly released payload can diffuse into and kill EDB+FN-negative cancer cells that were insensitive to non-cleaved MICVO without requiring ADC internalization, demonstrating MICVO's non-cellular associated antigen targeting and extracellular linker cleavage in the acidic TME as an effective mechanism to deliver a cytotoxic payload to cancer cells.
- ADCC may moderately contribute to the antitumor activity of MICVO, consistent with EDB+FN being a non-cellular target.
- These data support MICVO development as an investigational therapy for HNSCC patients with difficult-to-treat cancers in the ongoing clinical trials as a monotherapy (NCT05720117) and in combination with pembrolizumab (NCT06795412). Monotherapy R/M HNSCC clinical data to be presented during Plenary Session 3 (3LBA).

References

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