IDENTIFICATION OF EXOSOME MODULATORS IN 3D BREAST CANCER MODELS USING EXOSCREEN AND CELL PAINTING TECHNOLOGIES

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INTRODUCTION

Three-dimensional (3D) spheroid cell culture models recapitulate the tumor micro-environment[1] better than their adherent two-dimensional (2D) counterparts[2]. Spheroid models represent a valuable breast cancer research tool, allowing optimized drug selection and improved tumor distribution; furthermore, 3D breast cancer models (mammospheres) will reduce the number of animals employed and drug screening costs[3-5]. Of note, the molecular complexity of breast cancer, especially when targeting metastasis, will require combinatorial drug treatments[6,7].

Exosomes - extracellular vesicles (EVs) that play essential roles as in intercellular communicators[8] - help form the pre-metastatic niche[9] and support drug resistance[10]. EV markers include tetraspanins[11]; however, current methodologies to purify exosomes remain time-consuming and challenging to translate to clinical practice. We recently optimized a quick and reliable high-throughput screening (HTS) methodology[12] to identify exosome modulators that combines external signals measured by ExoScreen technology (a sensitive assay that measures protein-protein interactions) and internal exosomal markers in 2D models[13]. Our study employed MCF7 cells (Luminal A Breast Cancer subtype).

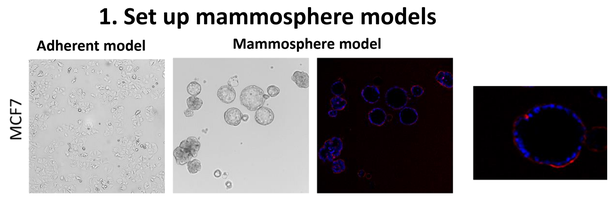
AIMS

1. Study the effect of polymer-drug conjugates (single drugs and combinations) on exosome release via the ExoScreen assay.
2. Combine ExoScreen and Cell Painting[14] assays to discover to elucidate the molecular mechanisms of action of polymer-drug conjugates as exosome modulators.

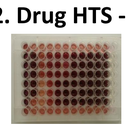
METHODS

MCF7 mammospheres were cultured with EGF2/B27 using low adherence plates and free or polymer-conjugated drugs were administered for 72 h. Supernatants were collected and analyzed by ExoScreen assay to quantify the number of released exosomes using anti-CD9 acceptor beads and a biotinylated-anti-CD63 antibody. Cell Painting strategies identified the modulation of various intracellular targets: (i) mitochondria (MitoTracker™ Red), plasmatic membrane (CellMask™ Green Plasma Membrane), and nucleus (Hoechst) in live cells, and (ii) endoplasmic reticulum [ER] (Concanavalin A, Alexa Fluor™ 488), intraluminal/extracellular vesicles (CD63-APC), and nucleus in fixed cells. IMARIS Demo Software was used to analyze obtained images. Cell viability by MTS assay was used to normalize the ExoScreen signal.

1. Set up mammosphere models

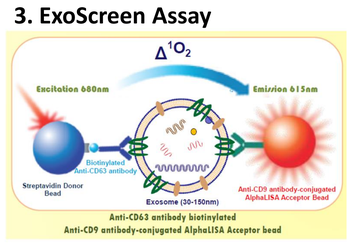


2. Drug HTS - MTS cytotoxicity assay 72h

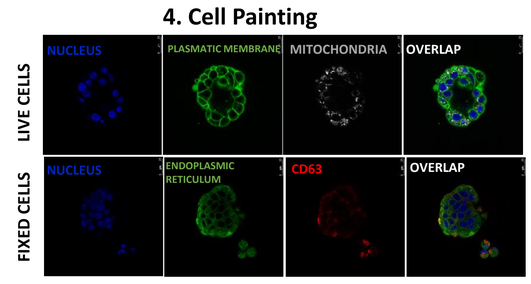


CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS)

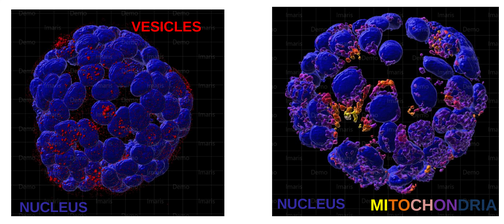
3. ExoScreen Assay



4. Cell Painting



5. Imaging Analysis - IMARIS Demo Software



Size/Number of Vesicles | Shortest Distance to Nuclei

RESULTS

1. Rationally designed polymer-based combination drug conjugates (Fig. 1). After validating identified drug combinations in patient-derived organoids, we synthesized and fully characterized rationally-designed polymer-based combination conjugates[15] to maintain drug synergistic activity against the primary tumor and metastasis. To study metastasis, we checked the ability of our conjugates to inhibit exosome release and regulate selected intracellular targets to help to elucidate mechanisms of action.

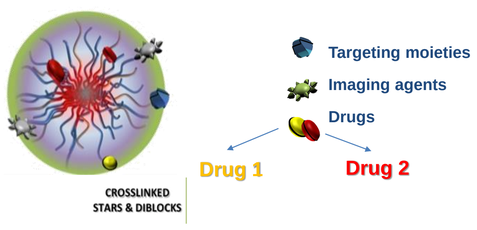


Fig. 1. Schematic representation of a rationally-designed polymer-based combination conjugate with selected drug combinations.

2. Evaluation of exosome modulation by ExoScreen assay (Fig. 2). We evaluated exosome modulation ($CD9^+CD63^+$) for two different anti-cancer drugs (1 and 2) as free drugs or polymer-based single/combination conjugates. We also included an exosome inhibitor as a positive control. Drug 1 inhibited while Drug 2 increased the release/biogenesis of exosomes as free drugs or conjugates.

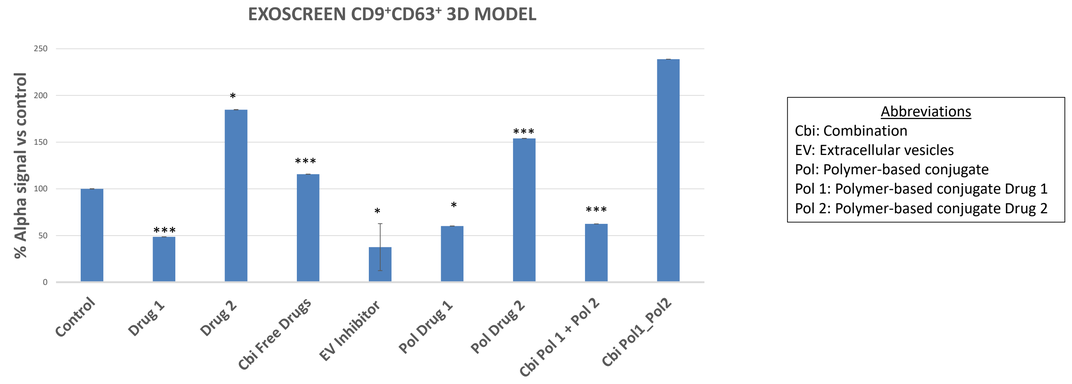


Fig 2. ExoScreen Assay in MCF7 mammospheres. Supernatants were analyzed by AlphaLISA beads (Perkin Elmer) using anti-CD9 antibody-conjugated acceptor beads and an anti-CD63 antibody biotinylated.

3. Evaluation of organelle modulation by imaging analysis (Fig. 3). (Preliminary data) **A) Cell Vesicle Size Distribution** - Most treatments increase the percentage of larger EVs (0.3-0.4 μm, as measured by CD63 marker, intracellularly). Free Drug 2 and polymer-based conjugate Drug 2 have a similar pronounced effect. **B) Mitochondrial organization** - Free Drug 1 and polymer-based conjugate Drug 2 reduce the distance of the mitochondria to the nucleus (Free Drug 2 excluded from analysis due to problems in staining). **C) ER modulation** – Drug 2, in all its forms, prompt a marked increase in signal intensity for the ER marker (green) according to its distance from the nucleus.

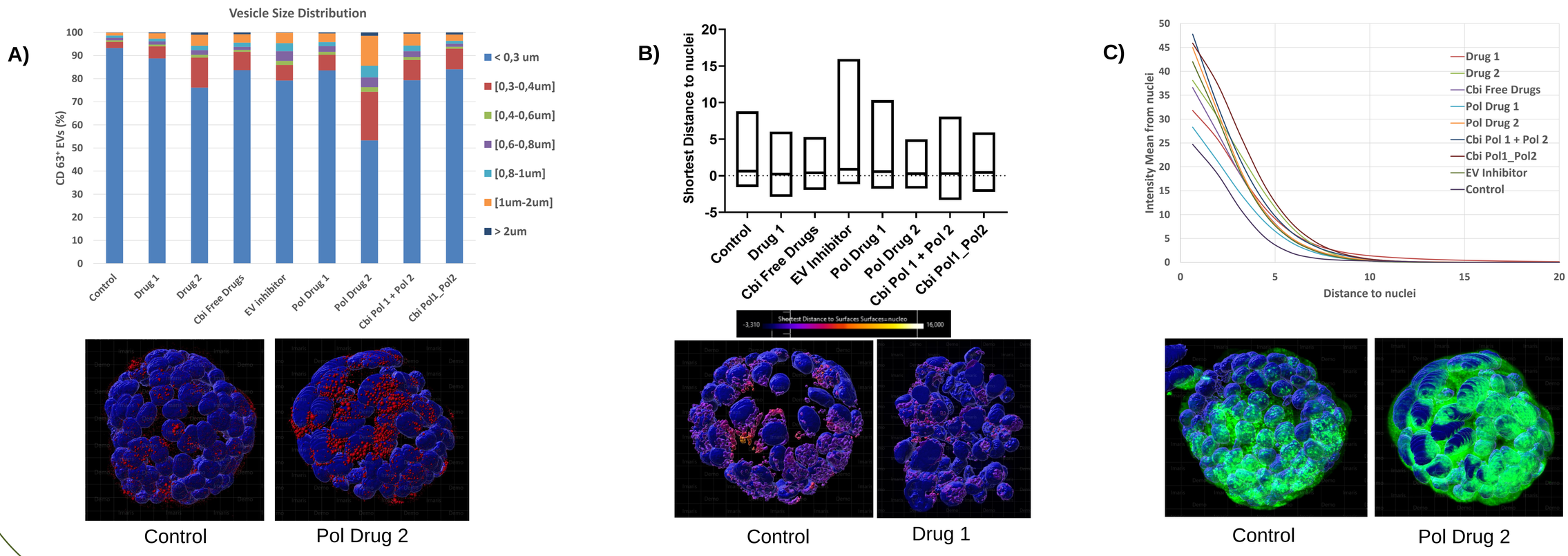


Fig. 3. Imaris representation of nuclei and different organelles. Surfaces were created with "surfaces" tool for nuclei and mitochondria and with "spots" for EVs using intensity-based methods. Size structure was set at 1.2 μm for mitochondria, and 8 μm for nuclei. Spots were created at 0.3 μm for EVs.

CONCLUSIONS

- We established 3D breast cancer mammospheres as an improved approach to developing anti-tumor therapeutics.
- Studies of EVs suggest that free and conjugated forms of Drug 1 and Drug 2 function similarly in 2D[13] and 3D culture conditions - Drug 1 acts as an exosome biogenesis/release inhibitor and Drug 2 increases the levels of extracellular exosomes. Preliminary data provided by Cell Painting suggests that released exosomes remain trapped within mammospheres after administration of Drug 1; meanwhile, Drug 2 administration increases exosomes biogenesis, leading to an increase in intra- and extra-cellular levels.

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