Additional mechanisms of action of Enfortumab vedotin, an anti-Nectin-4 ADC demonstrating bystander effect and immunogenic cell death antitumor activity in models of urothelial carcinoma

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Introduction

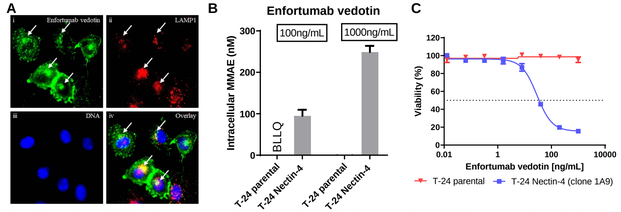
- Enfortumab vedotin (EV) is an investigational antibody-drug conjugate (ADC) that is comprised of a Nectin-4 targeting human monoclonal antibody conjugated to a microtubule-disrupting agent, monomethyl auristatin E (MMAE), via a protease-cleavable linker
- Nectin-4 is a cell adhesion protein highly expressed in several solid tumors, including urothelial, breast, gastric, and lung carcinomas
- EV delivers MMAE to Nectin-4 positive cells, leading to cell cycle arrest and cell death
- PADCEV™ (enfortumab vedotin-ejfv) is FDA approved for adult patients with locally advanced (la) or metastatic urothelial cancer (mUC) who have previously received a PD-1 or PD-L1 inhibitor, and a platinum-containing chemotherapy in the neoadjuvant/adjuvant, locally advanced or metastatic setting; achieving an objective response rate of 44% (EV-201; NCT03219333). This indication is approved under accelerated approval based on tumor response rate. Continued approval for this indication may be contingent upon verification and description of clinical benefit in confirmatory trials
  - Rosenberg, J.E., *et al*. Journal of Clinical Oncology (2019) vol. 37(29), pp. 2592-2600.
- EV is active in combination with pembrolizumab in 1L metastatic or locally advanced UC, achieving an objective response rate of 73.3% (EV-103; NCT03288545)
- Breakthrough Therapy designation was granted to EV plus pembrolizumab based on these data, providing rationale for further investigation of the combination in patients with 1L la/mUC
  - Rosenberg, J.E., *et al.* Journal of Clinical Oncology (2020) vol. 38, (suppl 6; abstract 441); ASCO 2020 Genitourinary Cancers Symposium
- EV is also being evaluated in other solid tumor indications, including HR+/HER- breast cancer, triple negative breast cancer (TNBC), squamous non-small cell lung cancer, non-squamous non-small cell lung cancer, head and neck cancer, gastric, and esophageal cancer (EV-202, NCT04225117)

Objectives of this Study

- Develop *in vitro* and *in vivo* Nectin-4 expressing bladder models for studying enfortumab vedotin mechanisms of action
- Provide mechanistic data by which enfortumab vedotin induces hallmarks of immunogenic cell death (ICD) as a mechanism underpinning the clinical benefit observed with enfortumab vedotin as monotherapy or in combination with pembrolizumab in mUC
- Describe additional mechanisms of action, including the bystander effect, to support clinical studies in cancers with heterogenous Nectin-4 expressing tumors

Primary Mechanism of Cytotoxicity for Enfortumab vedotin

Rapid internalization & trafficking to lysosomes | ***MMAE accumulation in Nectin-4 cancer cells*** | ***Cytotoxicity to Nectin-4 expressing T-24 cells***



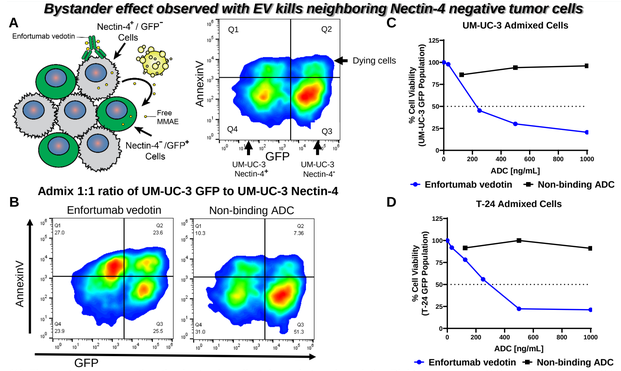
The T-24 and UM-UC-3 bladder cancer models were transduced with human Nectin-4. (A) T-24 Nectin-4 (clone: 1A9) cells were stained after 2 hours of treatment with enfortumab vedotin and stained for enfortumab vedotin (**i, iv;** green), lysosomal marker LAMP1 (**ii, iv;** red) and Hoescht DNA stain (**iii, iv;** blue). White arrows or merged yellow staining show the areas where enfortumab vedotin is colocalized with LAMP1 vesicles. (B) To measure the intracellular concentration of MMAE delivered enfortumab vedotin, T-24 parental and T-24 Nectin-4 (clone 1A9) cell lines were treated for 24 hours with 100 and 1,000 ng/mL ADC. Mass spectrometry analysis (LC-MS/MS) was then used to determine that enfortumab vedotin released 95 nM MMAE and 249 nM MMAE in T-24 Nectin-4 (clone: 1A9) cells at 100 ng/mL (IC50 concentration) and 1,000 ng/mL (IC90 concentration) dose levels, respectively. BLLQ, below lower limit of quantification. (C) Enfortumab vedotin directly kills the T-24 Nectin-4 model while the parental T-24 cell line lacking Nectin-4 is insensitive to enfortumab vedotin. Cell viability was measured 120 hours post-treatment with enfortumab vedotin using CellTiter-Glo.

Cell line	Cell Surface Nectin-4 expression (copies per cell)	Enfortumab vedotin Cytotoxicity IC50
T-24	<2000	>1000 ng/mL
T-24 Nectin-4 Clone 1A9	~650,000	33 ng/mL
UM-UC-3	<2000	>1000 ng/mL
UM-UC-3 Nectin-4 Clone 1D11	~680,000	6 ng/mL

Table indicating the cell surface expression of Nectin-4 and the cytotoxicity to enfortumab vedotin.

Bystander Effect Observed with Enfortumab vedotin

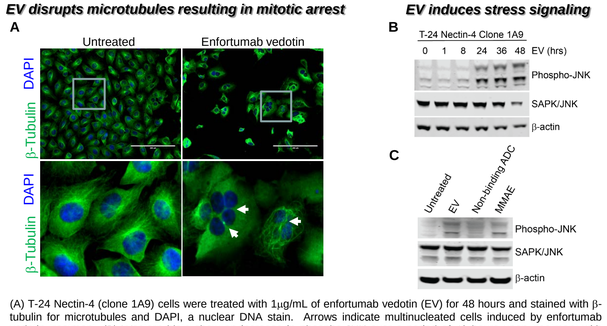
Bystander effect observed with EV kills neighboring Nectin-4 negative tumor cells



(A) The cartoon representing the bystander effect for enfortumab vedotin. Right panel, UM-UC-3 Nectin-4 (clone 1D11) bladder cancer cells were co-cultured with GFP$^+$-Nectin-4$^-$ bladder cells. Q1 (EV killed Nectin-4$^+$ cells); Q2 (bystander effect killed Nectin-4$^-$ cells); Q3 (GFP$^+$, Nectin-4$^-$ live cells); Q4 (GFP$^-$, Nectin-4$^+$ live cells). Admixed GFP positive and negative cancer cells at a 1:1 ratio and treated drug for 72 hours. Cell death was measured by AnnexinV staining between the two populations. (C,D) The percentage of cells in Q3 representing the Nectin-4 negative live cell population was measured after 168 hours of treatment in a 1:1 co-culture with varying concentrations of enfortumab vedotin or non-binding ADC control for the UM-UC-3 and T-24 bladder cancer models. GFP, green fluorescent protein.

Disruption of Microtubules and Induction of ER Stress by Enfortumab vedotin

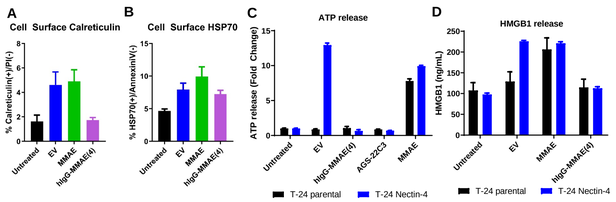
EV disrupts microtubules resulting in mitotic arrest | ***EV induces stress signaling***



(A) T-24 Nectin-4 (clone 1A9) cells were treated with 1μg/mL of enfortumab vedotin (EV) for 48 hours and stained with β-tubulin for microtubules and DAPI, a nuclear DNA stain. Arrows indicate multinucleated cells induced by enfortumab vedotin treatment. (B) Western blots show an increase in phospho-JNK over a period of 48 hours upon treatment with enfortumab vedotin (EV) at 1μg/mL. (C) Phosphorylation of JNK is observed in treatment with enfortumab vedotin and MMAE but absent in untreated or non-binding ADC control. ER, endoplasmic reticulum

Enfortumab vedotin Induces Hallmarks of Early Immunogenic Cell Death

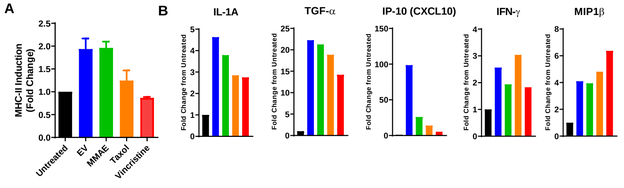
EV induces the translocation or secretion of intracellular components to promote and activate immune cells



(A) The percentage of T-24 Nectin-4 (clone 1A9) cells containing calreticulin on the cell surface (calreticulin+) and propridium iodide negative (PI-) was measured 48 hours post-treatment with enfortumab vedotin (EV, 1μg/mL), MMAE (100nM) and ADC control (hIgG-MMAE (4), 1μg/mL). (B) The percentage of T-24 Nectin-4 (clone 1A9) cells that stained HSP70 on the cell surface and were AnnexinV negative after treatment with drugs for 48 hours. (C) Extracellular release of ATP was measured 48 hours post treatment with EV (1μg/mL), Nectin-4 Ab (AGS-22C3, 1μg/mL), MMAE (100nM), and ADC control (hIgG-MMAE (4), 1μg/mL). (D) Extracellular release of HMGB1 was collected after 48 hours of treatment.

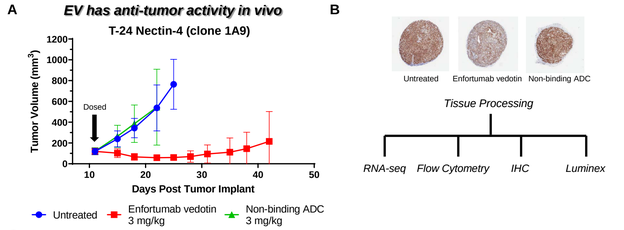
Enfortumab vedotin Treated Tumors Stimulate and Activate Human Macrophages

Cells killed by EV activate macrophages and stimulate the release of cytokines



T-24 Nectin-4 (clone 1A9) cells were treated with drug for 24 hours. Cell debris material was collected and incubated with macrophages from PBMCs. (A) Macrophages were collected and stained for activation markers by flow cytometry such as cell surface expression of MHC-II. (B) Cytokine profiling was performed using Luminex Human Cytokine array and select cytokines showed activation.

Enfortumab vedotin *In vivo* Immunogenic Cell Death

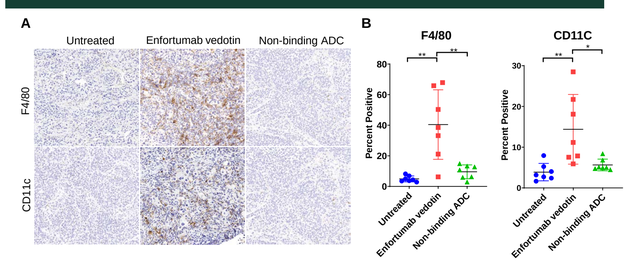


EV induced gene signatures associated with microtubules and ER stress

Change Direction	Signature	*p-value*
Up	Autophagy Immunogenic Cell Death	0.00699
Up	GO Response to Endoplasmic Reticulum Stress	0.0175
Up	GO Intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress	0.0262

(A) The T-24 Nectin-4 (clone 1A9) cells were implanted into nude mice and passaged via trocar, allowed to reach approximately 200mm^3 tumor volume, and subsequently treated with a single IP dose of enfortumab vedotin (3mg/kg) or non-binding ADC (3 mg/kg) with 5 animals per treatment group. (B) Follow-up ICD studies with this model involved collecting tumors 5 days post treatment for downstream analysis by RNA-seq, flow cytometry, immunohistochemistry (IHC), and Luminex. Tumors from each treatment shown were stained for Nectin-4 (C) RNA-seq differential gene expression analysis indicated that EV treated cells produce gene signatures consistent with microtubule disruption, ER stress, and immunogenic cell death. RNA gene signatures from 1267 differentially regulated genes were used to identify signatures that went up or down between the EV treatment vs untreated samples (n=7). The *p*-value is calculated using the Wilcoxon test.

Enfortumab vedotin Treatment Recruits Immune Cells to the Tumor Site

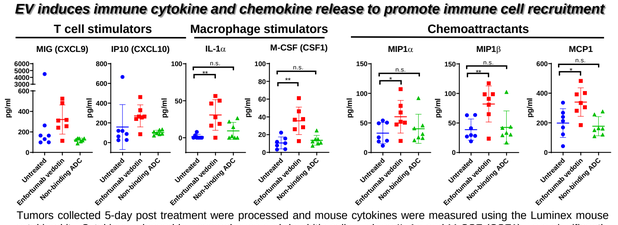


EV demonstrates the potential to turn "cold" tumors "hot" in bladder cancers

Tumors from the T-24 Nectin-4 (clone 1A9) xenograft were collected at Day 5 post treatment and divided for downstream analysis by IHC, flow cytometry, cytokine analysis and RNA-seq. (A) IHC staining of the tumors shows enriched immune cell infiltration by F4/80 and CD11c staining in the Enfortumab vedotin treatment group compared to untreated or non-binding ADC control. (B) Dissociated tumors were stained for immune infiltration by measuring the percentage of CD45 expressing cells and evaluated by flow cytometry. Statistical analysis was performed using an unpaired t test. *p-value;* *** <0.001; ** <0.01; * <0.05.

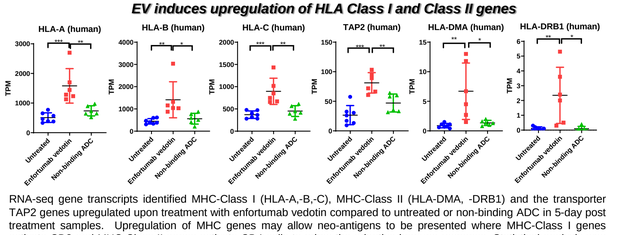
Immune Cell Activation Induced by Enfortumab vedotin

EV induces immune cytokine and chemokine release to promote immune cell recruitment



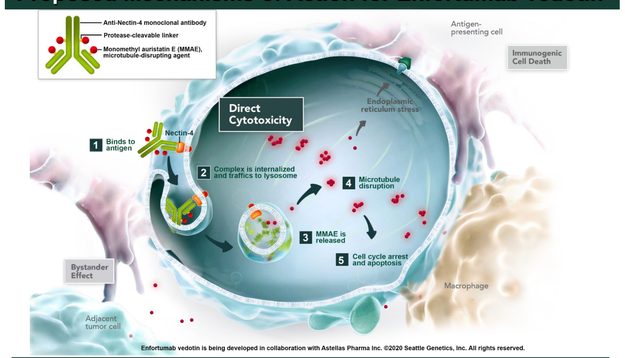
Tumors collected 5-day post treatment were processed and mouse cytokines were measured using the Luminex mouse cytokine kit. Cytokines released by macrophages and dendritic cells such as IL-1α and M-CSF (CSF1) were significantly elevated in the EV treatment group compared to untreated samples. Other cytokines such as T-cell stimulators (MIG & IP10) and chemoattractants (MIP1β & MCP1) were also elevated in this analysis. RNA-seq analysis confirms elevated gene transcripts associated with these cytokines (data not shown). Statistics were determined using a student t-test. *p-value* *** <0.001, ** <0.01, * <0.05, n.s. not significant .

EV induces upregulation of HLA Class I and Class II genes



RNA-seq gene transcripts identified MHC-Class I (HLA-A,-B,-C), MHC-Class II (HLA-DMA, -DRB1) and the transporter TAP2 genes upregulated upon treatment with enfortumab vedotin compared to untreated or non-binding ADC in 5-day post treatment samples. Upregulation of MHC genes may allow neo-antigens to be presented where MHC-Class I genes activate CD8 and MHC-Class II genes activate CD4 cells to prime the adaptive immune response. Statistical analysis was performed using an unpaired t test. *p-value;* *** <0.001; ** <0.01; * <0.05.

Proposed Mechanisms of Action for Enfortumab vedotin



Conclusions & Future Directions

- Beyond targeted auristatin delivery, cell cycle arrest, and apoptosis, the following antitumor mechanisms of action of enfortumab vedotin in urothelial cancer have been demonstrated:
  - The bystander effect activity supports clinical studies in heterogenous Nectin-4 expressing tumors
  - Induction of early hallmarks of immunogenic cell death result in the recruitment and activation of innate immune cells in bladder cancer models
  - Potential to promote immune cell recruitment (turning "cold" tumors "hot") at the tumor site in a mouse bladder xenograft model
  - Increased expression of HLA/MHC-Class I and Class II to activate the adaptive immune response as a potential mechanism for neoantigens display
- Future experiments include demonstrating the anti-tumor activity of the combination of enfortumab vedotin and anti-PD-1 inhibitor and confirming immunogenic cell death and immune cell memory *in vivo* utilizing a vaccination-based approach
- These data provide rationale for the clinical combination of enfortumab vedotin and a PD-1/L1 inhibitor. This combination has previously demonstrated clinical activity in 1L la/mUC (EV-103 dose escalation and cohort A) and is currently being evaluated in a randomized, phase 3 study in 1L la/mUC (EV-302; ClinicalTrials.gov Identifier: NCT04223856)

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