Extracellular proteolytic cleavage of peptide-linked antibody-drug conjugates promotes bystander killing of cancer cells

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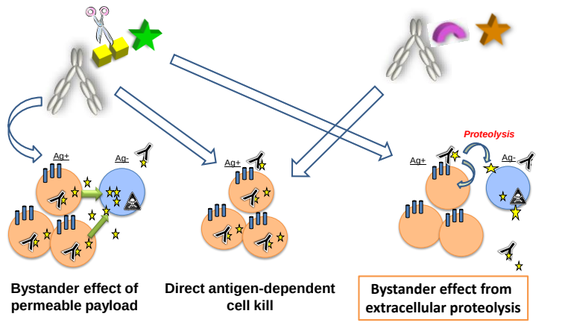
AACR 2014 #4837

Oncology
A Pfizer Research Unit

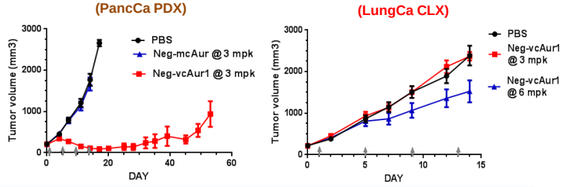
BACKGROUND & ABSTRACT

- Antibody drug conjugates (ADCs) are designed to deliver cytotoxic payloads to tumor cells via binding of antibody to surface antigen followed by internalization and intracellular drug release. Immunoconjugate linkers are typically categorized as non-cleavable or cleavable; a cleavable linker example is Y_mcValCitPABC_X, with antibody Y, a dipeptide sequence with self-immolative PABC spacer, and cytotoxic payload X. This linker is known to be cleaved by endosomal/ lysosomal proteases such as cathepsins, releasing attached payload.
- In addition to intracellular processing of this linker, we report that conditioned media of cultured tumor cell lines is sufficient to promote extracellular cleavage of ADCs with dipeptide-linked payloads. ADCs incubated with conditioned media from cultured tumor cell lines causes cytotoxicity of antigen-negative recipient cells. Conditioned media also promoted cleavage of a dipeptide-based cleavable substrate with fluorescent probe. An ELISA also confirmed the presence of cathepsins in conditioned media.
- In all cases, the magnitude of the response was greatest when donor cells were grown in 3D culture. In contrast, minimal response was observed using conditioned media from other cancer cell lines.
- Complementing these studies, we demonstrated proteolytic activity in the interstitial fluid derived from tumors grown in athymic mice. Fluid extracted from xenograft tumors (cultured cancer cell lines and patient-derived tumors) demonstrated proteolytic activity using a cleavable-fluorescent linker-probe in a plate assay.

Hypothesis: The effect of some cleavable linker-payloads may be due to extracellular proteolysis



Observation: Some tumor xenografts in mice are inhibited with ADCs containing non-targeting IgG and cleavable-linked payloads

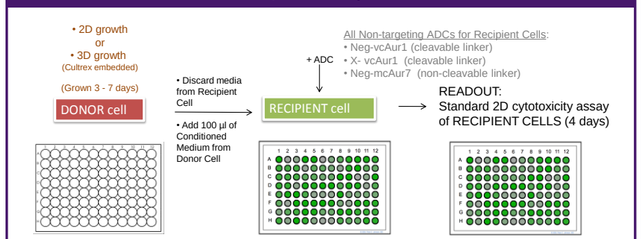


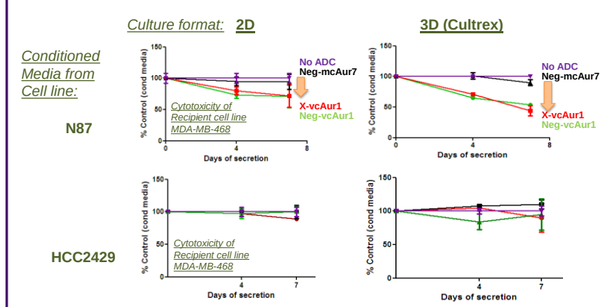
Relative magnitude of effect of non-targeting vcADC:	++	-/+

Left panel: Pancreatic patient derived tumor fragment (PA0165) was implanted subcutaneously into athymic mice. At ~200 mg staging, mice were treated q4d x 4 with PBS vehicle (*black line*), 3 mg/kg of Neg-vcAur1 (non-targeting antibody with cleavable-linked auristatin, *red line*), or 3 mg/kg Neg-mcAur (non-targeting antibody with non-cleavable-linked auristatin, *blue line*).

Right panel: Lung cancer cell line (HCC2429) was implanted subcutaneously into athymic mice. At ~200 mg staging, mice were treated q4d x 4 with PBS vehicle (*black line*), 3 mg/kg (*red line*) or 6 mpk (*blue line*) of Neg-vcAur1 (non-targeting antibody with cleavable-linked auristatin). *All animal experiments were reviewed and approved by Pfizer IACUC.*

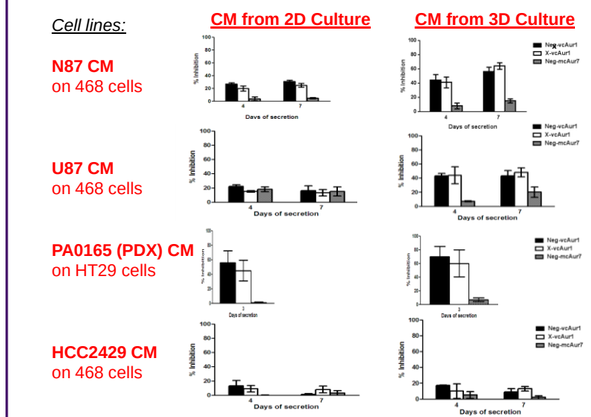
Non-targeting ADC with cleavable-linker, in presence of conditioned media from donor cell line N87, inhibits recipient cells





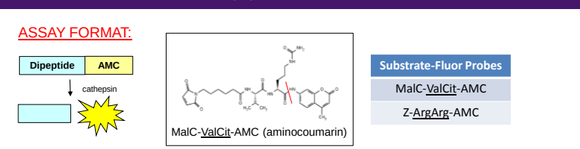
Conditioned media from donor N87 or HCC2429 cells is collected on day 4 and 7, then incubated with 100 nM of the indicated non-targeting ADCs in the presence of recipient MDA-MB-468 cells for 4 days, followed by cytotoxicity assessment of recipient cell by MTS assay. Cytotoxicity of recipient cells up to 50% is observed in presence of vcAur1 ADCs incubated with media from N87 cells grown in embedded 3D culture, suggesting cleavage of ValCitPABC by released N87 proteases. Data are normalized to individual conditioned media with no ADC. In contrast, there is no apparent cleavage of ValCitPABC or release of free Aur1 when using HCC2429 cell conditioned media (grown 2D or 3D, *lower panels*)

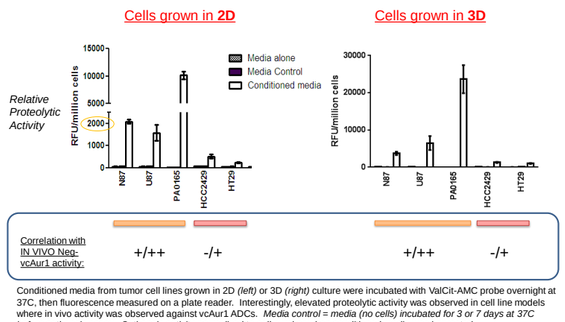
Increased bystander cell kill using conditioned media (CM) from certain tumor cells in presence of mcValCitPABC-Aur1 ADCs. Effect is amplified using CM from 3D culture.



Conditioned media from the indicated cell line collected after 3, 4, or 7 days. All ADCs are non-targeting for the recipient cells, and are dosed at 100 nM payload concentration. Cytotoxicity assays of recipient cells (468 and HT29) were conducted for 4 days with MTS endpoint. Data are the mean ± SD of 3 – 5 independent experiments.

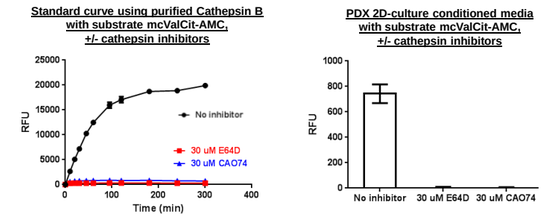
Conditioned media shows proteolytic activity in plate-based assay with dipeptide-AMC substrate





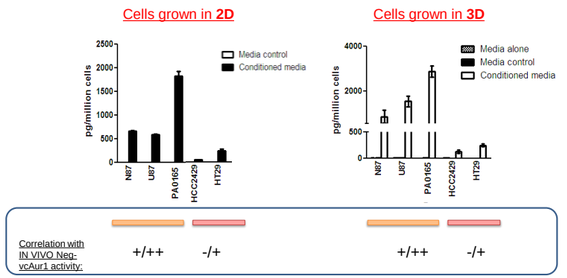
Conditioned media from tumor cell lines grown in 2D *(left)* or 3D *(right)* culture were incubated with ValCit-AMC probe overnight at 37C, then fluorescence measured on a plate reader. Interestingly, elevated proteolytic activity was observed in cell line models where in vivo activity was observed against vcAur1 ADCs. *Media control = media (no cells) incubated for 3 or 7 days at 37C before cathepsin assay. Cathepsin activity normalized to cell number when conditioned media was harvested.*

Cathepsin inhibitors attenuate proteolytic activity of cell line conditioned media



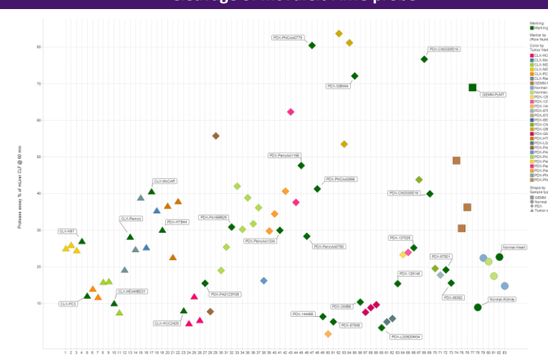
Left panel: Purified cathepsin B was incubated with vcAMC probe +/- 30 uM cathepsin inhibitors E64D or CA074, demonstrating complete inhibition of proteolysis in plate-based assay. *Right panel:* Proteolytic activity in conditioned media from pancreatic PDX PA0165 grown in 2D is also inhibited by cathepsin inhibitors (30 hr proteolysis timepoint). *Assay buffer: 60 uM mcValCit-AMC in 50 mM sodium acetate, pH 5.0, 2 mM DTT.*

Cathepsin ELISA detects Cathepsin B protein in conditioned media



Conditioned media from tumor cell lines grown in 2D *(left)* or 3D *(right)* culture were tested in an ELISA specific for human cathepsin B. *Media control = media without cells incubated for 3 or 7 days at 37C before ELISA. Cathepsin levels normalized to cell number.*

Protease activity in Interstitial Fluid: Cleavage of mcValCit-AMC probe



Interstitial fluid collected from the indicated PDX or cell-line xenografts or normal tissue (500 ug/ml total protein) was evaluated in the proteolysis assay with ValCit-AMC probe. Data are represented as percent signal compared with equivalent total protein from a mouse liver lysosomal preparation. Color by replicate samples to view inter-animal variation.

Several cell lines exhibit extracellular proteolysis, enhanced in 3D culture, which correlates with in vivo profile

Cell line	Bystander Cytotox (2D CM)	Bystander Cytotox (3D CM)	vc-Probe proteolysis (3D CM)	Cathepsin B ELISA (3D CM)	In vivo efficacy observed with Neg-vcAur1 ADC
N87 (GastricCa CLX)	+	++	+	++	+
U87 (Glio CLX)	-	++	+	++	+
PA0165 (Panc PDX)	++	++	++	++	++
HCC2429 (LuCa CLX)	-	-/+	-	-	-/+
HT29 (CoCa CLX)	-	-/+	-	-	-

CONCLUSIONS

- Different levels of proteolytic activity were detected in the conditioned media of cultured cancer cell lines, assessed by cytotoxicity studies, proteolysis assays with ValCit-containing fluorescent substrate, and by cathepsin ELISA. These effects were enhanced when donor cells were grown in 3D cultures.
- Proteolytic activity was detected in the interstitial fluid from cancer cell line xenografts and patient-derived xenografts implanted in athymic mice.
- These data are consistent with the reported secretion of cathepsins by cancer cells, and we now show that these proteases may mediate extracellular release of cytotoxic payloads from ADCs containing peptide-based cleavable linkers.
- Efficacy associated with non-targeting ADCs is sometimes attributed to pinocytosis and other non-specific uptake mechanisms; these extracellular proteolysis data suggest an alternative explanation for biological activity observed with non-targeting ADCs.
- Released permeable payload from extracellular cleavage of ADCs may promote the killing of proximal antigen-negative cancer cells in a heterogeneous tumor mass, providing a beneficial debulking effect.

ACKNOWLEDGEMENTS

We gratefully acknowledge the contributions from our colleagues in the ORU, WWMC, GBT, PDM, and DSRD.