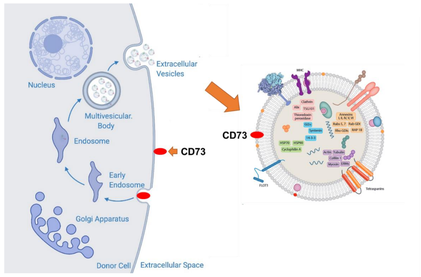
A CD73 enzyme activity assay for stability and dose-finding of therapeutically active EVs and CDVs

D. Auer [1], T. Schally [1], C. Arzt [1,2], H. Fang [1,2], E. Grasmann [1,2], C. Kals[1], E. Bayer [1], E. Rohde [1,2,3], H.-C. Lau [4], S.W. Oh [4], & M. Gimona [1,2,5]

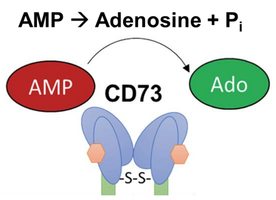
[1] GMP Unit, Spinal Cord Injury and Tissue Regeneration Center Salzburg (SCI-TReCS), Paracelsus Medical University Salzburg, AUSTRIA
[2] Extracellular Vesicles-Theralytic Technology (EV-TT) Transfer Center , Paracelsus Medical University Salzburg, AUSTRIA
[3] University Institute for Transfusion Medicine, Paracelsus Medical University & Salzburger Landeskliniken GmbH, Salzburg, AUSTRIA
[4] BioDrone Research Institute, MDimune Inc. #602 KOLON Digital Tower-III, 49 Achasan-ro, Seongdong-gu, Seoul 04790, KOREA
[5] Research Program "Nanovesicular Therapies", Paracelsus Medical University Salzburg, AUSTRIA

BACKGROUND

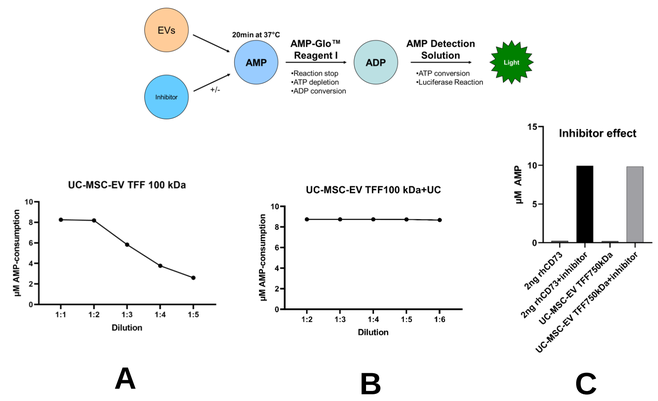
The translation of nanovesicle-based therapeutics into clinical application requires the definition and quantitative, reproducible analysis of criteria of bioactivity and stability. For naive unmanipulated extracellular vesicles (EVs) or cell-derived vesicles (CDVs) generated by serial extrusion of cells the definition of the active substance(s) remains difficult and the intrinsic heterogeneity of nanovesicle preparations prevents dose determination that is solely based on particle number or total protein mass. The GPI-anchored 5'-ectonucleotidase CD73 is an established identity marker for multipotent (mesenchymal) stromal cells (MSCs) and is abundantly present on umbilical cord MSC-derived EVs and CDVs. The exposed position at the vesicle membrane and the delicate nature of the enzymatic activity position CD73 as a suitable surrogate molecule to establish a biochemical assay that meets the above criteria and that may assist in the control of storage stability and dose definition.



1 Extracellular Vesicle Biogenesis
UC-MSCs expose CD73 on their cell surface (red dot) and UC-MSC-derived EVs as well as extruded CDVs share the same characteristic.[1]



2 Basic principle of CD73 function
CD73 hydrolyzes AMP to adenosine and inorganic phosphate.[2]



3 CD73 activity assay principle
Umbilical cord-derived MSCs were expanded in human platelet lysate (HPL; Helios Pure GI GMP grade from Aventacell).
EVs were harvested from conditioned growth medium containing EV-depleted HPL by Tangential Flow Filtration using a 750 kDa cutoff.

CDVs were extruded from expanded and freshly harvested UC-MSCs by serial extrusion through 10µm, 3µm and 0.4 µm and purified by Tangential Flow Filtration using a 750 kDa cutoff.
CD73 enzymatic activity of UC-MSC-EV and CDV preparations was determined by incubating 10µL of EVs in assay buffer (10mM HEPES, 2mM MgCl2) with 10µM AMP for 20min at 37°C. After completing the enzymatic reaction the level of AMP was detected with the AMP-Glo™ Assay Kit (Promega). Addition of the AMP-Glo™ Reagent I terminates the reaction, removes any ATP, and converts AMP to ADP. Addition of AMP Detection Solution drives the conversion of ADP to ATP and the detection of ATP through the luciferase reaction. The AMP concentration is proportional to the emitted light and can be extrapolated using a standard curve.

2ng rhCD73 was used as positive control and 10µM AMP-CP as CD73 inhibitor. The µM AMP consumption was calculated by the difference Δ(EVs+AMP+Inhibitor) - (EVs+AMP). With the Δ calculations from the inhibitor, it is possible to accurately calculate the consumption and ensure reproducibility. Dilutions were performed (A&B) for more precise calculation of the AMP consumption/particle (C).

RESULTS

Reproducibility and stability of the assay system was confirmed over a range of buffers (including phosphate-containing systems) and the sensitivity was suitable for both naturally secreted EVs and extruded CDVs. The determined activity/particle and correlates with CD73 abundance (Figure R1). We reproducibly obtained values of 2-4 x 10^{-8} µM Adenosine production / particle. Enzymatic activity was abolished by incubation above 65°C in both EV or CDV preparations while EV aggregation or loss of particles was not observed by this treatment (Figure R2). The measured enzymatic activity in correlation to the particle number was independent of the total protein concentration of the preparation (Figure R3).

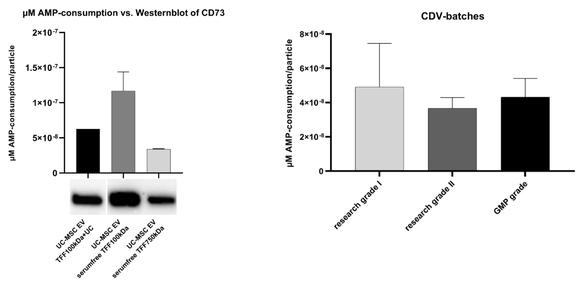


Fig. R1
Enzymatic activity/particle in nanovesicle preparations of in UC-MSC-EV and UC-MSC-CDVs
Both EVs and CDVs exhibit enzymatic activity in the assay.

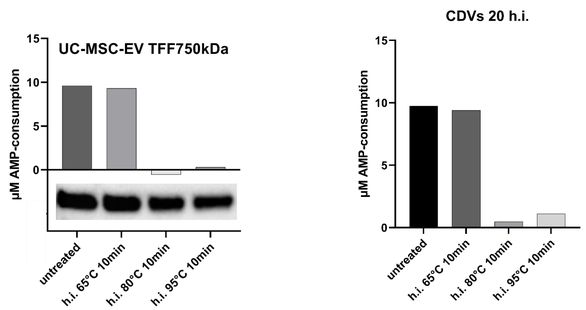


Fig. R2
Effect of heat inactivation on CD73-activity in UC-MSC-EV and UC-MSC-CDVs
During heat inactivation the particle number and protein concentration remained constant, but the activity was abolished after 10 minutes at in 80°C. A corresponding Western blot for CD73 (8x10^8 particle/sample) shows only minor reduction in protein content.

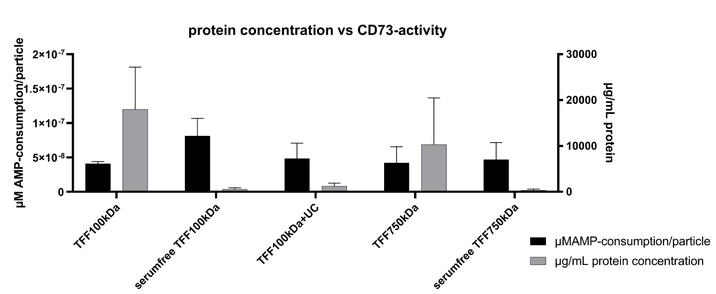
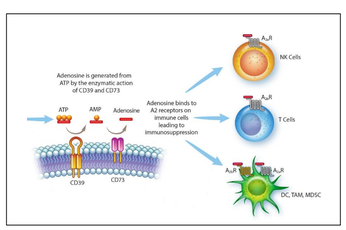


Fig. R3
Effect of protein concentration
The activity measurement and the enzymatic activity itself is not affected by the total protein concentration in a given sample. In addition an elevated protein concentration does not lead to an increased CD73 activity.



Potential Mode of Action of UC-MCS-derived nanovesicles: Immunomodulation and trophic support via Adenosine signaling

Nucleotides can behave as trophic, differentiating, and immunomodulatory molecules in many physiological and pathological events through autocrine and paracrine mechanisms. Purinergic ligands are thus potent candidates to mediate cellular crosstalk and to promote cell growth and survival, regulate inflammation and contribute to local tissue homeostasis and repair.

CONCLUSION

The biological activity of a GPI-anchored enzyme is sensitive to denaturing conditions and could indicate the deterioration of a given $CD73^+$ nanovesicle preparation (EVs or CDVs) during the storage process. Excipients and co-purifying protein components in the preparations do not seem to affect the activity and thus allows this assay to be used for a wide range of buffers and purification strategies.

Supported by the Projects P1812596 "EV-TT" and 20102-F1900731-KZP "EV-TT-Bpro".

[1] Modified from: Jurgielewicz, Stice and Yao (2021) Therapeutic Potential of Nucleic Acids when Combined with Extracellular Vesicles Aging and Disease 2021 Sep 1;12(6):1476-1493 and Kourembanas S. (2014) Exosomes: Vehicles of Intercellular Signaling, Biomarkers, and Vectors of Cell Therapy. Annu Rev Physiol
[2] Modified from :https://journals.physiology.org/doi/full/10.1152/ajpcell.00285.2019