Aim 1: Development and Characterization of Exosome-based Therapeutic for PARPi and CSF-1R Inhibitors.
Sub-aims:
1.1. Generate exosomes from iPSC-MSCs and load them with PARPi cargo.
1.2. Conjugate the surface of the exosomes with CSF-1R inhibitors and markers specific to TAMs (CD68, CD163) and cancer cells (MM2, EpCam, HER2, CA125).

Based on the research findings highlighted by La Greca et al. [1], the proteomic composition of extracellular vesicles (EVs) derived from induced pluripotent stem cells (iPSCs), iPSC-derived mesenchymal stem cells (iPSC-MSCs), and conventional mesenchymal stem cells (MSCs) varies significantly. This suggests a nuanced evolution of protein content as iPSCs transition into iPSC-MSCs, with the resulting EVs exhibiting a distinct proteomic signature that is more specific and likely reflects their specific functions within the stem cell microenvironment. This includes roles in supporting stem cell maintenance, facilitating differentiation, and mediating intercellular communication within tissues.

Given these insights, our approach will involve harnessing iPSC-MSCs to generate EVs for therapeutic purposes. To meet the demands for high yield and potency necessary for clinical applications, we will employ innovative culture strategies, particularly bioreactors, which offer continuous culture capabilities and enable real-time monitoring of crucial parameters such as oxygen levels and pH. Recent work by Cao et al. [2] has demonstrated that EVs derived from 2D cultures and hollow fiber bioreactor (HFB)-cultured MSCs exhibit comparable surface marker profiles, size, and morphology, with the latter yielding up to a 19.4-fold increase in production.

For the culture of EVs, our methodology will involve utilizing a bioreactor system with a 48-hour harvest interval supplemented with human platelet lysate (HPL) as a culture medium. HPL not only supports xeno-free MSC culture, aligning with clinical trial requirements, but also enhances translational potential. It's worth noting that while HPL contains exogenous serum derived EVs along with other nanoparticles such as growth factors and protein aggregates, it still represents a superior serum alternative within this context.
After the EVs have been released into the culture medium, for EV isolation, we will use a microfluidic system which can isolate exosomes with high purity, minimizing contamination from other extracellular vesicles or protein aggregates. The process is more efficient and requires less time than ultracentrifugation techniques (gold standard), it can be scaled up and the same system can be used for exosome modifications.
Using Western Blot, Elisa, or Sem analysis, we will then proceed to the characterization of the MSCs assessing the presence of protein markers, including CD9, CD63, CD81, CD59, as well as cytosolic proteins such as ALIX, TSG101, and Hsp70/90 [3] (Fig. 1 and 2).

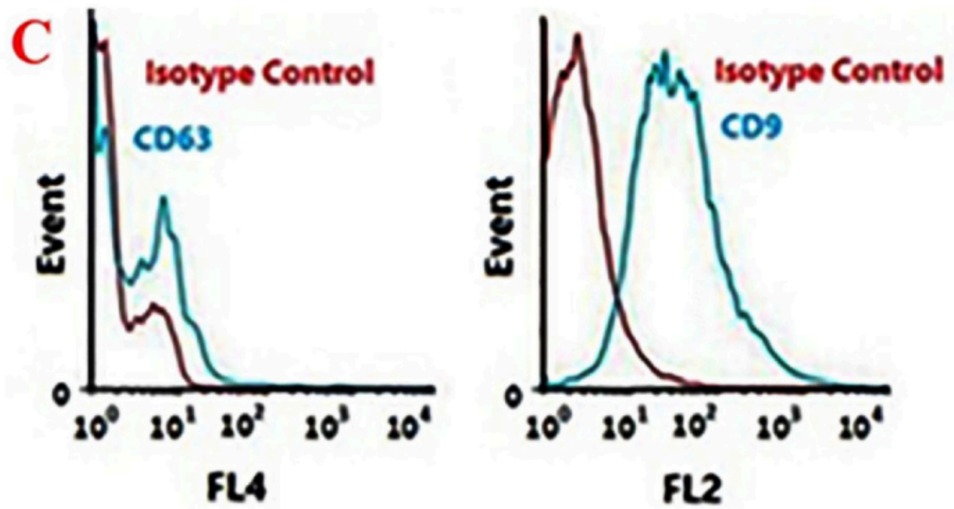


Figure 1. Flow cytometry analysis of the positive marker at the surface of the exosomes – Ref: [4]

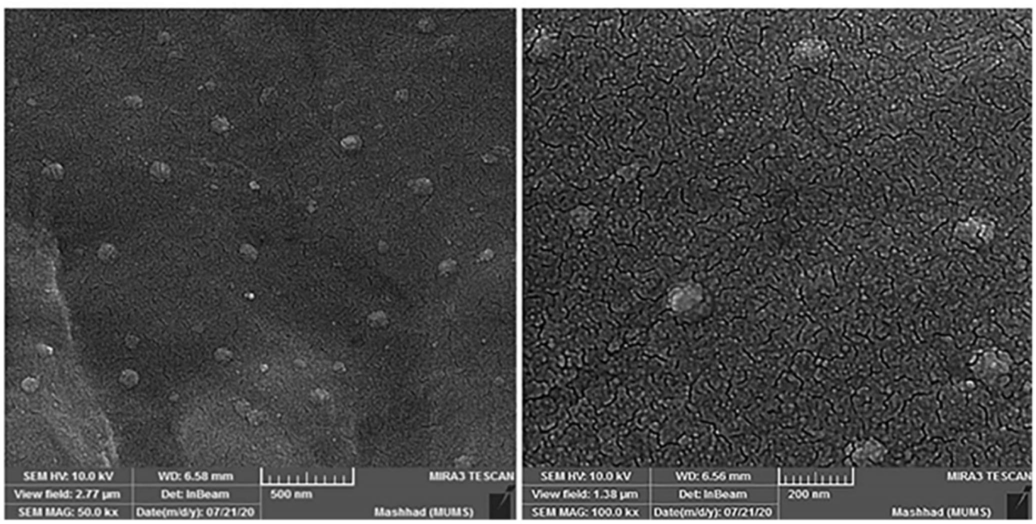


Figure 2. SEM image of exosome with 500 and 200 nm scale bars – Ref: [4]

To enhance the specificity of iPSC-MSC-derived exosomes for targeting tumor-associated macrophages (TAMs) and cancer cells, we must delicately balance the need for efficient TAM targeting with the imperative to evade uptake by macrophages and leukocytes in the Mononuclear Phagocyte System (MPS) organs. This optimization is critical to ensure that the engineered exosomes maintain sufficient circulation time to effectively reach and target tumors.
Given the limitations associated with PEGylation, we are exploring alternative strategies such as "Self" peptide conjugation. This approach involves modifying the exosome surface with self-peptides that mimic endogenous proteins, potentially reducing recognition by MPS cells. A study by Pial et al. [5], demonstrated an inverse correlation between nanobead uptake by the immune system and in vivo persistence. The authors

demonstrated that "self" CD47 nanobeads had longer bloodstream circulation and likewise, our engineered exosomes, designed to minimize immune cell uptake, will be more likely to evade the MPS and reach their target tissue (Fig. 3 and 4).

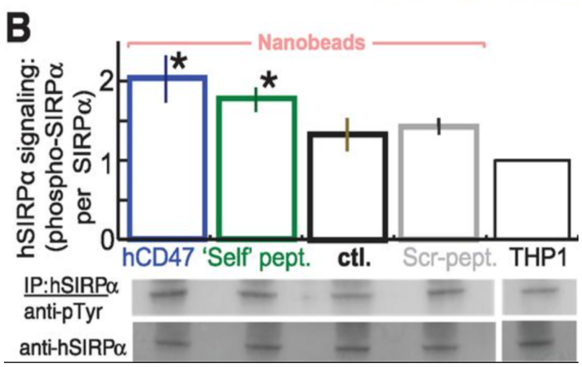


Figure 3 - Binding of hCD47 and Self peptide increases phosphor-SIRPα. Ref: [5]

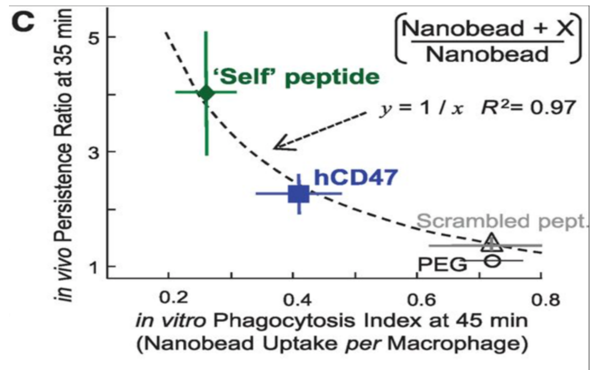


Figure 4 - Inverse correlation between in vivo persistence ratio and in vitro inhibition of phagocytosis by hCD47 and Self peptide at 45 min – Ref: [5]

The next steps in our research will involve:

- **Combining CD47 expression:** and incorporating TAM-specific ligands, such as antibodies against TAM markers (e.g., CD68, CD206, CD163), on the exosome surface along with CD47 expression. This dual targeting approach enables selective TAM targeting despite CD-47-mediated immune evasion.
- **Conjugating the exosome surface with CSF-1R inhibitors** to enrich the M1 population of TAMs and modulate TAM recruitment and distribution However, the largest macrophage polarization effects have been observed for agonists of the toll-like receptors 7 and 8 (TLR7/8) [6], and we may want instead to investigate these therapeutics. These small molecules can be modified for conjugation with exosomes, enhancing their antitumor efficacy.
- **Fine-tuning CD47 Expression Levels**: on exosomes to balance immune evasion with TAM targeting. This optimization can enhance exosome biodistribution and maximize TAM targeting.
- **Integrating a pH-Sensitive components:**
  Like in the design of a micelle for drug delivery by Pia et al. [7], we will modify the PARPi cargo to include a functional group compatible with click chemistry; and attach it via a peptide linker to matrix metalloproteinase-2 (MMP-2), a protein only found in tumors. This will allow selective release of the PARPi cargo near the cancer cells.

Aim 2: In Vitro Evaluation of Therapeutic Efficacy and Specificity
Sub-aims:
2.1. Assess the cytotoxic effects of the engineered exosomes on a panel of cancer cell lines in vitro.

We will prime a variety of human cancer cell lines, including A549 lung cancer, SK-OV-3 ovarian cancer, and MDA-hyb1 breast cancer cells, to our engineered exosomes treated with sub-lethal doses PARPi for 24 hours. Then, using LC-MS/MS, we will quantify the amount of PARPi delivered to the cancer cells by the MSC-derived exosomes. This measurement, as illustrated in previous research by Merlzer et al [8] (Fig. 5), will help to compare the exosome uptake by the cancer cells with control groups, such as untreated cancer cells, or cancer cells treated with free PARPi. Furthermore, it will aid in the evaluation of the specificity and efficacy of the engineered exosomes in delivering PARPi to the cancer cells.

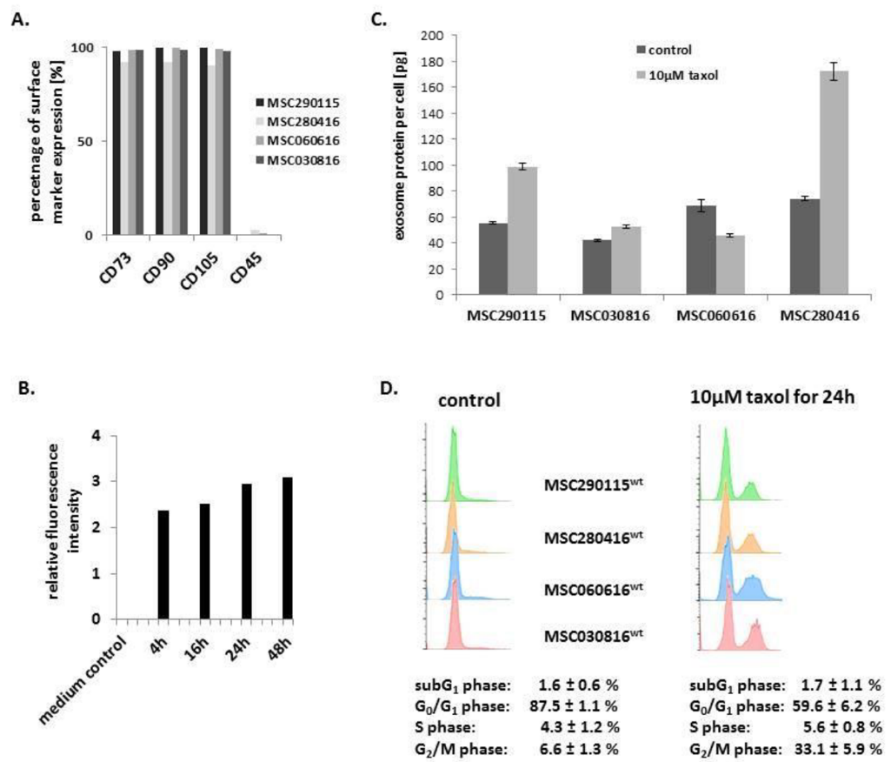


Figure 5 – Ref: [8]

A) Characterization of the four different MSC investigated populations.

B) Kinetic of exosomes production by MSC^{GFP},
progressive exosome release increase to reach a plateau after 24h.

C) Quantification of exosome produced per cell within a specific timeframe.

D) Apoptotic/necroptotic subG1 phase cells remained at equally low levels in control and Drug-treated MSC populations, confirming no detectable cytotoxic effects.

The cytotoxic effects will be assessed using a fluoroscan assay, where the reduction in florescence will indicate cell death in the drug-loaded cancer cells. We will analyze cell growth and viability and compare the results with those of our control cells (Fig. 6 and 7).

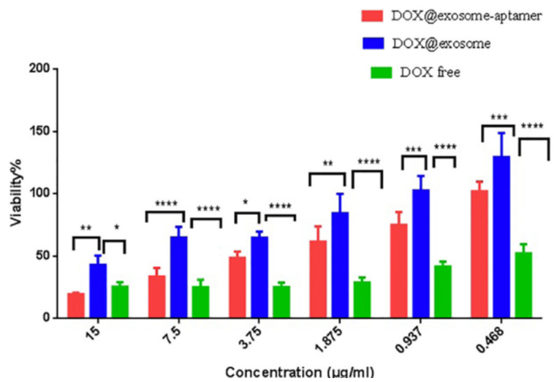


Figure 6 – Ref: [4] - Cellular toxicity assessment.

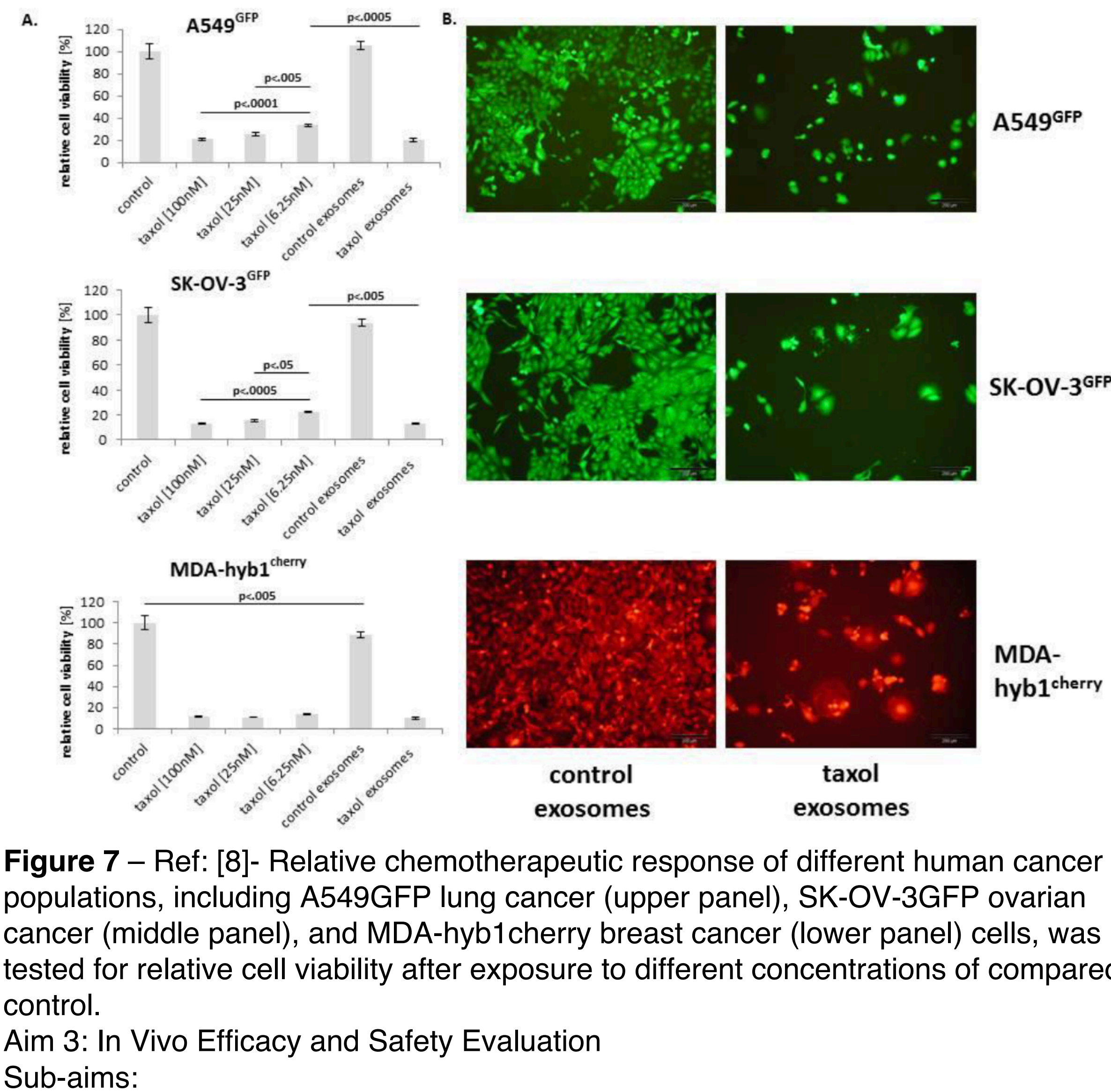


Figure 7 – Ref: [8]- Relative chemotherapeutic response of different human cancer cell populations, including A549GFP lung cancer (upper panel), SK-OV-3GFP ovarian cancer (middle panel), and MDA-hyb1cherry breast cancer (lower panel) cells, was tested for relative cell viability after exposure to different concentrations of compared control.

Aim 3: In Vivo Efficacy and Safety Evaluation

Sub-aims:

3.1. Evaluate the recruitment and activation of immune cells by treated cancer cells, focusing on CD4+ and CD8+ T cells and the impact on TAMs.
3.2. Conduct preclinical trials using relevant animal models to assess the therapeutic efficacy of the exosome-based delivery system.
3.3. Evaluate the safety profile and potential off-target effects of the treatment in animal models.

Based on study from [9], hematolymphoid humanized mouse models are the most promising animal models to test the antitumor effects of checkpoints blockers and immunotherapy strategies. If needed to align our therapeutic design with the unique characteristics of a cancer tissue, we may opt for different murine models, such as a GEMM, including the KPC (KrasLSL-G12D/+; Trp53R172H/+; Pdx-1-Cre) model for pancreatic cancer or the APC (adenomatous polyposis coli) model for colorectal cancer. Each mouse will be treated with either only PARPi substance or MSC-derived exosomes (control or drug-treated MSC). After a month, animals will be sacrificed, and their tumors will be dissected to measure their volume before and after treatment. Furthermore, metastases to distant organs such as lung, liver, spleen, and kidney, will be monitored with the expectations of observing reduced metastasis with the administration of PARPi-loaded exosomes with dosage lower than free PARPi injection to the animals.CD73 can serve as a marker for identifying cancer cells that have spread from the primary tumor to distant organs. Its presence, alongside MCherry transcripts, in PCR analysis can indicate the presence of metastatic cancer cells, providing insights on metastasis progression and drug efficiency; metastasis must be reduced with drug-loaded-MSC-engineered (Fig. 8,9 and 10).

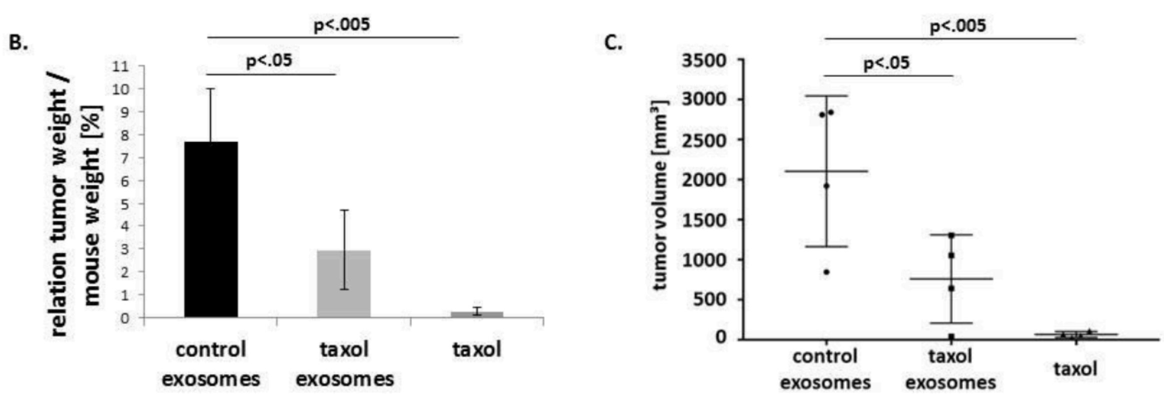
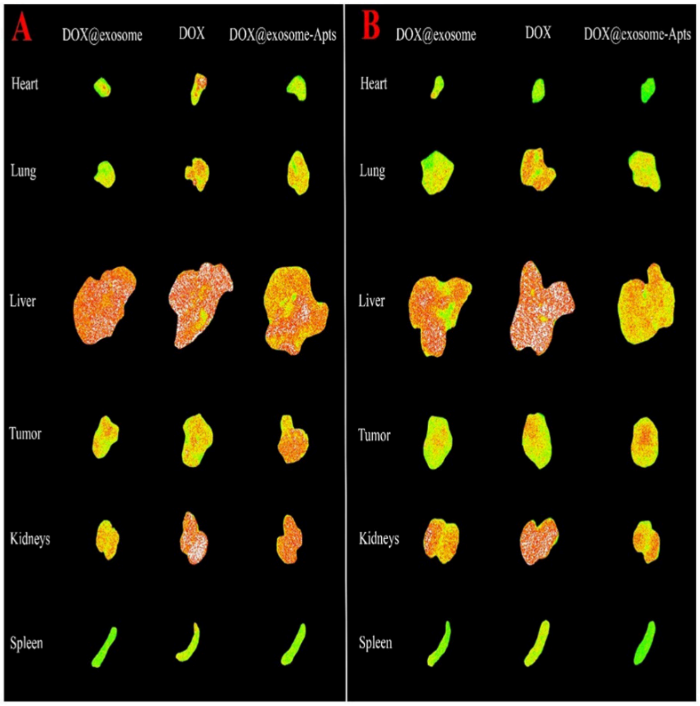


Figure 8 – Ref [8]:
On the left: comparison of ratio of tumor weight to mouse weight between control exosomes, drug exosome-treated tumors and drug-treated tumors- On the right: average tumor volume comparison between the three treatments.



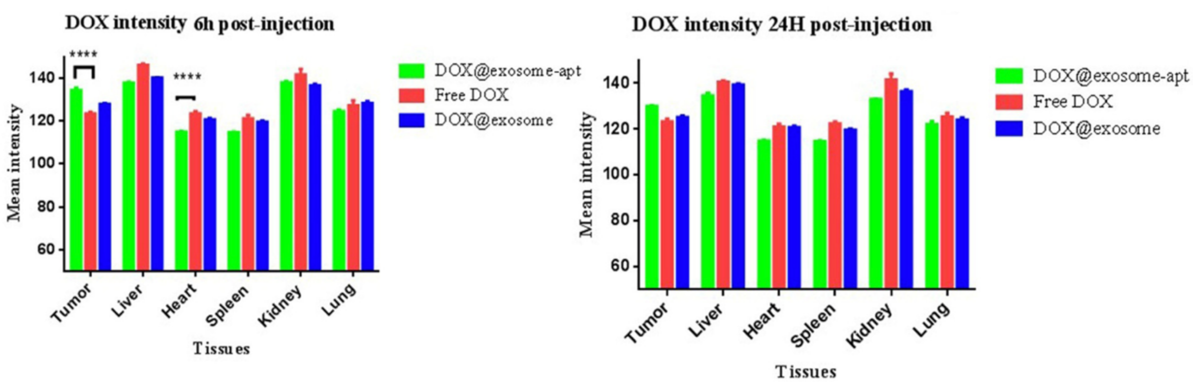


Figure 9 and 10 – Ref:[4] – On the top: fluorescence images of tumor, liver, spleen, kidneys, heart, and lungs of tumor-bearing mouse models. On the bottom: mean fluorescence drug analysis 6h and 24h post injection

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