Targeting Tumor-Associated Macrophages with PI3Kγ Inhibitor-loaded Nanocarriers for Cancer Therapy

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Abstract

Macrophages exist in virtually all organs and are central players in normal immune functions. They are also key regulators in the pathogenesis of various human diseases, including cancer. Once in tumors, these cells can adopt a tumor-promoting phenotype named tumor-associated macrophages (TAMs). TAMs contribute to tumor growth in many ways, such as immunosuppression, angiogenesis, and metastasis. [1]

PI3Kγ is a signal-transducing enzyme that mediates the key cellular functions in cancer and immunity. It is highly expressed in TAMs and plays an important role in the pro-tumor function of these cells. Inhibit its activity can lead to the expression of pro-inflammatory factors, then stimulate cytotoxic T cell activation, anti-tumor immunity, and promote tumor regression.[2] IPI549 is a potent and highly selective inhibitor of PI3kγ. It can repolarize TAMs toward a pro-inflammatory phenotype, which suppresses tumor growth. It also demonstrates robust inhibition of PI3K-γ mediated neutrophil migration *in-vivo*. [3]

CRV is a macrophage-targeting peptide. It can specifically bind to the RXRB receptor, which expresses in TAMs. After systemic administration, rapid and efficient homing of CRV to the TAMs can be observed. [4] Since the PI3kγ expressed in both tumor and healthy tissues, CRV could be utilized as the ligand, which specifically targeting to TAMs and concentrates the IPI549 at TAMs.

Due to the low solubility and potential side effects of IPI549, nanocarriers (liposomes and exosomes) which are decorated with CRV peptide, are utilized to load IPI549. It was expected to enhance the specific uptake of the compound by TAMs *in-vivo*.

Methods and Results

Two strategies were used to enhance the loading efficiency. First, 1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol) (DSPE-PEG2000) and Egg phosphatidylcholine (EPC) were used to build the phospholipid bilayer. The unsaturated and saturated phosphatidylcholines, with a significant difference in phase transition temperature and chain stiffness, created bilayer "pockets" in which the bulky IPI549 is embedded. Meanwhile, DSPE-PEG2000 micelles encapsulate IPI549 in a hydrophobic cavity, then fusion with the phospholipid bilayer of liposomes further increases the drug loading. Exosomes secreted by RAW cells were isolated and collected by ultra-centrifuging. CRV was decorated on the surface of nanocarriers (exosomes and liposomes) through incubation methods.

Conclusion

25% IPI549 to phospholipid molar ratio, a relatively high drug loading compared to other insoluble drugs, was attained during liposome preparation. After systemic administration, rapid and efficient homing of CRV-IPI549 liposomes to the tumors could be observed. The decorated ligands（TAT and RPAR）of the exosomes still keep the combination activity with their receptors (heparin and neuropilin 1 receptor b1b2 domain protein). The decorated ligands do not inhibit the uptake of exosomes by macrophages.

Keywords: TAM; PI3Kγ inhibitor; CRV peptide; liposomes; exosomes.

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Abbreviations

TAM: Tumor-Associated Macrophage

P13kγ: Phosphatidylinositol 3-kinase-gamma

RXRB: Retinoid X receptor beta

PEG: Polyethylene glycol

DSPE: 1,2-Distearoyl-sn-glycerol-3-phosphorylethanolamine

EPC: L-α-phosphatidylcholine (egg PC)

DMEM: Dulbecco's Modified Eagle Medium

DIL: 1,1'-dioctadecyl-3,3,3' ,3'-tetramethylindocarbocyanine perchlorate

SR101: Sulforhodamine 101

FAM: Carboxyfluorescein

DLS: Dynamic light scattering

CHAPTER 1. INTRODUCTION

IPI549 is a potent and highly selective PI3Kγ inhibitor. It has been used to reshape tumor immune microenvironments and promote cytotoxic T-cell-mediated tumor regression through switching immune suppression to stimulation of macrophages. A major difficulty in developing IPI549 as a therapeutic agent is its poor water solubility. The study aimed to develop novel TAM targeting nanocarriers that are capable of incorporating high P13kγ inhibitor content. CRV decorated liposomes and exosomes were synthesized. Additionally, the *in-vivo* distribution and *in-vitro* cell uptake was evaluated. In the first chapter, the background of TAM, P13kγ, and its inhibitor, CRV, and nanocarriers will be introduced.

1.1 Tumor associate macrophage

Macrophages exist in all organs and are central players in normal immune functions. They are also key regulators in the pathogenesis of various human diseases, including cancer.

Macrophage means "big eater" in Greek. Macrophages are large phagocytic cells. Macrophages develop in the bone marrow as "monocytes," which circulate in the bloodstream and then relocate to different tissues. When inflammation occurs, monocytes become macrophages, targeting, and eliminating specific cells. Some macrophages function as scavengers, removing necrotic cells, while others engulf microbes to provide host immunity. Macrophages are given different names depending on the tissue location [5]. For example, they are called microglia in the central nervous system. Macrophages hold critical roles in immune defense and in regulating homeostasis. Their dysfunction and dysregulation are linked with many diseases, such as obesity, cardiovascular disease, and cancer.[6](Figure 1.)

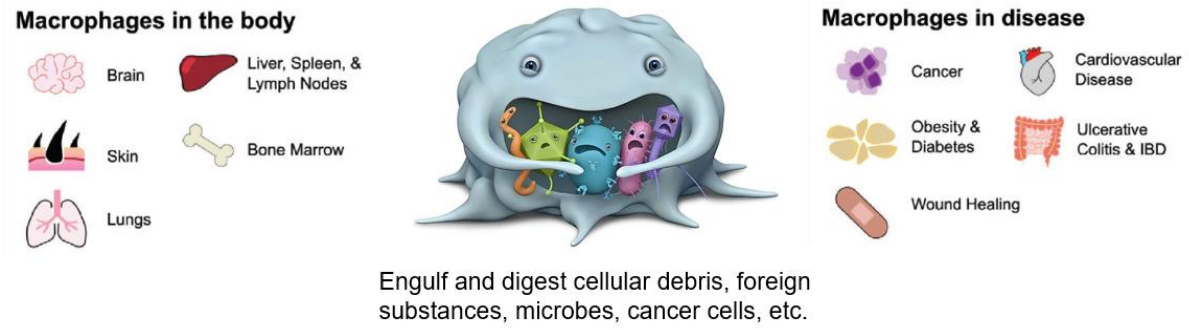

Figure 1: Macrophages are present in all organs throughout the body. This figure is adapted from [6]

Once in tumors, macrophages can adopt a tumor-promoting phenotype named tumor-associated macrophages (TAMs). TAMs are a major component of the tumor microenvironment, comprising up to 50% of the tumor mass.[7] (Figure 2) TAMs contribute to tumor growth in many ways, such as immunosuppression, angiogenesis, and metastasis. [1] Increased numbers of TAMs correlate with tumor progression and poor prognosis.[8]

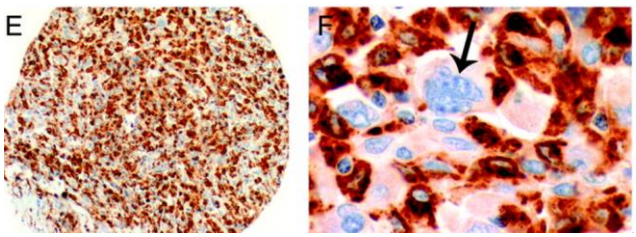

Figure 2: TAMs comprising up to 50% of the tumor mass. Brown marker: TAM marker (CD68) Blue marker: Anaplastic carcinoma cell surrounded by TAM.This figure is adapted from[7]

Within the continuum of the polarization status of macrophages, two extreme phenotypes can be defined, M1 pro-inflammatory/anti-tumor and M2 anti-inflammatory/pro-tumor.[9] M1-like macrophages, activated by pro-inflammatory cytokines, have the ability to kill tumor cells, inhibit angiogenesis, and promote adaptive immune responses. At the other extreme, M2-like macrophages, induced by anti-inflammatory cytokines, promote tumor initiation, progression, and survival. They also inhibit immune-stimulatory signals and are devoid of cytotoxic activity. Unfortunately, TAMs mostly exhibit an "M2-like" phenotype.[10](Figure 3.) In figure 4, the positive staining of CD86 (M1 marker) and CD206 (M2 marker) were observed mostly in the cytoplasm of TAMs. The average levels of CD86 and CD206 positive staining cells was 57 and 61 cells/field repectively. [11]

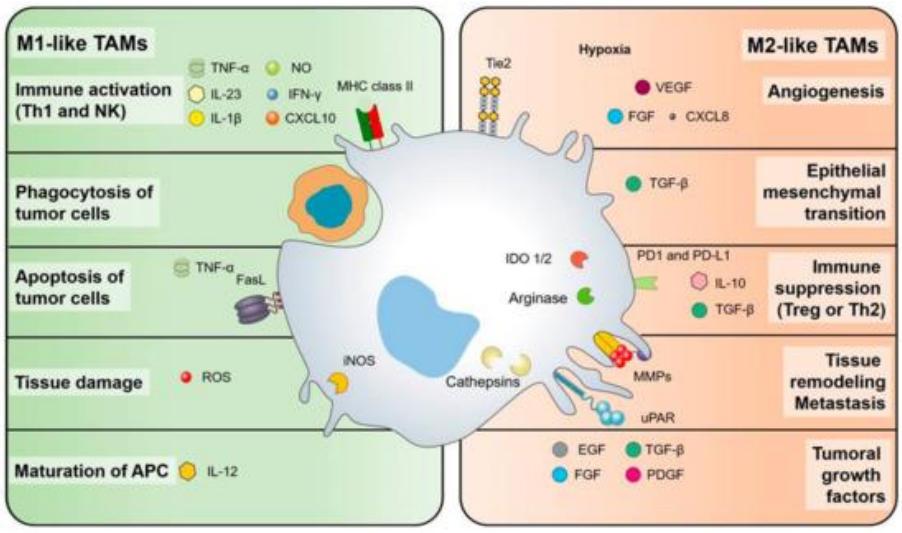

Figure 3: Anti-tumor and pro-tumor functions of tumor-associated macrophages (TAMs)

This figure is adapted from[10]

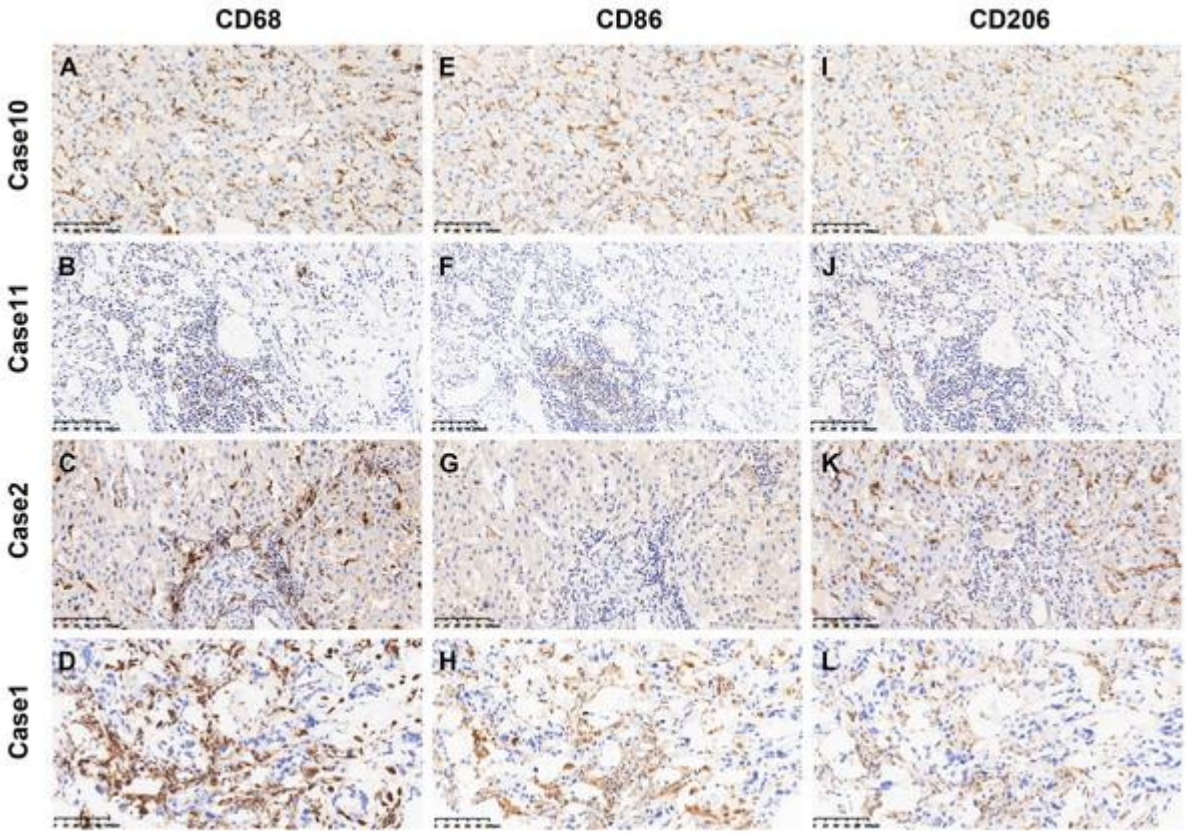

Figure 4: Representative images of CD68+, CD86+ and CD206+ immunostaining in intrahepatic cholangiocarcinoma ICC [11]

TAM infiltration in tumors has been correlated with poor prognosis. Furthermore, numerous investigations have revealed that TAMs are primarily responsible for resistance to classical anti-tumor treatments (i.e., chemotherapy or radiotherapy), and they also limit the efficacy of new

immunotherapies (i.e., anti-PD1). These findings called attention to TAMs as promising targets of novel anti-tumor therapeutic approaches.[12]

Figure 5 shows how TAMs home to the tumor site and promote the metastatic spread. First, macrophages accumulate in tumors by proliferation from tissue-resident precursors or by trafficking from bone-marrow-derived precursors. [6] Once in tumors, these cells can adopt a tumor-promoting phenotype and, after that, induce immunosuppression, angiogenesis, tumor growth, and metastasis.

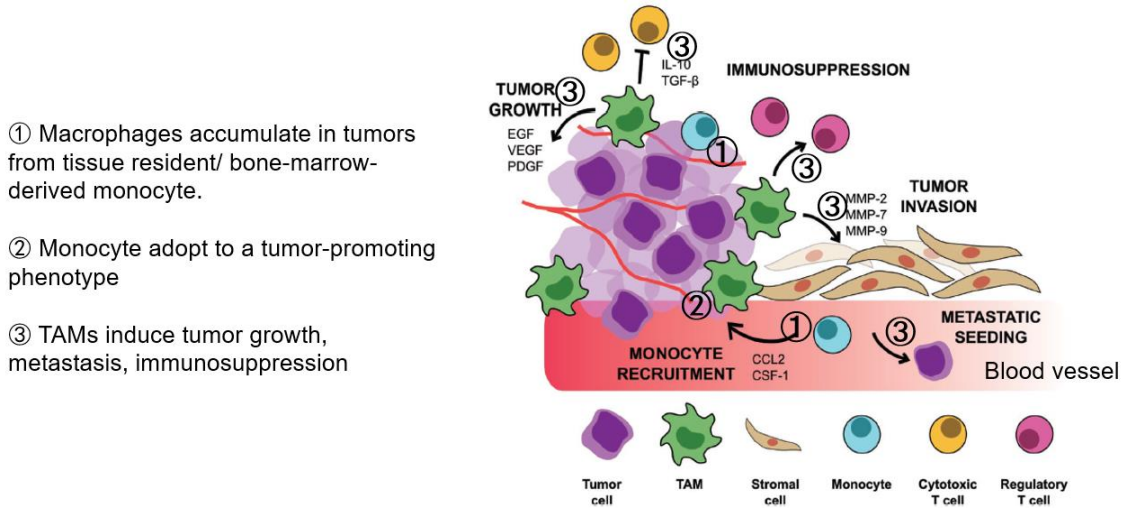

Figure 5: Tumor-associated macrophages potentiate tumor growth and invasion. This figure is adapted from [6]

It was found that TAM receptor signaling skews macrophage polarization. (Figure 6.) TAM receptor binding and downstream signaling dampens M1 polarization and promotes M2 polarization.[13]

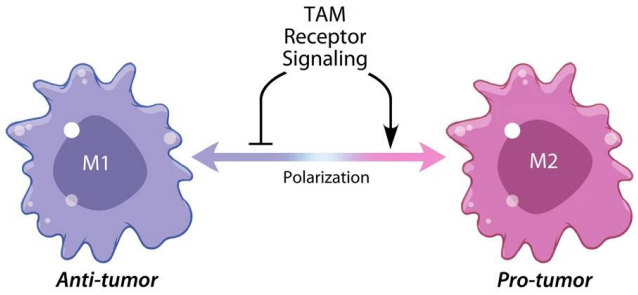

Figure 6: TAM receptor signaling skews macrophage polarization.

This figure is adapted from[13].

1.2 PI3Kγ

PI3Kγ is a signal-transducing enzyme that mediates the critical cellular functions in cancer and immunity. It is highly expressed in TAMs and plays an essential role in their pro-tumor process. Inhibiting its activity can lead to the expression of pro-inflammatory factors, which leads to stimulation of cytotoxic T cell activation, anti-tumor immunity, and promotion of tumor regression.[2]

IPI549 is a potent and highly selective inhibitor of phosphoinositide-3-kinase gamma (PI3kγ) (Figure 8.) [14] with IC50 of 16 nM.[14] IPI549 can repolarize TAMs toward a pro-inflammatory phenotype and inhibit the neutrophil migration, which suppresses tumor growth. (Figure 7.) [3][15] IPI549 is considered a candidate for anti-tumor therapy.

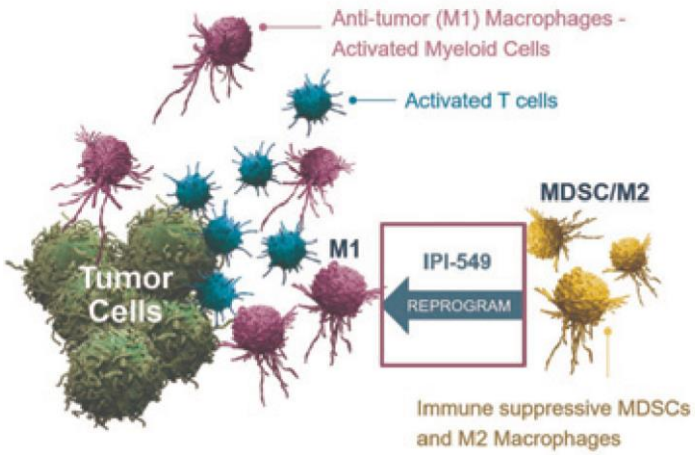

Figure 7: IPI549 repolarizes TAMs toward a pro-inflammatory phenotype, which suppresses tumor growth. This figure is adapted from[3].

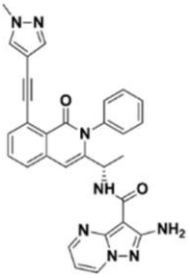

Figure 8: Chemical structure of IPI549

This figure is adapted from[14].

1.3 Nanocarriers

1.3.1 Liposomes

Liposomes were first reported as a drug carrier in 1974.[16] Over the years, many reports have recognized its potential as effective drug carriers. Liposomes are spherical vesicles formed by one or more lipid bilayers. They can entrap hydrophilic molecules in the inner aqueous core, and hydrophobic drugs can reside at the surface or in the palisade region of the phospholipid bilayer. The addition of a relatively inert PEG coating can provide a longer circulatory life *in-vivo*. Different targeting ligands, like monoclonal antibodies, vitamins, specific antigens, or targeting peptides were developed to enhance the targeting efficiency and minimize systemic toxicity. [17](Figure 9)

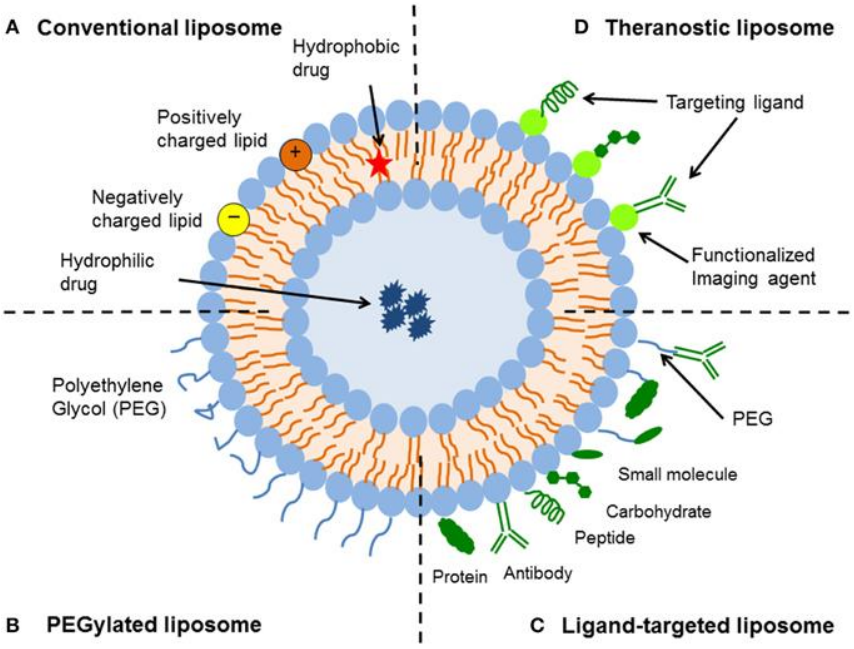

Figure 9: Schematic representation of the different types of liposomal drug delivery systems. This figure is adapted from[17].

1.3.2 Exosomes

Exosomes are membrane-bound extracellular vesicles that are produced in the endosomal compartment of most eukaryotic cells. They range in diameter from 30-150 nm. [18] Exosomes can not only be identified in blood, urine, cerebrospinal fluid but also are released *in-vitro* by cultured cells. (Figure 10)[19] Importantly, exosomes secreted by tumor cells can signal surrounding cells and promote cancer cell survival and metastasis.[20]

Exosomes naturally occur in humans. They are non-immunogenic and show high biocompatibility. They also display lower clearance rates, which are contributed by their natural origin in comparison with other drug delivery systems. The long-term accumulation in organs and tissues of exosomes is lower, which reduces systemic toxicity and facilitates cellular uptake.[21] Exosomes have been considered as vehicles for drug delivery, which offers significant advantages compared with other artificial nanocarriers. [22]

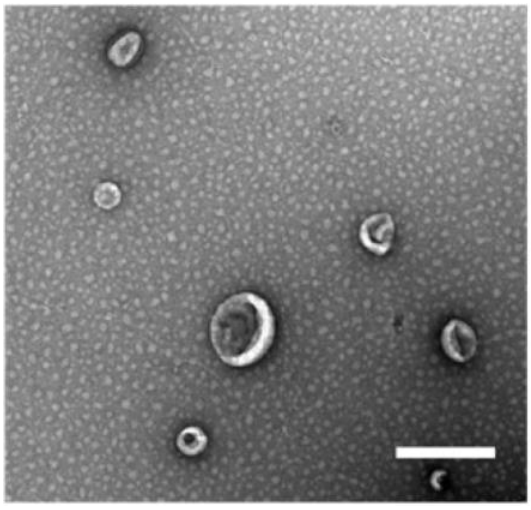

Figure 10: Transmission electron micrograph of exosomes isolated from urine. The bar is 400 nm. This figure is adapted from[19].

1.4 CRV

CRV is a macrophage-binding peptide that binds to the retinoid X receptor beta (RXRB), which is largely limited to TAMs.[23]. [4] With systemic administration, rapid and efficient homing of CRV to the TAMs can be observed. (Figure 11.)[4] Since PI3kγ is expressed in both tumor and healthy tissues, CRV could be used as the ligand, which specifically targets TAMs and concentrates the IPI549 at TAMs.

Since PI3kγ is expressed in both tumor and healthy tissues, the cell surface markers can be exploited to concentrate the drug at the TAM surface. Some receptors, e.g., CD206 and folate receptor β, are examples of TAM markers that have been widely used for ligand-directed delivery. However, most of these surface markers are also found on healthy monocytes/macrophages, and more importantly, are critical components in normal immune function. It thus comes as no surprise that targeting these receptors has shown side effects. In the previous report of our lab, CRV-pSiNPs (CRV peptide conjugated porous silicon nanoparticles) delivered more cargo (SR101) into the tumors than pSiNP without CRV. (Figure 12.) [4] CRV could be utilized as the ligand, which specifically targeting to TAMs and concentrates the IPI549 at TAMs.

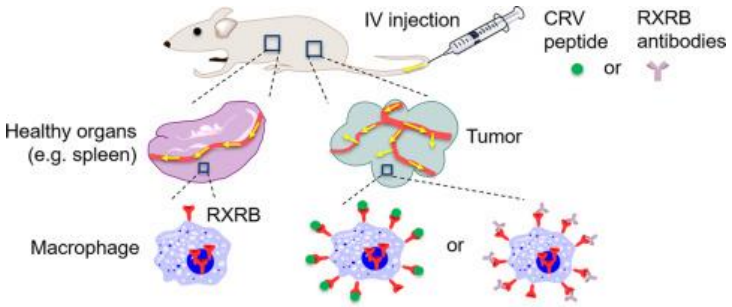

Figure 5: CRV targeting TAM through recognition of RXRB. This figure is adapted from[4].

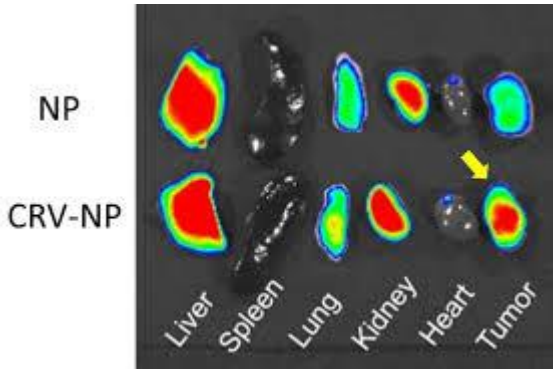

Figure 6: CRV facilitates the delivery of nanomaterials to tumors. This figure is adapted from[4].

1.5 Specific Aims and Hypothesis

The overall goal of this study was the development of a novel TAM targeting nanocarrier formulation, which is capable of incorporating high IPI549 content. The influence and mechanism of different phospholipid combinations on loading efficiency were evaluated. Additionally, *the in-*

vivo distribution of the IPI549 liposome administered systemically in 4T1 tumor-bearing mice and the *in-vitro* nanocarrier cell uptake and receptor binding were evaluated.

IPI549 has very poor water solubility, which presents a challenge for the administration. In the previous report, 5% NMP with 95% PEG 400 were used to dissolve IPI549 for gavage.[24] However, this vehicle could not be used for systemic treatment. PI3K-γ is also involved in other tissues or diseases like neurons, inflammatory, and cardiovascular diseases.[25] Therefore, the formulation of IPI549 should ideally provide adequate solubility as well as minimize off-target side effects.

The liposome is a possible solution to the above problems. It has many advantages over other formulations, such as biocompatibility, the ability to carry large drug payloads, and a wide range of physicochemical and biophysical properties that can be modified to control the biological characteristics.[26]

Due to the poor solubility and potential side effects of IPI549, nanocarriers that are decorated with CRV peptide are utilized to load IPI549. It was expected to enhance the specific uptake of the compound by TAMs *in-vivo.*

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

IPI549 was purchased from Active Biochem LTD. Egg phosphatidylcholine (EPC), hydrogenated egg phosphatidylcholine (HEPC), distearoyl-phosphatidylethanolamine-poly(ethylene glycol) 2000 (DSPE-PEG2000), and cholesterol (Chol) were purchased from Avanti Polar Lipids, Inc. Carboxyfluorescein-conjugated peptides (FAM-cys-CRV and FAM-cys-GGS, etc.) were purchased from LifeTein, LLC (CRV sequence: CRVLRSGSC). All other chemicals were purchased from Sigma or Merck. RAW264.7 and 4T1 cells were obtained from American Type Culture Collection (Manassas, VA). Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), and 1% Penicillin-Streptomycin were purchased from Glibco. Hoechst 33342 were purchased from Thermo Fisher.

2.2. Preparation of nanocarriers

2.2.1 Liposomes

2.2.1.1 Conventional liposomes synthesis

IPI549 and lipid constituents were weighed out in the desired molar ratio and solubilized in chloroform. The solution was dried by a stream of N_2 gas, and the residual chloroform was removed by vacuum for one h such that a thin lipid film was deposited on the wall of the tube. Aliquots of 10% (w/v) sucrose were added to the tube, which was then mixed by a vortex mixer to form large multilamellar liposomes. The sample was then extruded above 41℃ (the phase-

transition temperature of DSPE-PEG2000) by passing the sample several times through polycarbonate filters of 100-nm pore size with extruder syringes to remove possible IPI549 precipitates which fail to be encapsulated and yield small unilamellar liposomes.

2.2.1.2 Peptide conjugation

For the synthesis of FAM-CRV conjugated IPI549 liposomes, DSPE-PEG2000 Maleimide was used instead of DSPE-PEG2000 at the same molar ratio in the formulation. Carboxyfluorescein-cysteine was conjugated through an aminohexanoic acid linker to the N-terminal amino group of CRV (FAM-cys-CRV). FAM-cys-CRV solution (1 mg/mL in PBS) or FAM-cysteine control (FAM-cys-GGS) was added to the IPI549 liposomes solution and mixing at four °C overnight. The mole concentration of FAM-cys-CRV was the same as the DSPE-PEG2000 maleimide in the reaction system. The dispersion was dialyzed overnight with PBS using 5 kDa MWCO tubing to remove the free FAM-cys-CRV.

2.2.2 Exosomes

2.2.2.1 Isolation

Exosomes were isolated and purified by differential ultracentrifugation using the process depicted in Figure11 [27]. The initial steps are designed to eliminate large dead cells and debris by successive centrifugations at increasing speeds (steps 1 to 5 below). In each of these steps, the pellet is discarded, and the supernatant is used for the following step. The final supernatant is then ultra-centrifuged at 100,000 × *g* to pellet the small vesicles that correspond to exosomes. The pellet

is washed in a large volume of PBS to eliminate contaminating protein and centrifuged one last time at the same high speed.

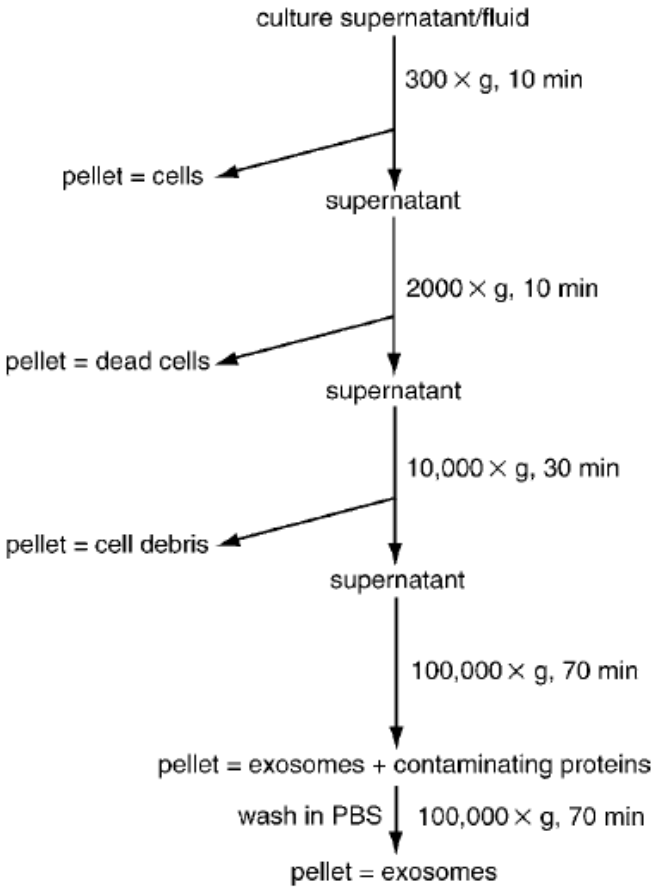

Figure 7: Flow chart for the exosome purification procedure based on differential ultracentrifugation. This figure is adapted from [27].

2.2.2.2 Peptide decoration

Synthesis of DSPE-PEG2000-Peptides

DSPE-PEG-MAL was mixed with FAM-Cys-Peptide (TAT,iRGD, RPAR) at a 1:1 molar ratio in PBS (pH = 7.4). The reaction mixture was gently stirred at 4 ℃ overnight, after which the resulting reaction mixture was placed in a dialysis bag (molecular weight cut off :5,000 Da) and dialyzed in PBS for 24 h to remove small molecules. The final solution in the dialysis bag was lyophilized and stored at −20°C until needed.

Decoration of exosomes with DSPE-PEG2000-Peptides

The washed exosomes were resuspended in PBS and then incubated with DSPE-PEG-Peptide, and DIL at 37 °C for 30 min to form DIL loaded DSPE-PEG2000-Peptide-inserted exosomes.[28] (Figure 14). The inserted exosomes were then washed three times with PBS before further use.

2.3 Characterization

2.3.1 Size distribution

The liposomes and exosomes were diluted using PBS at pH 7.4. A DLS Particle Analyzer (Microtrac NanoFlex) was used to measure the particle size distribution of the liposomes and exosomes at 25℃ according to the protocol. The samples were measured two times on the DLS Particle Analyzer according to the protocol. The data were collected, and the results are reported as number and volume mean distribution.

2.3.2 Incorporation efficiency

Drug incorporation efficiency, representing the retention of IPI549 in the filtered liposomes relative to the added drug, was measured by BioTek Fluorescence Microplate Reader. Plate map with a standard curve from 0 to 10 μg/ml with samples and standards in duplicate. The linear regression curve fit was used to determine unknown concentrations. Triton-X (0.3%) was used to release the capsulated drug. The fluorescent intensity was measured at Ex/Em: 360 nm/450 nm on a Tecan Infinity M1000 Pro. The liposomes were sealed in the vial and stored at 4 ◦C for further shelf stability test.

2.4 Cell culture and tumor model

4T1 mouse breast cancer cells, RAW 264.7 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. [29] Six to eight-week-old BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). To generate 4T1 tumors, 1×10^6 tumor cells (suspended in 100 μL of PBS) were orthotopically injected into the mammary fat pad of female BALB/c mice. All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at UMN.

2.5 *In-vitro* study

2.5.1 Cell uptake

Treatment

For the cell uptake assay, RAW cells in DMEM were plated in a 96-well cell culture plate (10^4 cells per well). Each well was treated with the different groups of exosomes, liposomes, or peptides and dye as control. After incubating for 4 hours at 37℃, a microscopic image was acquired of the cells after staining.

Cell staining

Cells were cultured as described above. After 4 h, the media was removed, and the cells were quickly rinsed with PBS solution (pH 7.4) and fixed in 4% formaldehyde in PBS (pH 7.4) for 10 min at room temperature. Then, the cells were washed twice with PBS (pH 7.4) and incubated for 10 min with Hoechst 33342 1:2000 in PBS (pH 7.4). Finally, the cells were washed three times in PBS (pH 7.4) at RT.

2.5.2 Plate binding

Neuropilin 1 receptor protein b1b2 (NRP-1 b1b2) coated 96-well plate preparation

In each well of a 96 well plate, 30 ul of NRP-1 b1b3 (5ug/ml, 1%BSA in PBS) were added. For the control, 30ul 1% BSA was used. After incubating overnight at 4 ℃, plates were unwrapped, and the supernatant was decanted carefully. PBS was used to wash plates three times carefully, and the plates were sealed and stored at 4 ℃ overnight.

DIL loaded Exosomes-DSPE-PEG2000-Peptide plate binding assay

A 50 ul aliquots of DIL loaded Exosomes-DSPE-PEG2000-Peptides, and free exosomes (control group) were dispensed into triplicate wells of coated binding plates. (Figure 14) After incubating at room temperature for 1 hr, the plates were washed carefully three times with 300 ul PBS. To

each well, 100 ul PBS was added, and the plates were read on a fluorescent plate reader at Ex/Em: 540 nm/570 nm (DIL) on a Tecan Infinity M1000 Pro.

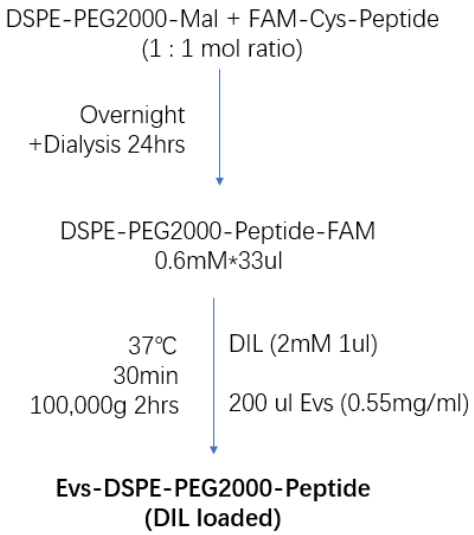

Figure 8: Flow chart of the preparation and plate binding process of DIL loaded exosomes-DSPE-PEG2000-Peptide

2.5.3 *In-vivo* homing studies

A 100 μL aliquot of FAM-CRV-IPI549 liposome or control FAM-GGS-IPI549 liposome was injected into the tail vein of 4T1 tumor-bearing mice. After two hours, the mice were sacrificed by transcardial perfusion with PBS. Tumors and healthy organs were collected and were subjected to immunofluorescence staining protocol and stained for FAM-CRV. [30]

2.6. Immunofluorescence staining

Frozen tissue sections were first treated with PBS containing 1% BSA and 0.1% Triton X100 (blocking buffer) at room temperature (RT) for one hour. The sections were washed three times with PBS and then incubated with primary antibodies diluted (anti-fluorescein antibody, 1:200) in blocking buffer at 4 °C overnight, followed by the appropriate secondary antibodies diluted (rat anti-rabbit 488, 1:200) in blocking buffer at RT for 1 h. After washing with PBS, sections were mounted in DAPI-containing mounting medium (Vector Laboratories, Burlingame, CA) with a coverslip and examined under a fluorescence microscope. Three random microscopic fields were selected.

CHAPTER 3. RESULTS

3.1 Liposomes

3.1.1 Preparation

3.1.1.1 Liposomes made of saturated PC

The saturated lipid, liposome formulation contained DPPC (50%), Chol (33.3%), DOTAP (10%), DSPE-PEG2000 (6.7%) by mol%. The results in Table 1 show that the liposomes made of saturated PC incorporated up to 16.6% IPI549 when the drug to phospholipid molar ratio was kept at 13%. However, drug incorporation efficiency fell to 7.4% when the drug to phospholipid molar ratio was increased to 21.3%. The saturated phospholipid liposomes exhibited a decline in drug incorporation as the drug to phospholipid molar ratio was increased. The loading capacity is limited by the formulation.

Table 1: Incorporation efficiency of IPI549 to saturated phospholipid liposomes

Liposome composition(mol)	Drug/PL (mole%)	Incorporation Efficiency(%)
DPPC/Chol/DOTAP DSPE-PEG2000 (7.5/5/1.5/1)	13.0	16.6
	21.3	7.4

Incorporation efficiency = IPI549 incorporated in liposomes/IPI549 added

3.1.1.2 Combinative formulation of saturated PC-PEG and unsaturated PC

A series of combinations of HEPC and EPC were investigated to develop a high loading capacity liposome formulation for IPI549. Crystals of IPI549 would precipitate in the drying process if the mole ratio of EPC to HEPC was lower than 7:2. This phenomenon is also found in the 3.1.1 saturated lipid section, which suggests that there is an upper plateau for the amount of encapsulated drug. The incorporation efficiencies of different lipid ratios are given in Table 2. When increasing the EPC: HEPC molar ratio from 7:2 to 8:1, the incorporation efficiency did not change. Thus, the maximum incorporation efficiency was 33.2%, when the drug to phospholipid molar ratio was 20%.

Table 2: IPI549 Incorporation efficiency of the liposomes made of EPC and HEPC.

Liposome composition (mol)	Drug/PL (mole%)	Incorporation Efficiency(%)
EPC/HEPC/Chol/DSPE-PEG2000 (14/4/1/1)	20	33.2
EPC/HEPC/Chol/DSPE-PEG2000(16/2/1/1)	20	32.8

3.1.1.3 Optimize DSPE-PEG/unsaturated phospholipid ratio

Replacing HEPC with DSPE-PEG2000, the loading capacity of the formulated liposomes with different lipids ratio are listed in Table 3. The molar ratio of IPI549 to phospholipid was from 25% to 62% in formulated liposomes. The drug to phospholipid molar ratio represents the originally added drug content. No precipitate was observed throughout the preparation. The characteristics of the formulated liposomes are given in Table 3. With increasing the DSPE-PEG2000 molar ratio, an increase in the average diameter of the liposomes was observed. (Figure 15) The incorporation

efficiency of IPI549 decreased compared with the 50% DSPE-PEG2000 group. The incorporation efficiency was maintained above 80%, and the particle size is around 100 nm diameter, which could get a high tissue uptake efficiency [31]when EPC and DSPE-PEG2000 are sustained on an appropriate ratio. Based on the above condition, the drug to phospholipid molar ratio was 25%.

Table 3: IPI549 Incorporation efficiency of the liposomes made of EPC and DSPE-PEG2000.

Liposome composition (EPC/ DSPE-PEG2000/Chol)		Drug/phospholipid		Volume distribution mean diameter(nm)	Incorporation Efficiency(%)
mol/mol	w/w	mol%	wt%		
7/2/1	50/50/4	25	11.5	77.1	85.6
23/20/7	25/75/4	33	11.5	115.8	24.6
0/97/3	0/100/4	62	11.5	1688	41.3

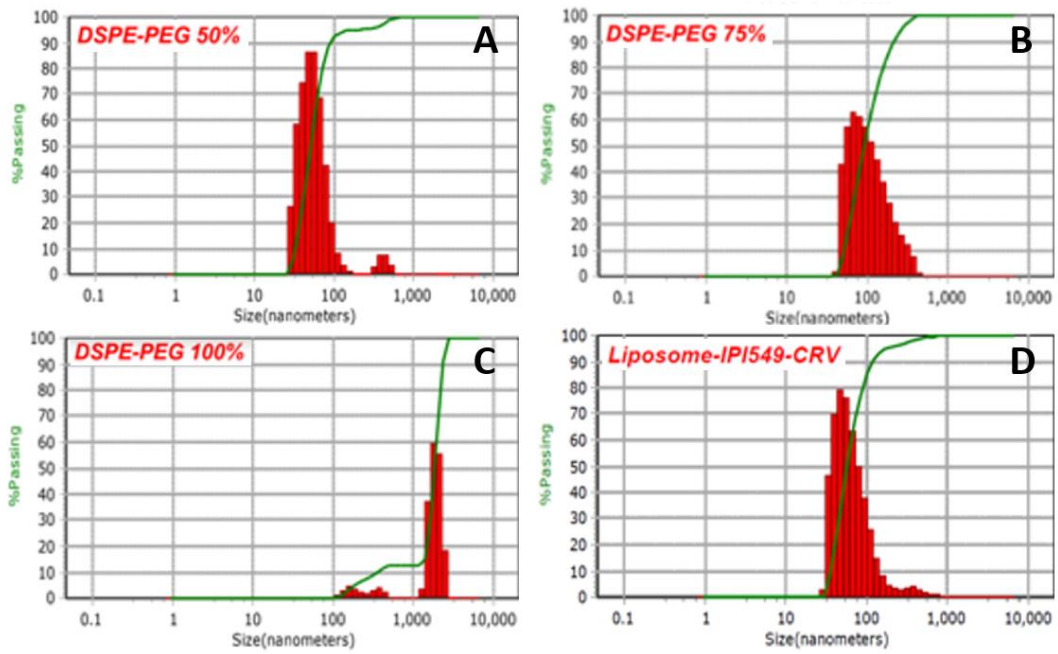

Figure 9: Volume distribution of A: 50%, B: 75%, C: 100% DSPE-PEG liposome, D: CRV-liposome (50% DSPE-PEG)

3.1.2 *In-vitro* and *in-vivo* studies

3.1.2.1 Cell uptake

The uptake of FAM-liposome-SR101 and FAM-CRV-liposome-SR101 by RAW cells were measured. SR101(RFP), FAM-Cys-CRV (GFP), FAM-Cys (GFP) were used as a control to exclude the effect of dye and free peptide. (Fig 16) Both FAM-liposome-SR101 and FAM-CRV-liposome-SR101 were taken up by RAW cells after 4 hrs of treatment. (Figure 17) CRV was fully conjugated, and this conjunction did not present an obstacle to the uptake by RAW cells.

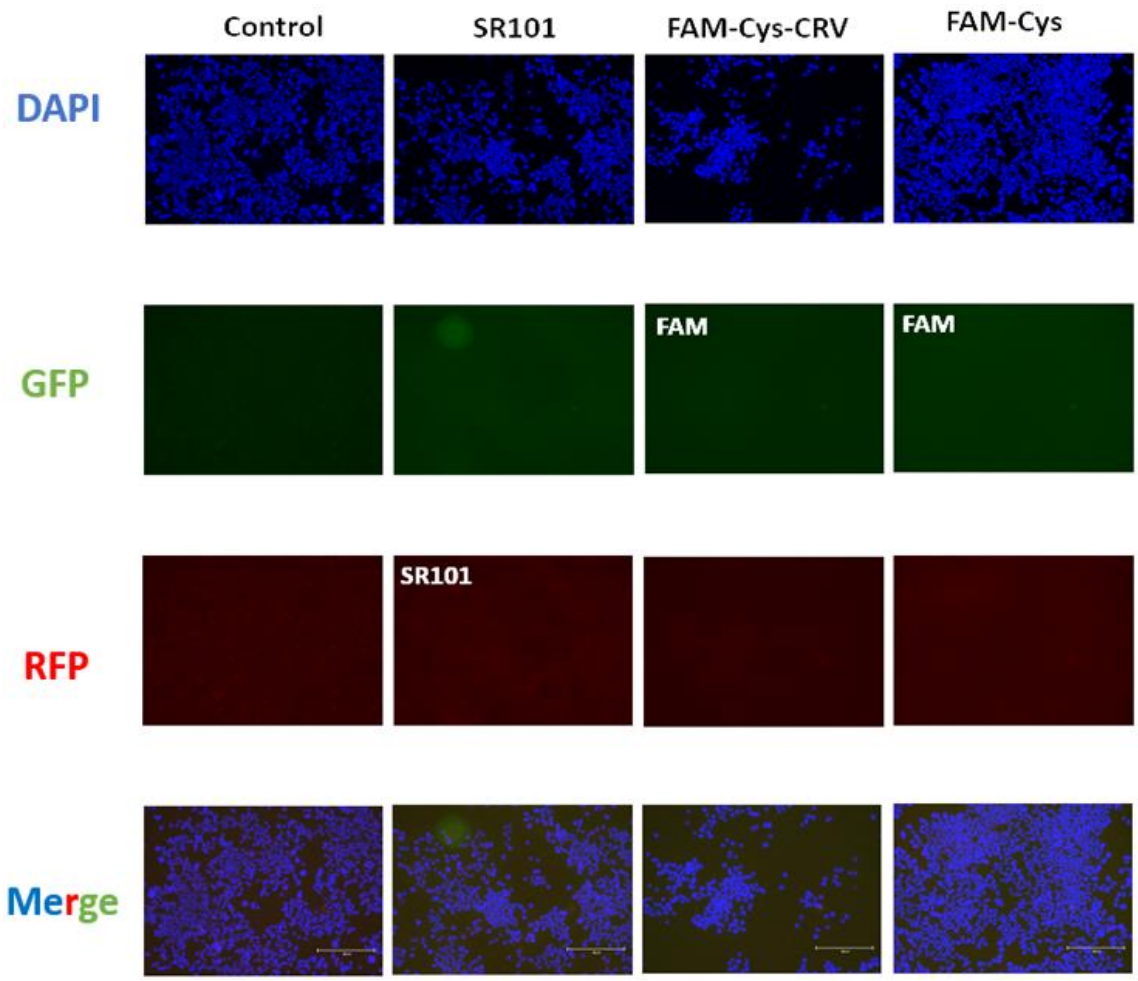

Figure 10: Cell uptake (4 hrs) of RAW cells. The cellular nucleus was stained with DAPI in blue, SR101(RFP), FAM-Cys-CRV (GFP), FAM-Cys (GFP) were observed. The scale bar is 150μm.

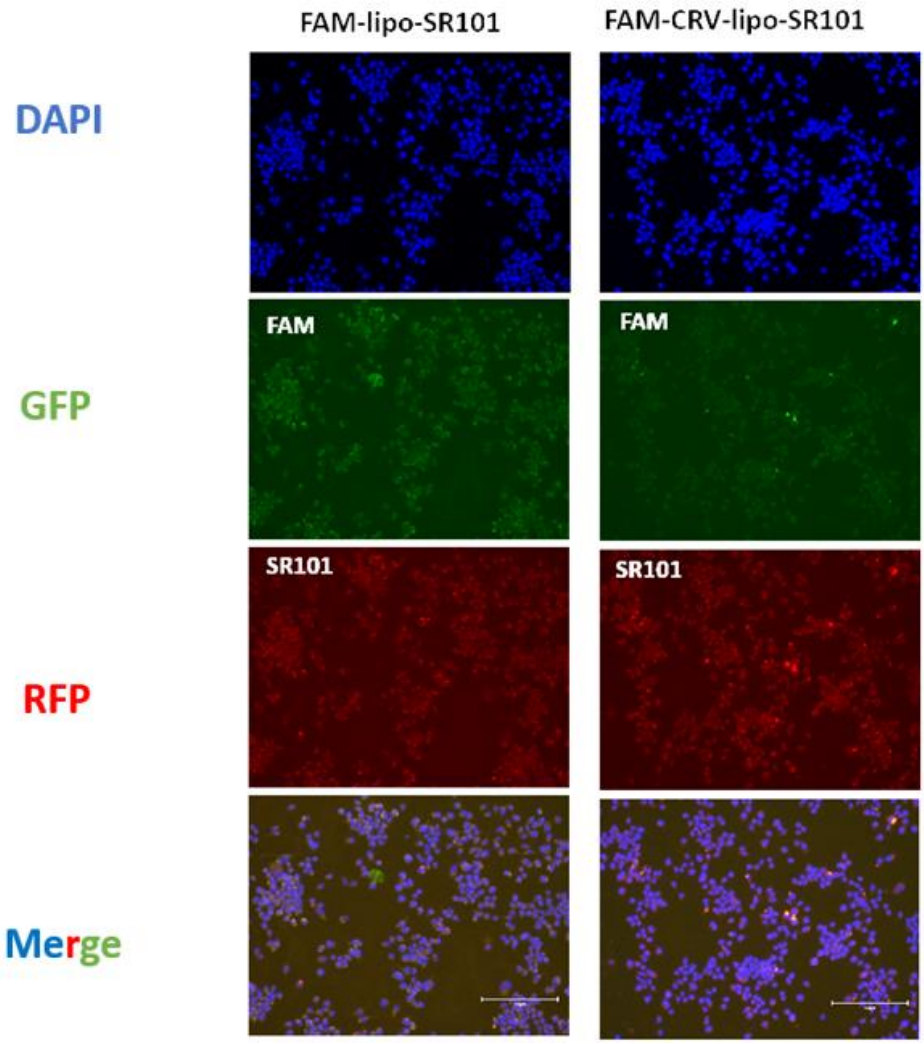

Figure 11: Cell uptake (4 hrs) of RAW. The cellular nucleus was stained with DAPI in blue, FAM-liposome-SR101, FAM-CRV-liposome-SR101 were observed.

3.1.2.2 Tissue distribution

Tissue distribution of IPI 549 loaded FAM-CRV-liposome was measured following IV injection. The dose of FAM and drug were 5.2 µg/kg and 200 µg/kg, respectively. Compared with liposomes without CRV decorate, IPI 549 loaded FAM-CRV-liposome had more rapid and greater accumulation at the tumor site. (Fig 18-23)

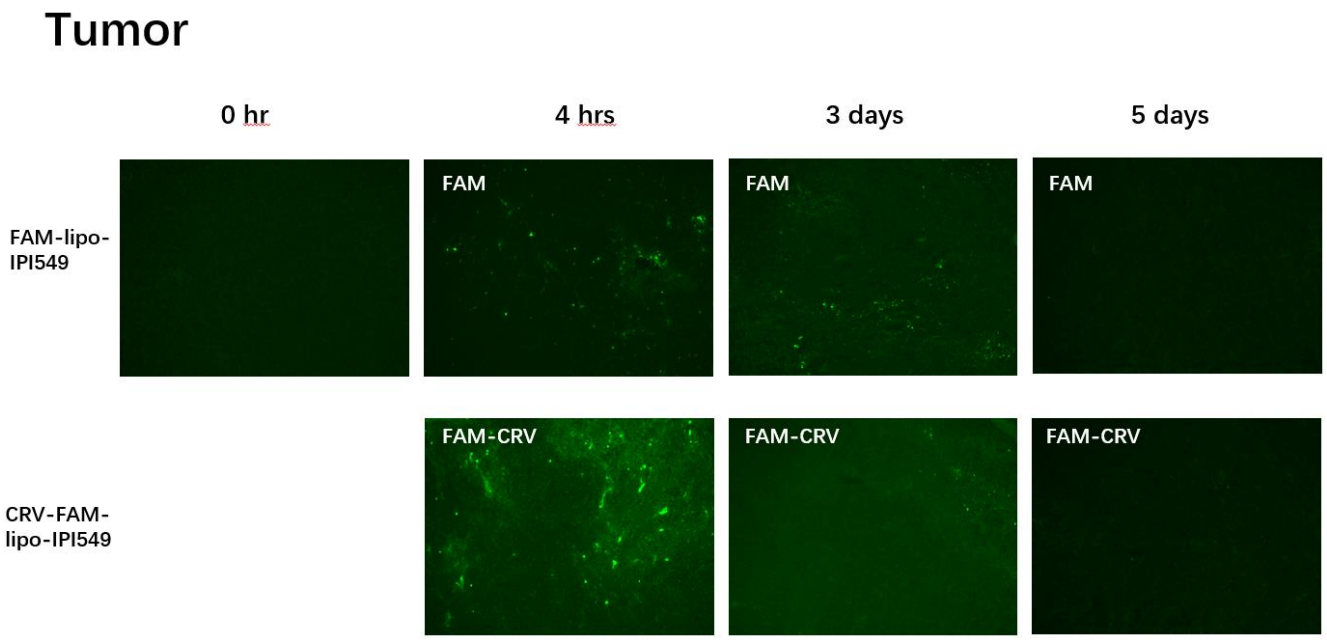

Figure 12: IPI 549 loaded FAM-CRV-liposome target to the tumor site (0, 4 hrs, 3 days, 5 days)

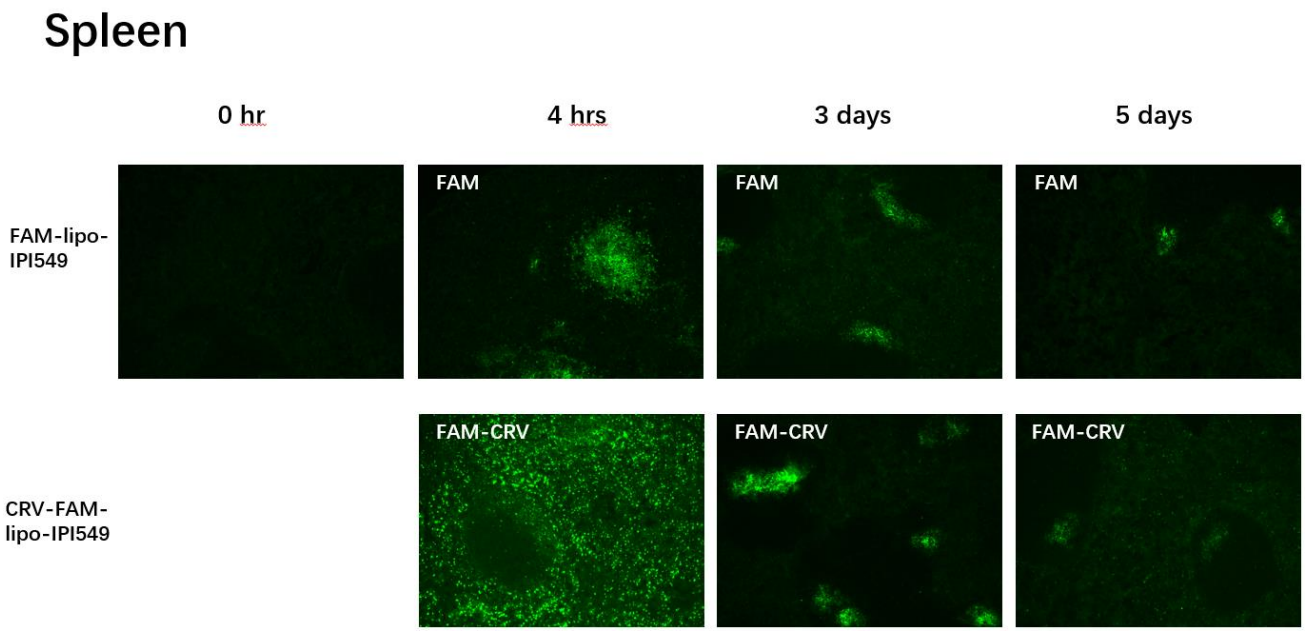

Figure 13: IPI 549 loaded FAM-CRV-liposome target to the spleen (0, 4 hours, 3 days, 5 days).

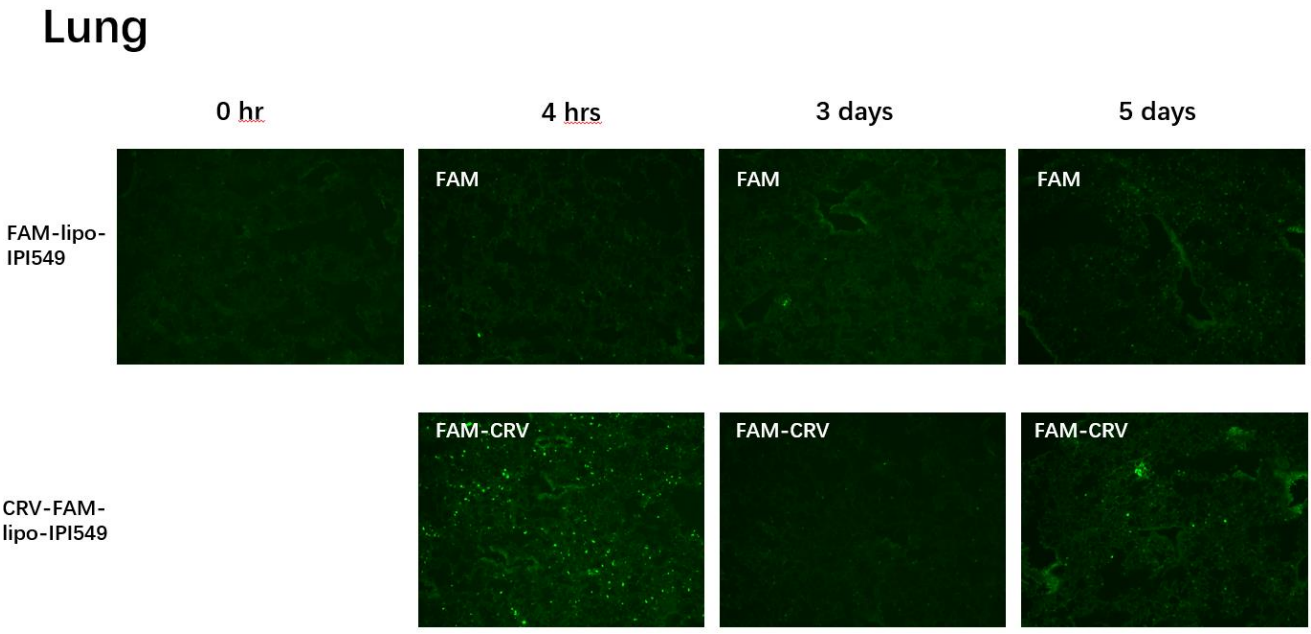

Figure 20: IPI 549 loaded FAM-CRV-liposome target to lung (0, 4 hours, 3 days, 5 days).

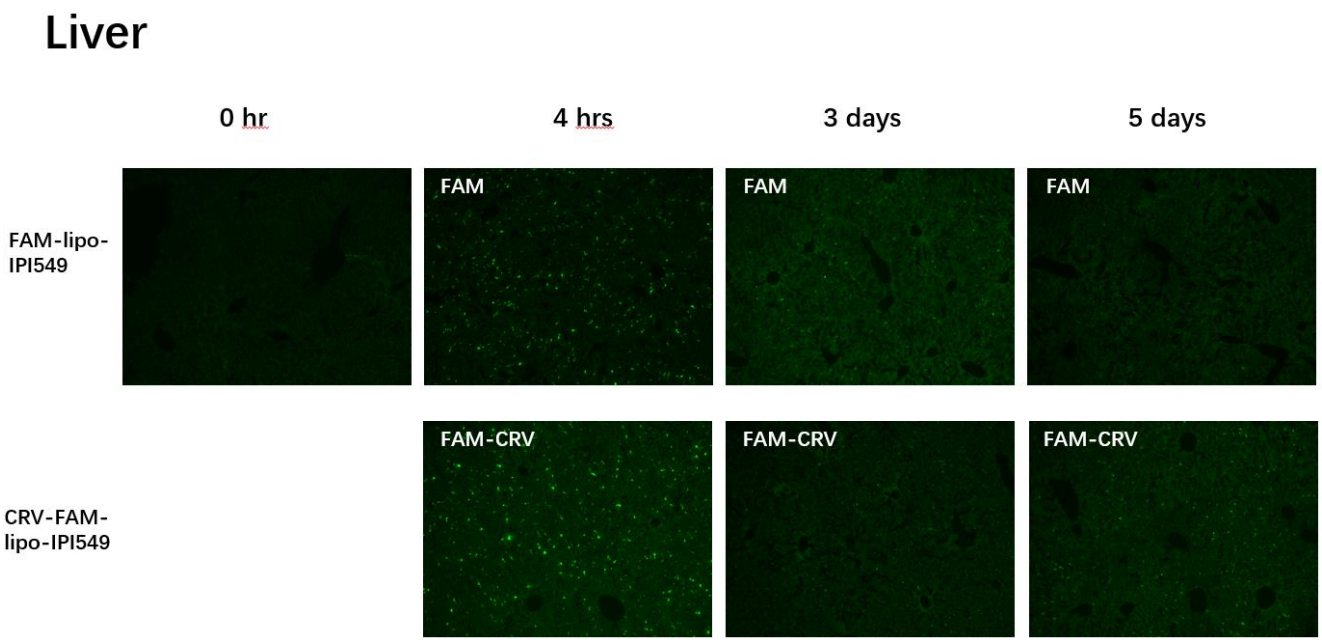

Figure 14: IPI 549 loaded FAM-CRV-liposome target to the liver (0, 4 hours, 3 days, 5 days).

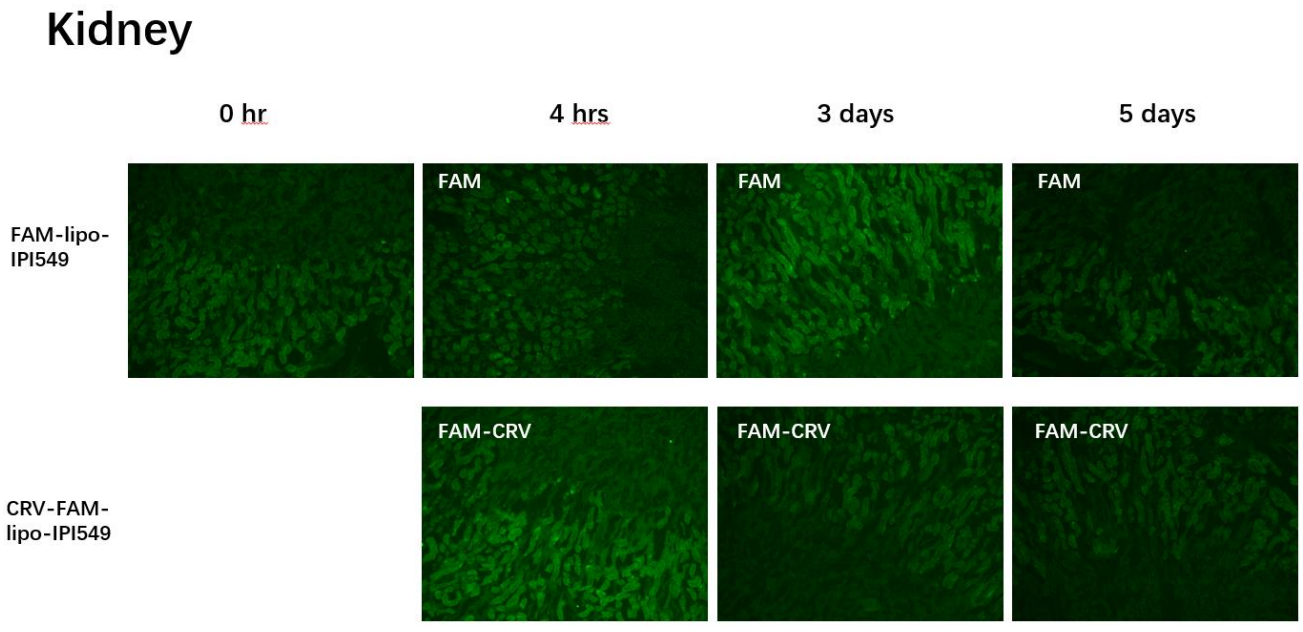

Figure 15: IPI 549 loaded FAM-CRV-liposome target to kidney (0, 4 hours, 3 days, 5 days)

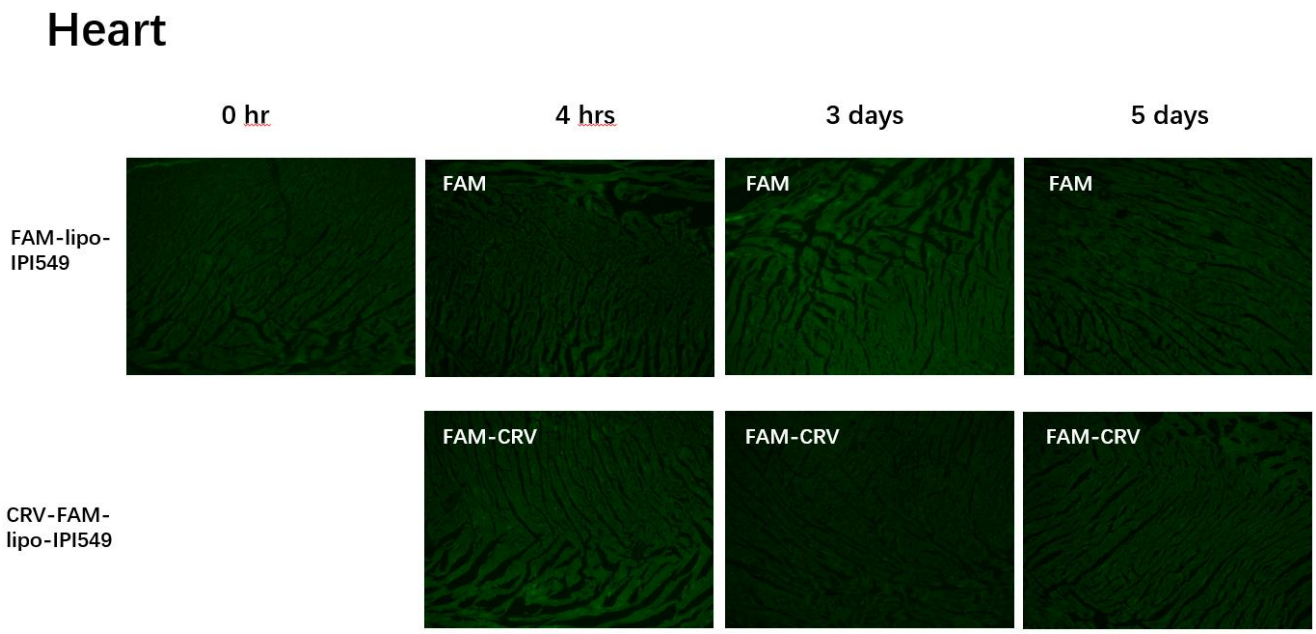

Figure 16: IPI 549 loaded FAM-CRV-liposome target to heart (0, 4 hours, 3 days, 5 days)

3.2. Exosomes

3.2.1 Preparation and size distribution

The measure means volume diameter of 4T1 and RAW exosomes was near 100 nm as measured by DLS (Figure 24).

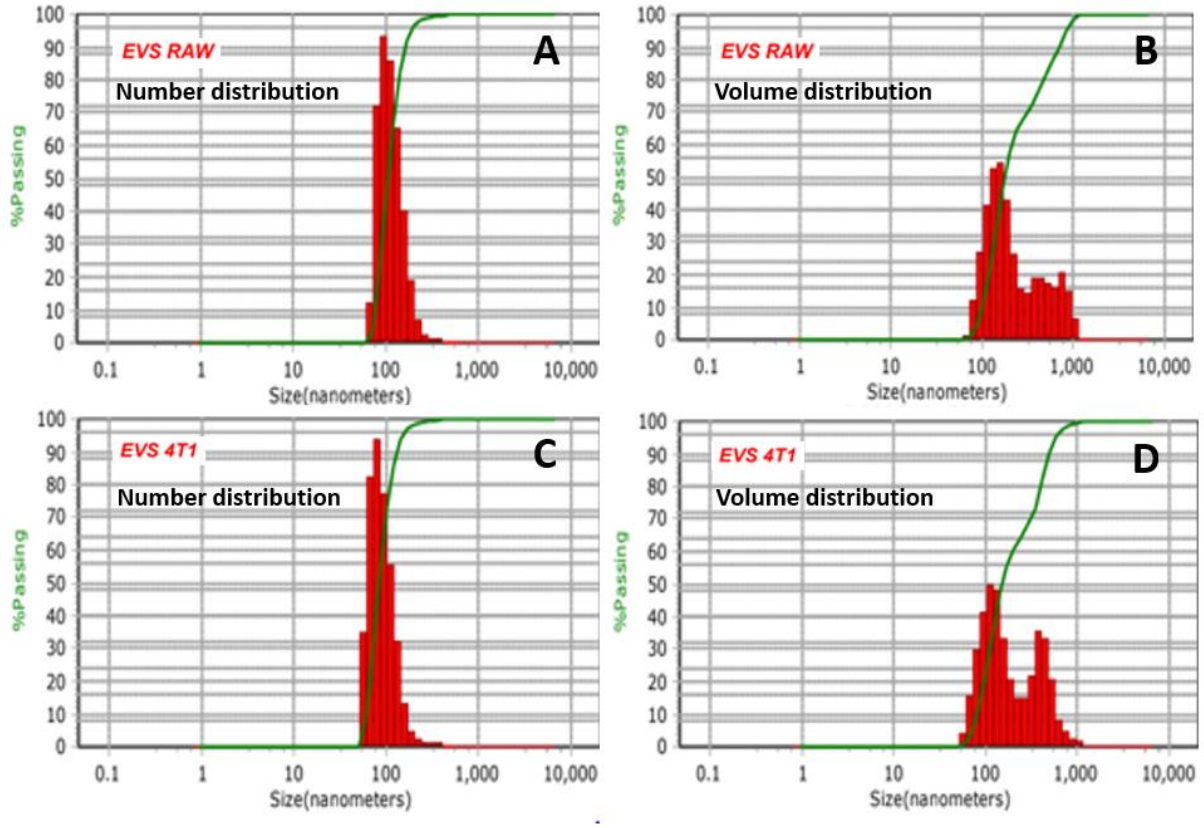

Figure 17: Volume distribution and number distribution of exosomes secreted by RAW and 4T1 cells.

Exosomes conjugated with peptides

After incubating with DIL and FAM-Peptide-PEG and centrifuging, the DIL loaded FAM-Peptide-PEG were obtained (pellet). (Figure 25) The ultracentrifuge was used to remove the unconjugated peptide. The yellow supernatant was the unconjugated FAM-Peptide-PEG. Control: DIL loaded exosomes; FAM: DIL loaded FAM-PEG conjugated exosomes; RPAR: DIL loaded FAM-PEG-RPAR conjugated exosomes; CRV: DIL loaded FAM-PEG-CRV conjugated exosomes

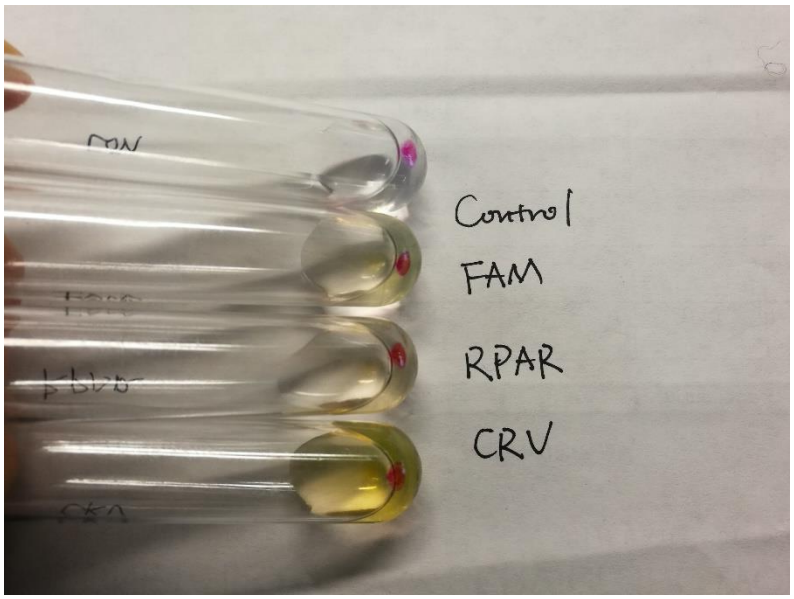

Figure 18: Appearance of DIL loaded exosomes conjugated with different dye or peptides (FAM, RPAR, CRV)

3.2.2 Plate binding

Table 4 shows the results from the binding study of TAT and RPAR with heparin or neuropilin 1 receptor b1b2 domain protein-coated plate. Free exosomes had low binding with plate coating protein compared with the peptide decorated exosomes. This result suggests that exosomes do not block peptide binding with its specific binding site.

Table 4: Binding effect of TAT-PEG-exosomes and RPAR-PEG-exosomes. EVs are control groups.

	TAT-PEG2000-exosomes		**RPAR-PEG2000-exosomes**	
	Exosomes	TAT-exosomes	Exosomes	RPAR-exosomes
Abs	13.3	354.7	44	199.5

Cell uptake of RAW cells to DIL loaded FAM-CRV-Exosomes was measured. DAPI, DIL(RFP), FAM(GFP) signal overlapped, indicating that the exosomes are fully decorated, and the modified exosomes were still able to be taken up by RAW. (Fig 26)

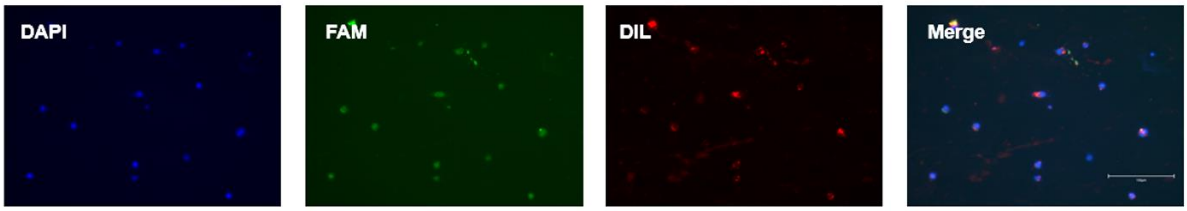

Figure 19: Cell uptake (2hrs) of cells. The cellular nucleus was stained with DAPI in blue, DIL(red fluorescent), FAM -CRV-Exosomes-DIL (green fluorescent and red fluorescent) were observed.

CHAPTER 4. DISCUSSION

4.1 Hydrophobic drugs and their delivery dilemma

Liposomes exhibit a variety of advantages, including high drug loading efficiency, controllable release kinetics, biocompatibility, facile decorations with polymers or ligands to enhance the targeting. As such, they offer an attractive means to improve the therapeutic efficacy of anticancer agents by enhancing the accumulation in tumors and reducing the non-selective distribution to normal tissues.[26, 32, 33] However, liposomes are predominantly used as carriers of hydrophilic molecules, which are encapsulated in the aqueous inner volume.[34]. Hydrophobic drugs are localized within the lipid bilayer of the liposomes, which limits the mass of encapsulating drugs. [35]

Because many anticancer drugs have low water solubility, formulation development is challenging. [36] A typical example is Paclitaxel, which has limited encapsulation in liposomes at about 3-4 mol%.[37] This can explain why currently, there are 16 liposome-based therapeutic products approved for clinical use, but only 6 of them are anticancer drugs.[33] Low water solubility is a major cause of failure in new drug development.[38][34] The slow drug absorption leads to variable bioavailability and gastrointestinal mucosal toxicity in the oral route. [39] The use of potentially toxic solubilizing agents, such as detergents or polymers, can not be used in IV injection formulations.

4.2 Micelle plus "lipid pocket" can encapsulate more

IPI549 is a new compound with low water solubility. In order to improve the poor drug payload of the IPI549 liposomes, two strategies were used. One was reducing the stiffness of the membrane by forming small "pockets." (Figure 27) [37, 40]. Two types of lipids, EPC, and DSPE-PEG2000, which have a significant difference between their phase transition temperatures, were used. EPC contains a high content of unsaturated fatty acid chains and has a lower phase transition temperature of −8 ◦C. DSPE-PEG2000, a saturated lipid with a polar PEG chain, has a phase transition temperature of approximately 41◦C.[41] At a physiological temperature of 37 ºC, the former would be in the fluid, liquid crystalline phase, whereas the latter would be in the solid-like gel phase. When both are present, phase separation is likely resulting in different domains in the bilayer. Moreover, the boundary region between these two phases or segregated microdomains can provide a small "pocket" for the solubilization of drugs, such as IPI549. [40]

And the cholesterol is another inducement of the stiff bilayer. Cholesterol was incorporated into the liposomes to enhance the stability of the liposomes. Commonly, 40 mol% cholesterol is added to sustain the membrane's stability. In another case of PTX liposome, 5 mol% cholesterol was used to balance the membrane stability and encapsulation capacity.[42, 43] In this paper, lower cholesterol content also was applied.

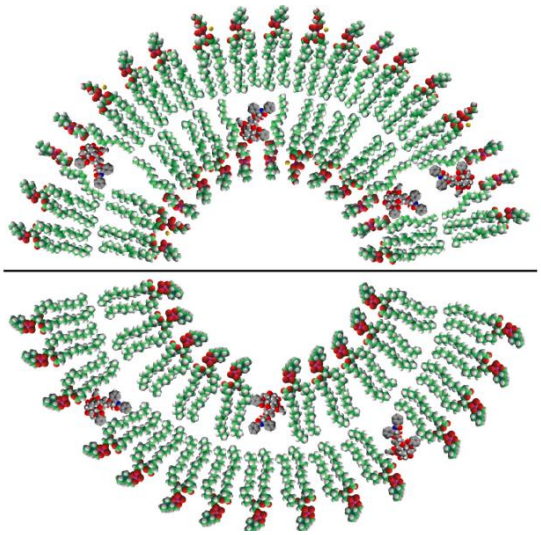

Figure 20: Arrangement of the encapsulated PTX in different lipid membrane bilayers of the liposomal carrier. This figure is adapted from[40].

Another strategy to enhance the loading capacity of IPI549 liposomes was to use DSPE-PEG2000 micelles. DSPE-PEG has been used to assemble either liposomes or micelles. In liposomes, it can induce the formation of hydrophobic cavities into the liposomes through fusion with lipid bilayers.[44] In this study, an attempt was made to enhance the liposome loading capacity of hydrophobic drugs by taking advantage of this property. During the hydration step, DSPE-PEG could form micelles and encapsulate part of the hydrophobic drug in 30 nanoseconds in the solution before the liposomes formed since the concentration of DSPE-PEG2000 during the hydration is much higher than its critical micelle concentration (CMC). (Figure 28) [45] Then the fusion of liposomes and drug-loaded micelles and drug transfer process is completed and followed by hydration and extrusion steps.

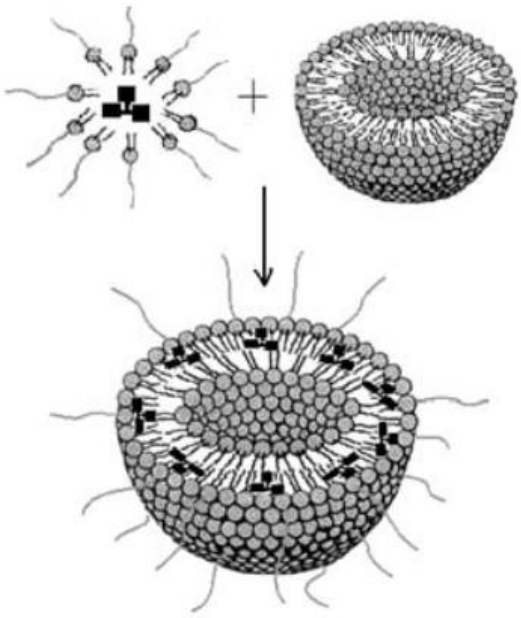

Figure 21: DSPE-PEG/ hydrophobic drug-loaded micelles added to the outer leaflet of preformed liposomes. This figure is adapted from[45].

4.3 CRV has the potential to direct PI3Kγ inhibitor target TAM

TAMs are a promising new target for tumor therapy.[46] It is a tumor-promoting phenotype of macrophages that forms approximately 50% of the tumor mass, and hybrids with tumor cells then contribute to tumor growth in many ways, such as immunosuppression, angiogenesis, and metastasis. [1, 47] Phosphoinositide-3-kinase gamma (PI3Kγ) is a highly expressed signal-transducing enzyme in TAMs, which plays an essential role in the pro-tumor function of these cells. Inhibiting its activity can lead to the expression of pro-inflammatory factors, then stimulate cytotoxic T cell activation, anti-tumor immunity, and promote tumor regression.[2]

Since the PI3kgama is expressed in both tumor and healthy tissues, we should concentrate the drug at the TAMs surface by cell surface markers. CD206 and folate receptor β are TAMs markers that have been widely used for ligand-directed delivery.[48, 49] However, these surface markers are nonspecific and could gather drug to the health macrophages, which will induce side effects.[50] CRV is a TAMs-targeting peptide. It is a novel cell surface marker and targeting tools, which can specifically bind to the RXRB receptor, which expresses in TAMs. After systemic administration, rapid and efficient homing of CRV decorated nanocarriers to the TAMs can be observed. [4] Since the PI3kγ expressed in both tumor and healthy tissues, CRV could be adopted as the ligand, which specifically targeting to TAMs and concentrates the IPI549 at TAMs. In this study, CRV was used to improve the delivery of IPI549 liposomes into solid tumors.

4.4 Exosomes targeting mediated by donor cells

Loading and targeting are the keys to the therapeutic potential of exosomes. Systemically delivered exosomes mainly accumulate in the kidney, liver, and spleen.[51] However, in most cases, including our study, therapeutic exosomes are more desirable to target the specific type of cells or tissue. Several factors determine the distribution of exosomes. Donor cells are one of them.

Exosomes express certain lipid and cellular adhesion molecules, which are the same as the parent cells that secrete them. It has been shown that exosomes have a natural targeting capability related to donor cells. In previous studies, miRNAs (miR-124) could be delivered into U87 glioblastoma cells by exosomes from mesenchymal stem cells. [52] Exosomes could take advantage of the different cell-binding receptor proteins or integrins expressed by different organs to fulfill their specific binding. For example, integrin αVβ5 targets exosomes exhibit tropism for the liver; α6β4

and α6β1target exosomes to the lung.[53, 54] This character has been considered to develop into a new strategy that utilized the exosomes derived from specific cells as a vehicle. [55]However, there are also potential risks that tumor or tumor-associated cells derived exosomes may induce the metastasis of tumors since it also expressed some molecules which we do not expect. Removing these molecules and do some modification on exosomes surfaces to avoid off-target effects is the right solution.

4.5 Decoration of targeting ligand on exosomes surface

Targeting exosomes can be acquired by constructing targeting molecules, such as antibody fragments or peptides, which can recognize the target antigens on the surface of exosomes. Targeting ligands are added to the surface of exosomes. The most common technique is to attach targeting proteins to donor cells, which will then secrete exosomes containing targeting proteins. In recent years, the most common means to achieve accurate targeting of exosomes is to use the Lactadherin C1C2 domain with synthetic tag and Lamp2b with synthetic tag.

The most widely used method to generate targeting exosomes is constructing antibodies or peptides, which can recognize the target antigens on the surface of the exosomes. Attaching targeting proteins onto donor cells and let them secrete exosomes containing targeting proteins is the most commonly used technique. It has been reported that the iRGD-targeting protein carrying immature dendritic cell exosomes, which loaded with doxorubicin, was found to have a specific effect on breast cancer cells, which express the αV integrin.[56]

Another method that could get a precise targeting of exosomes is combining exosomes and various nanoparticles by chemically editing exosomes membrane. Researchers loaded doxorubicin into

exosomes from A33 positive LIM1215cell. The surface carboxyl superparamagnetic iron oxide nanoparticles were coated with A33 antibody to bind to A33-positive exosomes so as to target A33-positive colon cancer cells.[57]

4.6 Decorate ligand on exosomes surface directly

Most of the exosome's modifications are based on the modification of donor cells. The changes of the donor cells may affect the protein composition and the function of exosomes. Directly decorate on the surface of exosomes is a solution. In our experiment, DSPE-PEG-Peptide was inserted into the membrane of exosomes directly, which could avoid the protein change, which induces by the donor cell modification. Based on the *in-vitro* study, it has been proved that peptide decoration does not affect exosomes binding to its target. And the plate binding study rule out the concern that exosomes will affect the peptide bond with its receptor. Exosomes combine with peptide prospects to attend a more accurate targeting effect.

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