# Affimer® Drug Conjugates (AffDC) targeting Fibroblast Activation Protein-α deliver highly toxic warheads to the tumor microenvironment by leveraging the pre|CISION® release mechanism

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## INTRODUCTION

### pre|CISION® Affimer® Drug Conjugates (AffDCs) are a Third Generation pre|CISION® therapeutic

**Antibody drug conjugates have revolutionized the treatment of multiple diseases in metastatic cancers, however there are limitations:**

- Non-specific release of the warhead leads to significant toxicities (e.g. interstitial lung disease/pneumonitis)
- Complexity of the bystander effect that results in killing of antigen-negative tumor cells
- Tumor penetration limitations with the large size of antibodies

**The Affimer® drug conjugates (AffDC) are a novel class of engineered biotherapeutics designed to deliver highly toxic warheads directly to the tumor microenvironment (TME) by combining two novel delivery systems:**

- The clinically validated pre|CISION® warhead release mechanism which has significant advantages over protease-cleavable linkers in that warhead release is only accomplished with FAP cleavage in the TME (*Banerjee et al. 2024, Twelves et al. 2024*)
- The pre|CISION® linker is specifically cleaved by membrane-bound FAP, but not by closely related or wider mammalian peptidases, and its conjugation renders a warhead inert by preventing cell uptake. This linker is cleaved by FAP on cancer associated fibroblasts, releasing active warhead to the TME
- The Affimer® protein is based on the human protein Stefin A and contains 2 antigen binding loops with antigen binding affinities similar to those achieved with antibodies
- Affimer® proteins have advantages over antibodies including smaller size (~1/10th the size of an antibody) which leads to better tumor penetration, a DAR of 2-3 for a monomer therapeutic and optimized manufacturing with production in *E. coli* vs. mammalian cell manufacturing

**The antigen binding domain (Affimer®) once selected is conjugated via conventional ADC methods to potent warheads using the pre|CISION® linker as a release mechanism. This novel therapeutic leverages tumor targeting and FAP-dependent activation of the warhead for highly specific delivery to the tumor**

**The first AffDC described herein comprises an Affimer® protein binding FAP conjugated to the pre|CISION® linker and Topoisomerase I inhibitor warhead combination**

**The pre|CISION® drug release mechanism has demonstrated clinical proof-of-concept in multiple patient populations with FAP-high diseases and doxorubicin sensitivity (AVA6000; pre|CISION®-Doxorubicin, a first generation pre|CISION® therapeutic, *Banerjee et al. 2024, Twelves et al. 2024*). A second generation pre|CISION® program with an Exatecan warhead, AVP6103, is described on poster #173 in this session**

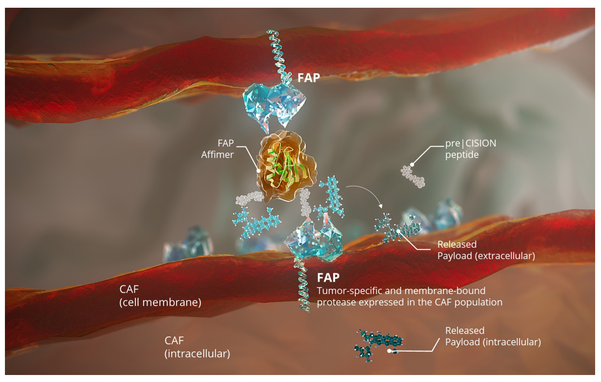



**FIGURE 1. pre|CISION® Affimer® Drug Conjugate: Mechanism of Action**

### AFFIMER® PROTEINS

- Affimer® proteins contain two loops that have the potential to bind target proteins with high affinity and high specificity. Loop insertions eliminate normal Stefin A biological activity
- Affimer® proteins are around 14kDa and may be further engineered as fusion proteins to incorporate a second antigen binding domain (e.g., an albumin-binding Affimer® domain may be incorporated into the final molecule to modulate PK properties)
- A simple structure allows the use of modelling software to support engineering, epitope mapping etc; *E. coli* expression allows rapid discovery cycle times

**TABLE 1. Advantages of Affimer® proteins as pre|CISION® delivery vehicle**

| Key Attributes | Affimer® | Antibody |
|---|---|---|
| Small protein, simple structure and folds, no disulphides or post-translational modifications | Y | N |
| Rapid discovery process with high-level expression in *E. coli* | Y | N |
| Rapid and flexible engineering to incorporate conjugation sites or to optimize affinity and biophysical characteristics | Y | N |
| Very high solubility (>250mg/ml PBS) with low viscosity | Y | N |
| High solvent stability for downstream processing (e.g. soluble in 25% DMSO or 15% DMA) | Y | N |
| High Tm for downstream processing | Y | Y/N |
| Simple, unencumbered IP | Y | N |

## TARGETING FAP IN TUMORS

- Affimer® proteins targeting FAP, conjugated to an Exatecan warhead via a pre|CISION® linker (AffDC), have been developed to enhance warhead delivery to the TME
- Fibroblast activation protein (FAP) is highly expressed in many tumors where it is expressed on cancer associated fibroblasts (CAFs). Figure 2 shows the intimate relationship of cancer epithelial cells, CAFs and blood vessels, providing an ideal architecture for warhead delivery
- pre|CISION® enabled compounds are designed to allow for FAP specific cleavage and release of the warhead, directly in the TME
- In the TME, CAFs with the highest expression of FAP are concentrated at the tumor-stroma interface and co-located with the blood vessels which delineates the "bystander effect" delivery
- The bystander effect of the pre|CISION® platform warhead delivery: The close proximity of blood vessels (●), FAP+ CAFs (●), and cancer cells (●), allows pre|CISION® compounds to be readily delivered, cleaved, and taken up by neighbouring cancer cells

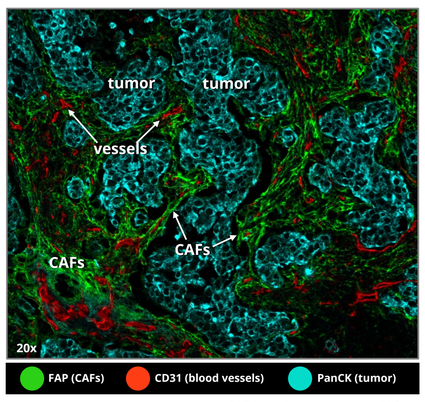


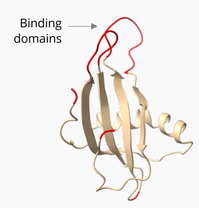

**FIGURE 2. CASE STUDY: 79–year-old male with SGC (ductal histology) with disease progression following first line therapy. Enrolled in the AVA6000 trial (Oct 2023) in the 385 mg/m² Q3W cohort with durable partial response (duration of response >18 weeks)**

In this case, tumor cells are negative for FAP (aqua) with prominent FAP-positive Cancer Associated Fibroblast (CAF) populations (green) observed at the tumor-stroma interface with vessels co-localized in stroma (red)

## ISOLATION, FORMATTING, AND ENGINEERING OF FAP-BINDING AFFIMER® PROTEINS

- Affimer® libraries have been generated with structural liabilities eliminated, no bias in amino acid distribution, optimized sequences for efficient screening processes and various loop lengths. Screening incorporates NGS to optimize hit identification
- Single-domain Affimer® proteins may be engineered to a two-domain protein to fine-tune affinity and epitope binding characteristics (Figure 3)
- Affimer® proteins normally contain no cysteine residues. Lead Affimer® proteins were engineered to introduce cysteine residues at multiple specific locations (Figure 3), offering various DAR permutations
- Multiple biologic, biophysical, and *in silico* tools were used to identify lead candidates binding to FAP (examples in Figure 4). Non-internalizing clones, as well as inability to inhibit FAP, are preferred characteristics to facilitate warhead cleavage by FAP in the ECM
- Binding specificity for FAP was also confirmed, with lead candidates unable to bind the related enzyme DPPIV or other cell surface proteins (data not shown)

**Structure of an Affimer® protein with the two binding loops indicated**




**Structure of an in-line fusion two-domain Affimer® protein showing 4 sites where cysteine residues can be engineered for efficient linker-payload conjugation**

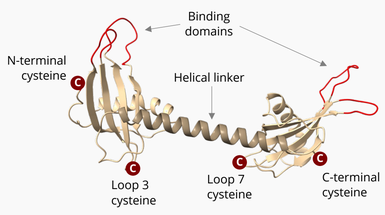
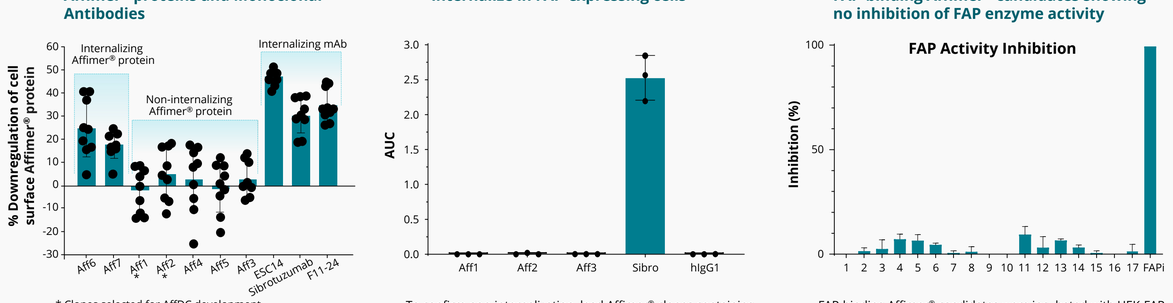



**FIGURE 3.**
**Affimer® proteins can be formatted into multi-specific fusion proteins and can be engineered to incorporate cysteine residues in different locations**

**Panel 1. Internalization of FAP-binding Affimer® proteins and Monoclonal Antibodies**

**Panel 2. Lead Affimer® clones do not internalize in FAP-expressing cells**

**Panel 3. Screening of a panel of 17 FAP-binding Affimer® candidates showing no inhibition of FAP enzyme activity**



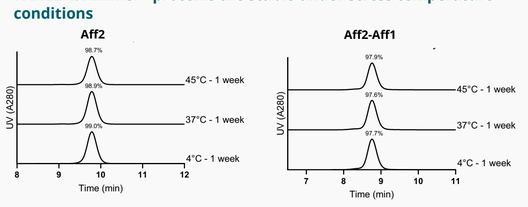

* Clones selected for AffDC development

Affimer® clones were evaluated for internalization based on down-regulation of Affimer® protein at the cell surface of FAP-expressing cells at 37°C vs. 4°C by cytometry. Antibodies (right-hand three columns) were used as positive internalizing controls.

To confirm non-internalization, lead Affimer® clones containing an engineered C-terminal cysteine residue were conjugated to the pH-sensitive pHrodo Red dye and incubated with HEK-FAP cells. Cell internalization of the dye conjugates was monitored for 24h using Incucyte. Dye-conjugated Sibrotuzumab antibody and human IgG1 were used as negative and positive controls, respectively.

FAP-binding Affimer® candidates were incubated with HEK-FAP cells to assess their ability to inhibit FAP activity. The FAP inhibitor SP-13786 (FAPi) was used as a control. FAP activity measured by its ability to cleave 3144-AMC to produce fluorescent AMC.

**PANEL 4. Affimer® proteins are stable under stress temperature conditions**




Aff2 single-domain (left panel) and Aff2-Aff1 two-domain (right panel) Affimer® proteins were incubated at 4°C, 37°C, or 45°C for 1 week. Protein aggregation in the samples was assessed by SEC. SEC profiles for the stressed samples are shown with the % purity for the monomeric species indicated.

**FIGURE 4.**
**Affimer® protein internalization was measured using two techniques (panels 1 and 2). FAP-binding candidate Affimer® clones were selected which do not internalize in FAP-expressing cells. Candidate clones also do not inhibit FAP enzyme activity (panel 3) and are stable under stress temperature conditions (panel 4)**

### BIOPHYSICAL ANALYSIS AND ENGINEERING

- Biophysical characterization identified candidate Affimer® proteins which bind distinct epitopes on FAP and which have high melting temperatures
- In-line fusion of two FAP-binding Affimer® proteins greatly increased avidity of binding to generate candidates with a wide range of binding affinities (Table 2)

**TABLE 2. Candidate Affimer® proteins have high affinity, high thermal stability, and bind distinct epitopes on FAP**

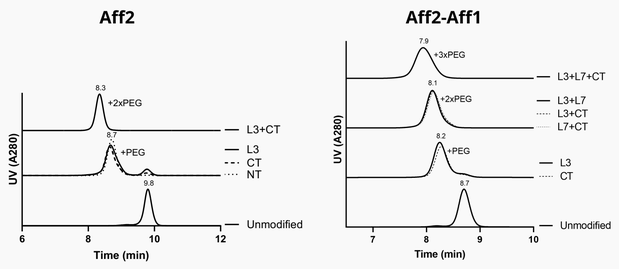
| Affimer® clone(s) | Affimer® format | Affinity ($K_D$) | Epitope group | Tm (°C) |
|---|---|---|---|---|
| **Aff1** | Single-domain | 188 nM | 1 | 85 |
| **Aff3** | Single-domain | 82 nM | 2 | 82 |
| **Aff2** | Single-domain | 16 nM | 3 | 78 |
| **Aff1-Aff3** | Two-domain ILF | 101 pM | 1, 2 | 78 |
| **Aff2-Aff1** | Two-domain ILF | 11 pM | 1, 3 | 78 |

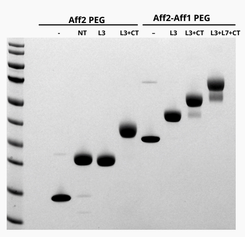
The FAP-binding affinities of single-domain and two-domain Affimer® proteins were assessed by SPR using single-cycle kinetics (Biacore 8K). Epitope binning experiments were performed using Biacore 8K and led to the identification of 3 epitope groups. The melting temperatures of Affimer® proteins were determined by DSC.

## CONJUGATION OF AFFIMER® PROTEINS WITH PRE|CISION® LINKER AND EXATECAN

- Single and two-domain Affimer® proteins with varying cysteine residues were initially conjugated using maleimide chemistry to 5kDa PEG to confirm efficient conjugation (Figure 5). Proteins were also tested to confirm binding affinity to FAP was not impacted (data not shown)
- Single and two-domain Affimer® proteins containing a single cysteine were conjugated using maleimide chemistry to a pre|CISION® linker-warhead carrying the Topoisomerase I inhibitor, Exatecan (Figure 6)
- Conjugation was efficient: resulting conjugates exhibited DAR=1. AffDCs were stable with expected biophysical characteristics (Figure 6 and Table 3)
- Linker-warheads were designed with optimal structure-activity relationships for FAP cleavage when attached to the Affimer® protein (kcat/KM kinetic analysis and FAP-enabled cytotoxicity)

**Engineered cysteine residues are efficiently conjugated to PEG-maleimide with specific DAR**

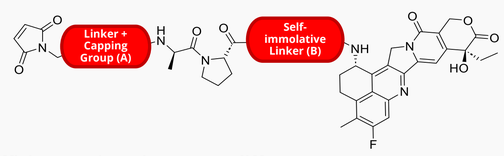


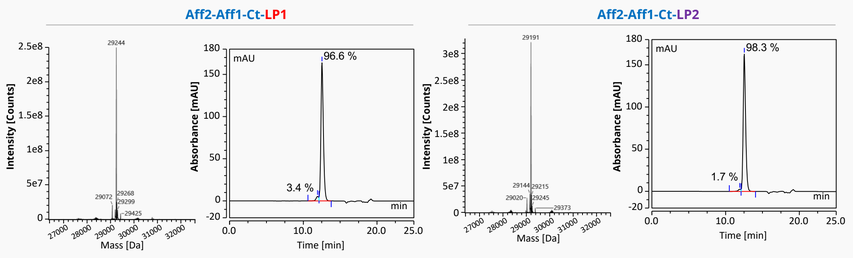

Aff2 single-domain and Aff2-Aff1 two-domain Affimer® proteins containing one engineered cysteine (DAR1) at the N-terminus, Loop 3 or C-terminus, or a combination of 2-3 cysteines (DAR 2-3) at the C-terminus, Loop 3 and Loop 7, were conjugated to PEG-maleimide. Conjugated Affimer® proteins were analyzed by SEC (left and central panels) and SDS-PAGE (right panel). Candidate Affimer® proteins with PEG-maleimide conjugations show SPR equivalent affinities as parent molecules.

**FIGURE 5. Affimer® proteins incorporating multiple cysteine residues in different locations (N-/C-termini, Loop 3/7) were initially tested to ensure efficient conjugation to PEG**

**Depiction of two pre|CISION® linker-warheads used to generate Exatecan AffDCs**




**Affimer® proteins show highly efficient conjugation to two different pre|CISION® linker-Exatecan warheads LP1 and LP2**




A two-domain Affimer® protein containing a C-terminal cysteine was conjugated to two different linker-warheads using maleimide chemistry. The conjugates were purified from the reaction mixtures using HIC and SEC purification. The integrity and DAR of the Affimer® conjugation products was monitored by LC-MS (left panels) and SEC (right panels), showing highly pure and stable species with a precise DAR=1.

**FIGURE 6. Affimer® proteins can be efficiently conjugated to different linker-warheads, resulting in final molecules with precise DAR and high purity**

**AffDCs retain the same binding properties as their parent Affimer® proteins**

**TABLE 3.**
**Two-domain Affimer® protein, conjugated to different Exatecan warhead moieties at the C-terminus or loop 3, show equivalent FAP binding affinities as parent molecule**

| Affimer® clone(s) | Affimer® format | Affinity ($K_D$) |
|---|---|---|
| **Aff2-Aff1** | Two-domain ILF parent | 11pM |
| **Aff2-Aff1-Ct-LP1** | Two-domain ILF: LP1 at C-terminus | 11pM |
| **Aff2-Aff1-Ct-LP2** | Two-domain ILF: LP2 at C-terminus | 10pM |
| **Aff2-Aff1-L3-LP1** | Two-domain ILF: LP1 in loop 3 | 5pM |
| **Aff2-Aff1-L3-LP2** | Two-domain ILF: LP2 in loop 3 | 4pM |

The FAP-binding affinities of AffDCs was assessed by SPR using single-cycle kinetics (Biacore 8K).

## FAP-TARGETED AffDC ARE CAPABLE OF KILLING FAP-EXPRESSING CELL LINES

- AffDCs conjugated to pre|CISION®-Exatecan exhibit greater cytotoxicity against HEK cells expressing FAP compared to parent cells which do not express FAP. Example data is shown for a two-domain Affimer® protein and one linker-payload species (LP2) conjugated at either loop 3 (upper plots) or the C-terminus (lower plots)
- AffDCs typically exhibit 30-fold higher cytotoxicity against HEK cells over-expressing FAP vs. parentals

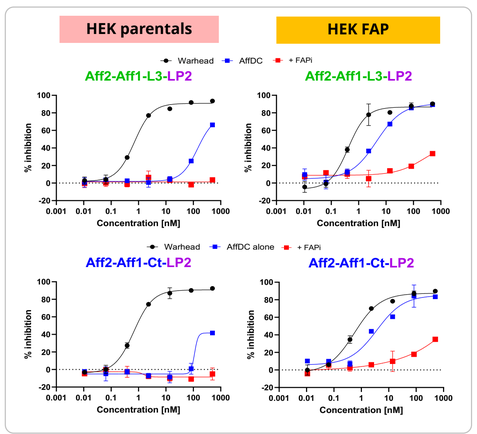



**FIGURE 7.**
**Inhibition of cell proliferation with AffDC molecules. Addition of FAP inhibitor (FAPi, red) prevents Exatecan release and hence prevents cytotoxicity. AffDC (blue) shows limited cytotoxicity in cells which do not express FAP (left plots) and greater cytotoxicity against cells expressing FAP**

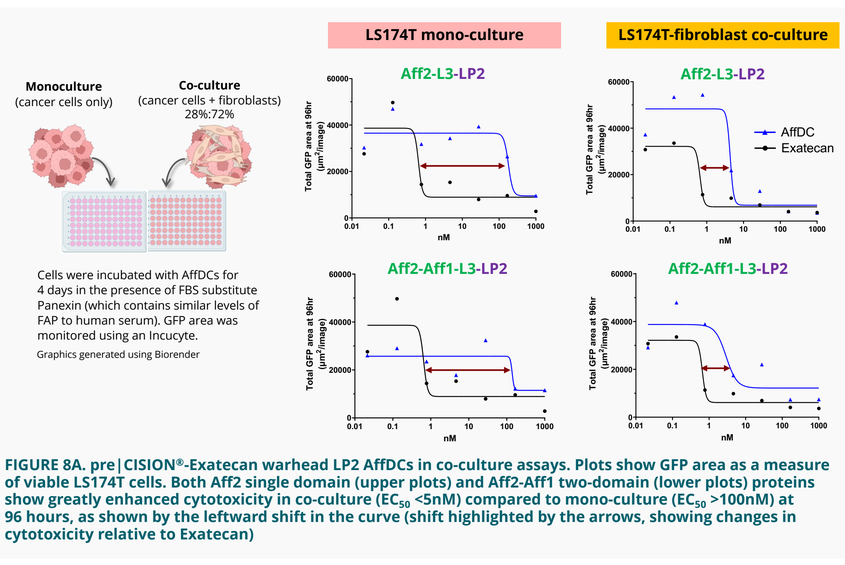
Cells were incubated with AffDCs for 4 days in the presence of FBS substitute Panexin (which contains similar levels of FAP to human serum). Viability was assessed with Cell Titer Glo

**TABLE 4. AffDCs show enhanced cytotoxicity against cells expressing FAP**

| Construct | HEK parental $EC_{50}$ (nM) | HEK FAP $EC_{50}$ (nM) | Fold change |
|---|---|---|---|
| **Linker warhead LP2 loop 3** | 143 | 4.5 | 32 |
| **Linker warhead LP2 C-terminal** | ~110 | 3.7 | 30 |

## CO-CULTURE ASSAY WITH FIBROBLASTS TO DEMONSTRATE BYSTANDER EFFECT

- In co-culture models with Green Fluorescent Protein (GFP)-expressing tumor cells (which do not express FAP) and matched fibroblast cells (which express FAP to levels typically observed in tumors), conjugates exhibit higher cytotoxicity activity towards the cancer cell line when fibroblast cells are present
- Models tested include pancreatic (MiaPaCa-GFP; 96-well assay) and colorectal (LS174T-GFP; 384-well assay). Example data shows single and two-domain Affimer® proteins conjugated to one linker-payload at loop 3




Cells were incubated with AffDCs for 4 days in the presence of FBS substitute Panexin (which contains similar levels of FAP to human serum). GFP area was monitored using an Incucyte.

Graphics generated using Biorender

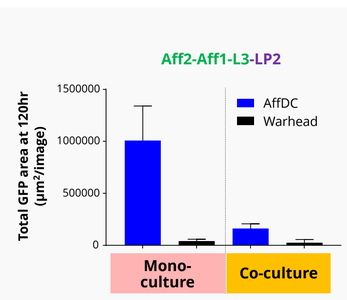



**FIGURE 8B. AffDC also elicits cell kill on MiaPaCa-GFP cells in the presence of matched fibroblasts. Assay at 120 hours with 10nM test article**

**FIGURE 8A. pre|CISION®-Exatecan warhead LP2 AffDCs in co-culture assays. Plots show GFP area as a measure of viable LS174T cells. Both Aff2 single domain (upper plots) and Aff2-Aff1 two-domain (lower plots) proteins show greatly enhanced cytotoxicity in co-culture ($EC_{50}$ <5nM) compared to mono-culture ($EC_{50}$ >100nM) at 96 hours, as shown by the leftward shift in the curve (shift highlighted by the arrows, showing changes in cytotoxicity relative to Exatecan)**

## CONCLUSIONS

- **Affimer® drug conjugates (AffDC) are an entirely new class of biologic medicines in cancer, that leverage novel antigen binders (Affimer® proteins) with the pre|CISION® linker to create highly specific and targeted biologic drug conjugated molecules that can overcome specific limitations of ADC:**
  - AffDC are third generation pre|CISION® therapies with tumor-specific and FAP-dependent warhead release that delivers toxic drugs directly to the tumor microenvironment while sparing normal tissues
  - With non-internalizing Affimer® protein binding and extracellular warhead release by FAP, the AffDC optimizes the bystander effect leading to effective killing of antigen-positive and antigen-negative tumor cells
  - AffDC have an antigen-binding region of 14kDa (single-domain) or 28kDa (two-domain) that is 10-20% of the size of an antibody, optimizing tumor penetration. AffDCs may be rapidly engineered for optimal biophysical and functional characteristics
- **The FAP AffDC molecule exhibits potent FAP binding (to single-digit pM) combined with highly tumor-specific warhead release by leveraging the pre|CISION® linker to release warhead directly in the tumor microenvironment**
  - The proprietary pre|CISION® linker in the FAP AffDC conjugate has been shown in the clinic to specifically release warhead directly in the tumor microenvironment and targeting that delivery to tumors with low FAP expression
- **The AffDC pre|CISION® warhead release mechanism leverages the bystander effect in that release occurs extracellularly in co-culture assays, killing non-FAP expressing cells as warhead is release in the extracellular milieu**

**REFERENCES**

Twelves et al. A Phase I trial of AVA6000, a Fibroblast Activation Protein (FAP)-released, tumor microenvironment (TME)-targeted doxorubicin peptide drug conjugate (PDC) in patients with FAP-positive solid tumors. Presented at the European Society of Medical Oncology Annual Meeting, September 14, 2024, in Barcelona, Spain

Banerjee et al. A Phase I trial of AVA6000, a Fibroblast Activation Protein (FAP)-released and tumor microenvironment (TME)-linked doxorubicin peptide drug conjugate in patients with FAP-positive tumors. Presented at the American Association for Cancer Research Annual General Meeting, April 2024 San Diego, CA USA

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**Poster #160 (Abstract #172)**