

BIRC5 Promoter-Driven Nanodrugs Suppress BIRC5-Positive Cancers Independent of ABCB1 Status and IDO1 Expression

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Introduction: BIRC5 (survivin), an inhibitor of apoptosis protein, is overexpressed in most tumors and is associated with drug resistance, proliferation, and metastasis, while being largely undetectable in normal differentiated tissues. This unique expression pattern makes BIRC5 an exceptionally selective therapeutic target, offering the potential to maximize anticancer efficacy while minimizing systemic toxicity to healthy tissues. However, few BIRC5-targeted agents have advanced to late-stage clinical trials.

Methods: We developed two nanodrug formulations using poly-L-lysine–modified NH₂-Fe₃O₄ magnetite nanoparticles (PL-MNPs) for selective targeting of BIRC5-positive cancer cells. We further evaluated their anti-cancer efficacy in vitro and in vivo (zebrafish xenograft model), using cancer cell models that expressed BIRC5 and exhibited ABCB1-mediated drug resistance and IDO1-induced immune therapy insensitivity.

Results: The PL-MNPs delivered plasmids driven by the *BIRC5* promoter (*pBIRC5*) encoding either antisense *BIRC5* mRNA (*As-BIRC5*) or a dominant-negative BIRC5 protein (*dN-BIRC5*), for tumor-specific BIRC5 inhibition. These nanodrugs demonstrated robust in vitro and in vivo anti-cancer activity in multiple BIRC5-positive cell lines (MIA PaCa-2, NTUB1, NTU0.017, SK-OV-3, KB, and KB-TAX50). The activity was preserved across cancer types and independent of ABCB1-mediated drug resistance, while maintaining cancer cell specificity, and was not affected by IDO1 expression, a factor associated with poor responses to immune therapy. PL-MNP uptake was partially mediated by clathrin-dependent endocytosis, with acidic intracellular environments facilitating efficient plasmid release. Conjugation of nanoparticles with Herceptin® (trastuzumab) significantly increased cellular uptake and anticancer activity, especially in clathrin-deficient SK-BR-3 cells that overexpress ERBB2.

Conclusion: These findings establish that the easily synthesized PL-MNP-*pBIRC5*/*As-BIRC5* and PL-MNP-*pBIRC5*/*dN-BIRC5* nanodrugs have strong potential to overcome BIRC5- and ABCB1-related drug resistance, representing a broadly applicable strategy against various malignancies. While the size of our nanodrug (~400 nm in hydrodynamic diameter) is compatible with reported effective nanoparticle sizes in some models, the extent to which the enhanced permeability and retention (EPR) effect contributes to tumor accumulation in human cancers remains uncertain and will require validation in more clinically relevant models and imaging modalities.

Keywords: ABCB1, BIRC5, ERBB2, HER2, survivin, iron oxide nanoparticle

Introduction

BIRC5 (Survivin) is a distinctive member of the inhibitor of apoptosis protein (IAP) family because of its widespread overexpression in a range of tumors, including colorectal, breast, lung, and liver cancers. It is undetectable or minimally

expressed in differentiated normal tissues (Figure S1A).^{1–3} Clinically, high BIRC5 expression is associated with poor prognosis across multiple cancer types (Figure S1B).^{4,5} At the cellular and molecular levels, BIRC5 inhibits caspase activity and is vital for the survival of cancer cells in different tumors (Figure S2).⁶ Its upregulation promotes tumor formation, metastasis, and resistance to chemotherapy.^{7–9} Although BIRC5 represents a promising target for cancer treatment, it is often considered “semi-druggable”,¹⁰ and only a limited number of BIRC5-targeting agents, such as small molecule inhibitors, locked nucleic acid-based antisense oligonucleotides, and vaccines, have reached Phase I clinical trials in recent years.^{11–14} Gene-based strategies targeting *BIRC5*, such as antisense oligonucleotides LY2181308 (18-mer) and SPC3042 (16-mer), were developed to suppress its expression in tumor cells. Despite promising preclinical results, clinical development of these agents was halted owing to limited efficacy, off-target effects, and severe adverse events.^{12,15} More broadly, translation of gene therapeutics to the clinic remains challenging, primarily due to inefficient tumor delivery, rapid systemic clearance, and poor specificity. These shortcomings highlight the urgent need for advanced delivery platforms and strategies that enhance stability, targeting, and precision in *BIRC5*-directed gene therapies.^{16,17}

Given BIRC5's unique expression and roles in cancer, a molecular therapeutic targeting the BIRC5 pathway, whose activation is driven by the *BIRC5* promoter, could enable cancer cell-specific activation and potent pro-apoptotic effects, providing a potential strategy for anticancer therapy. Compared with emerging strategies such as mRNA therapeutics and CRISPR-based genome editing, plasmid DNA offers several advantages. Plasmid DNA allows promoter-driven transcriptional regulation, enabling therapeutic gene expression to be selectively activated in *BIRC5*-expressing cancer cells. In contrast, mRNA therapeutics generally undergo immediate translation following cytoplasmic delivery and therefore lack tumor-specific control. Furthermore, while CRISPR-based approaches provide powerful genome-editing capabilities, they induce permanent genomic alterations and raise concerns about off-target editing. In comparison, plasmid DNA functions as a non-genome-integrating, transient expression system, thus offering a safer and more controllable platform for the development of BIRC5-targeted gene therapies.

We previously developed a plasmid DNA construct (p*BIRC5*/As-*BIRC5*) in which the *BIRC5* promoter drives expression of full-length antisense *BIRC5*. This construct induces apoptosis in MDA-MB-231 and PANC-1 cancer cells, but not in human umbilical vein endothelial cells, in vitro.¹⁸ We also generated a histidine-tagged, dominant-negative BIRC5 protein containing T34A and C84A mutations (T34A-C84A-dN-BIRC5-His) using bacterial expression. Transfection with this recombinant protein reduced the viability of MDA-MB-231 and MIA PaCa-2 cancer cells, also in vitro.¹⁹ The T34A mutation disrupts a key phosphorylation site in BIRC5, thereby activating the intrinsic apoptotic pathway and promoting tumor cell death, while the C84A mutation further impairs BIRC5's anti-apoptotic function. Despite their potent in vitro activity, the short half-life and poor stability of these agents have hindered their translation into effective in vivo therapy.

Iron oxide magnetic nanoparticles (MNPs) have been approved by the U.S. Food and Drug Administration (FDA) for clinical applications in magnetic resonance imaging and the treatment of iron-deficiency anemia.²⁰ Poly-L-lysine (PL), a well-established biocompatible and biodegradable polymer, is frequently used in nanoparticle synthesis due to its ability to promote stable nanoparticle formation and improve bioavailability.²¹ PL-based nanoparticles have been shown to protect plasmid DNA from nuclease-mediated degradation, thus enhancing gene delivery efficiency.²² Although PL coating can accelerate blood clearance through enhanced immune recognition, it also promotes cellular uptake and DNA delivery, improving therapeutic effectiveness at target sites.²³ Furthermore, lysosomal degradation of PL-MNPs into biocompatible iron ions supports favorable long-term pharmacokinetic safety.²⁴

Zebrafish (*Danio rerio*), a vertebrate model that bridges invertebrate and mammalian models in developmental biology and cancer research,²⁵ shares approximately 70% genetic homology with humans (82% for disease-related genes) and offers high fecundity, external fertilization, and optical transparency for real-time cellular imaging.²⁶ Their immature adaptive immune system supports efficient xenotransplantation of human cancer cells, enabling non-invasive monitoring of tumor growth, metastasis, angiogenesis, and drug responses without graft rejection.²⁷ In line with FDA initiatives promoting alternative models for drug evaluation (eg., AI-based approaches and organoids), the zebrafish xenograft model provides a rapid and cost-effective platform for preclinical anticancer drug testing. This model is particularly well-suited for evaluating nanoparticle-based therapeutics, as it allows real-time assessment of tumor targeting, therapeutic efficacy, and safety in a vertebrate context.

In this study, we designed and characterized two novel polymeric magnetite nanodrugs (PL-MNPs) for BIRC5-targeting. Both formulations utilize a PL coating and encapsulate plasmid DNA encoding a therapeutic agent under the control of the *BIRC5* promoter for cancer cell-specific activation: either antisense *BIRC5* mRNA (As-*BIRC5*) or a T34A-C84A dominant-negative BIRC5 protein (dN-BIRC5). These PL-MNPs displayed favorable physicochemical properties and potent anticancer activity in BIRC5-expressing cancer cells, regardless of the multidrug resistance protein ATP-binding cassette subfamily B member 1 (ABCB1/MDR1/P-gp) and indoleamine 2,3-dioxygenase 1 (IDO1) expression. They also remained non-toxic to differentiated, BIRC5-non-expressing normal cells, both in vitro and in vivo (zebrafish xenograft model). We demonstrated that a simple surface modification strategy, such as conjugation with cell surface protein-specific antibodies, can significantly enhance cellular uptake and antitumor efficacy of these PL-MNPs, particularly in clathrin-mutated cancer cells in vitro and in vivo.

Materials and Methods

Cell Lines and Cell Culture Conditions

Human cancer cell lines MIA PaCa-2 (pancreatic carcinoma) (ATCC, cat. no. CRL-1420TM), KB (cervical carcinoma) (ATCC, cat. no. CRL-3596TM), and SK-BR-3 (breast adenocarcinoma) (ATCC, cat. no. HTB-30TM) were obtained from the American Type Culture Collection (ATCC) (Table 1). MIA PaCa-2 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, cat. no. 31800-022) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, cat. no. 04-001-1A) and penicillin/streptomycin/glutamine (PSG) (Biological Industries, cat. no. 03-031-01B). KB cells were cultured in RPMI 1640 medium containing 5% FBS and PSG. The multidrug-resistant KB-TAX50 subline, which overexpresses ABCB1, was generated by paclitaxel selection as previously described^{18,28} and cultured in medium containing 50 nM paclitaxel. The ovarian cancer cell line SK-OV-3 was kindly provided by Dr. Meng-Ru Shen (Department of Pharmacology, National Cheng Kung University, Tainan, Taiwan) and cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Merck, cat. no. SAB-56498C) supplemented with 10% FBS and PSG. SK-BR-3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (BioConcept, cat. no. 1-26p02-L) supplemented with 10% FBS and PSG. The bladder cancer cell line NTUB1 was kindly provided by Dr. Jang-Yang Chang (Taipei Cancer Center, Taipei Medical University, Taipei, Taiwan) and cultured in RPMI 1640 medium containing 10% FBS and PSG. The multidrug-resistant NTU0.017 subline, which overexpresses ABCB1, was generated by paclitaxel selection and cultured in medium containing 17 nM paclitaxel. Human dermal microvascular endothelial cells (HMEC-1), kindly provided by Dr. Ben-Kuen Chen (Department of Pharmacology, National Cheng Kung University, Tainan, Taiwan), were maintained in MCDB131 medium (Sigma-Aldrich, cat. no. M8537) supplemented with 5% FBS, PSG, and 10 ng/mL epidermal growth factor (EGF) (Table 1). The WI-38 VA-13 subline 2RA human lung fibroblast cells were kindly provided by Dr. Wen-Tai Chiu (Department of Biomedical

Table 1 Anticancer Potency of p*BIRC5* Plasmid DNA-Adsorbed PL-MNPs Is Unaffected by ABCB1 Expression in Cancer Cells

Cell line	Tissue Origin	Cell type	ABCB1 Status
MIA PaCa-2	Pancreas	Cancer	Negative
KB	Cervix	Cancer	Negative
KB-TAX50	Cervix	Cancer	Expressing
NTUB1	Bladder	Cancer	Negative
NTU0.017	Bladder	Cancer	Expressing
SK-BR-3	Breast	Cancer	Negative
SK-OV-3	Ovary	Cancer	Negative
MCF 10A	Breast	Normal	Negative
HMEC-1	Capillary	Normal	Negative
WI-38 VA-13 subline 2RA	Lung	Normal	Negative
HaCaT	Skin	Normal	Negative
THP-1	Peripheral blood	Normal	Negative

Engineering, National Cheng Kung University, Tainan, Taiwan) and cultured in Minimum Essential Medium (MEM) (Gibco, cat. no. 11095080) containing 10% FBS and PSG. MCF 10A cells were kindly provided by Dr. Chih-Yang Wang (Ph.D. Program for Cancer Molecular Biology and Drug Discovery, Taipei Medical University, Taipei, Taiwan) and cultured in MEGM[®] Mammary Epithelial Cell Growth Medium (Lonza, cat. no. CC-3150). HaCaT cells were kindly provided by Dr. Chao-Kai Hsu (Department of Dermatology, National Cheng Kung University, Tainan, Taiwan) and cultured in DMEM containing 10% FBS and PSG. THP1 cells were kindly provided by Dr. Tzeng-Horng Leu (Department of Pharmacology, National Cheng Kung University, Tainan, Taiwan) and cultured in RPMI containing 10% FBS and PSG. All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Regular mycoplasma testing confirmed that the cell cultures were free of contamination. The use of these human cell lines was approved by the review board of the Ministry of Science and Technology (Taiwan) and the biosafety committee of National Cheng Kung University (Taiwan).

Kaplan-Meier Survival Analysis

The overall survival of patients with pancreatic, lung, breast, bladder, and cervical cancers, stratified by *BIRC5* mRNA expression levels (Affymetrix ID: 202094_at), was analyzed using the Kaplan–Meier Plotter (<https://kmplot.com/analysis/>) with publicly available data from the Cancer Genome Atlas (TCGA) database.²⁹

Plasmid Construction

The mammalian expression vector pDRIVE-h*BIRC5* (InvivoGen, cat. no. pdrive-hSurvivin), which contains the human *BIRC5* promoter driving the *LacZ* gene (a *BIRC5* expression-specific luciferase reporter system), was used as the vector backbone in this study (Figure S3). The control plasmid DNA, designated p*BIRC5*/Emp (Figure S3), was generated by removing the *LacZ* gene through double digestion with the restriction enzymes *Bsp*HI (Thermo Fisher Scientific, cat. no. FD1284) and *Eco*RI (Thermo Fisher Scientific, cat. no. FD0274). Then, a short DNA fragment containing multiple cloning sites was inserted into the pDRIVE-h*BIRC5* vector between the *Bsp*HI and *Eco*RI sites. The inserted sequence was 5'-CATGAGCGATCGCGGATCCACGCGTTAAG-3', which includes recognition sites for *Bsp*HI, *Asi*SI, *Bam*HI, *Mlu*I, and *Eco*RI restriction enzymes. Polymerase chain reaction (PCR) amplification was performed to obtain the *BIRC5*^{A100G-TG250GC} gene fragment, which encodes the T34A-C84A dominant-negative *BIRC5* protein. To facilitate cloning, the original *Asi*SI and *Mlu*I restriction sites present in the template plasmid pCMV6-AC-GFP-T34A-C84A dN-*BIRC5*³⁰ were replaced with *Bsp*HI and *Eco*RI sites, respectively. The PCR primers used were: forward 5'-AATCATGATGGGTGCCCCGACGTTGCCCCC-3' and reverse 5'-AAGAATTCTCAATCCATGGCAGCCAGCTGCTCGATGGCACG-3' (with the *Bsp*HI and *Eco*RI sites underlined). The PCR product (*BIRC5*^{A100G-TG250GC}) was initially cloned into the pJET1.2 vector. The presence of the inserted restriction sites and the sequence integrity of *BIRC5*^{A100G-TG250GC} were confirmed by DNA sequencing. Next, the *BIRC5*^{A100G-TG250GC} fragment was excised from pJET1.2 by using *Bsp*HI and *Eco*RI and then subcloned into the lacZ-deleted pDRIVE-h*BIRC5* vector. The resulting construct, called p*BIRC5*/dN-*BIRC5*, which contains the human *BIRC5* promoter and the *BIRC5*^{A100G-TG250GC} gene, was transformed into *Escherichia coli* DH5α competent cells for plasmid propagation and long-term storage.

DNA Transfection

Lipofectamine[™] 3000 (Thermo Fisher Scientific, cat. no. L3000015) was used to transfect plasmid DNA, purified with the ZymoPURE[™] II Plasmid Maxiprep Kit (Zymo Research, cat. no. D4203), into MIA PaCa-2, KB, KB-TAX50, HMEC-1, and WI-38 VA-13 subline 2RA cells. Cells were seeded in 96-well plates or 60-mm dishes and allowed to adhere overnight. Lipofectamine[™] 3000 was diluted in Opti-MEM[™] medium (Gibco, cat. no. 31985) without serum or antibiotics, and plasmid DNA was also diluted in Opti-MEM[™] without serum or antibiotics. The diluted Lipofectamine[™] 3000 and plasmid DNA were mixed at a 1:1 ratio and incubated at room temperature for 20 min. The transfection mixture was subsequently added to the cells in PSG-free culture medium for the indicated duration.

Beta-Galactosidase Staining

Cells were seeded in 6-well plates and cultured overnight. The following day, cells were transfected with pBIRC5/Emp or pBIRC5/LacZ plasmids for 24 h. β -Galactosidase activity was then assessed using the β -Galactosidase Staining Kit (Cell Signaling Technology, cat. no. 9860). Briefly, cells were fixed with Fixation Solution for 15 min at room temperature, rinsed with PBS, and incubated with β -Galactosidase Staining Solution for 16 h at 37°C. Stained cells were imaged by bright-field microscopy.

In vitro Cell Viability Assay

Cells were seeded at a density of 1,700 cells per well in 96-well plates and allowed to adhere overnight. The next day, cells were transfected with plasmid DNA or treated with plasmid DNA-loaded nanoparticles and incubated for 96 h. Subsequently, 200 μ L of MTT solution (VWR Life Science, cat. no. 0793), diluted 1:10 in phenol red-free RPMI medium, was added to each well and incubated for 4 h at 37 °C. The medium was then replaced with 100 μ L of MTT lysis buffer (500 mL/L dimethylformamide, 100 g/L sodium dodecyl sulfate) and incubated for 12 h. Absorbance was measured at 570 nm using a SpectraMax iD3 multi-mode microplate reader (Molecular Devices). Results for cell viability are presented relative to the untreated control (100%). All treatments were performed in duplicate wells and repeated in at least three independent experiments. The IC₅₀ value is defined as the concentration required to inhibit 50% of cells viability.

WST-1 Cell Proliferation Assay

Cells were seeded at a density of 1,700 THP-1 cells per well in 96-well plates and cultured overnight. The next day, cells were treated with plasmid DNA-loaded nanoparticles and incubated for 96 h. Ten microliters of WST-1 solution (Takara Bio, cat. no. MK400) was added to each well and incubated for 4 h at 37 °C. Absorbance was measured at 440 nm using a SpectraMax iD3 multi-mode microplate reader. Cell viability was calculated relative to untreated control cells, set at 100%. All treatments were performed in duplicate wells and repeated in at least three independent experiments.

Western Blot Analysis

Cells were lysed with CelLytic™ M cell lysis reagent (Sigma Aldrich, cat. no. C2978) supplemented with 1 mM phenyl-methylsulfonyl fluoride, 1 mM sodium fluoride, and a protease inhibitor cocktail (Roche, cat. no. 05892791001). Protein concentrations were measured using the BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein were resolved by SDS-PAGE and transferred to PVDF membranes (Merck Millipore). Membranes were blocked for 1 h at room temperature in 5% non-fat dry milk prepared in TBS containing 0.05% Tween-20 (TBST) and then incubated overnight at 4 °C with primary antibodies: anti-BIRC5 (R&D Systems, cat. no. AF886), anti-ACTA1 (Merck Millipore, cat. no. MAB1501), anti-BIRC4 (R&D Systems, cat. no. AF8221), anti- γ -H2AX (Merck Millipore, cat. no. 05-636), anti-CASP3 (GeneTex, cat. no. GTX110543), anti-PARP (Cell Signaling Technology, cat. no. 9532), and anti-IDO1 (GeneTex, cat. no. GTX634652). After washing three times with TBST, the membranes were incubated for 1 h at room temperature with the appropriate HRP-conjugated secondary antibody: goat anti-rabbit IgG (Merck Millipore, cat. no. AP132P), goat anti-mouse IgG (Merck Millipore, cat. no. AP124P), or rabbit anti-goat IgG (Merck Millipore, cat. no. AP106P). Protein bands were visualized using enhanced chemiluminescent reagents and detected with a FUJI LAS-1000plus luminescence imaging system (Fujifilm, Tokyo, Japan). All Western blot experiments were performed at least three times.

Fluorescence Resonance Energy Transfer (FRET) Protein-Protein Interaction Assay

Plasmids encoding pCMV6-mRFP-BIRC5 (receptor) and pCMV6-mGFP-dN-BIRC5 (donor) were co-transfected into MIA PaCa-2 cells to evaluate the interaction between wild-type BIRC5 and dominant-negative BIRC5 (dN-BIRC5). Briefly, MIA PaCa-2 cells were seeded onto coverslips and allowed to adhere overnight. Then, cells were co-transfected with pCMV6-mRFP-BIRC5 and pCMV6-mGFP-dN-BIRC5 using Lipofectamine™ 3000 transfection reagent, following the manufacturer's instructions, and incubated for 48 h. After transfection, cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Nuclei were counterstained with mounting medium containing DAPI. Fluorescence images were captured using a confocal microscope (FV1000, Olympus) to visualize the protein-protein interaction via FRET signals.

Preparation of Plasmid DNA-Absorbed Poly-L-Lysine-Modified $\text{NH}_2\text{-Fe}_3\text{O}_4$ Magnetite Nanoparticles (PL-MNPs)

$\text{NH}_2\text{-Fe}_3\text{O}_4$ magnetite nanoparticles (MNPs) were synthesized following previously described co-precipitation methods.^{31,32} Briefly, 1 M ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) and 2 M ferrous chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$) solutions were individually prepared by dissolving the salts in 2 M hydrochloric acid (HCl). The FeCl_3 solution was mixed with the FeCl_2 solution in a 4:1 volume ratio, followed by the addition of 1 mL glycine solution (0.5 g/mL). The mixture was vigorously stirred, and 5 M sodium hydroxide (NaOH) was added dropwise until the solution turned black. Stirring continued for an additional 15 min at room temperature. The precipitates were collected using a permanent magnet, washed several times with deionized water, and resuspended. Next, 3 g of glycine dissolved in 50 mL of HCl was added to the washed precipitates, stirred for 5 min, and sonicated for 30 min. Subsequently, a mixture of deionized water and acetone (volume ratio 5:2:3) was added, followed by centrifugation. The precipitates were washed with 7 mL of deionized water and 3 mL of acetone sequentially via centrifugation. The resulting $\text{NH}_2\text{-Fe}_3\text{O}_4$ MNPs were dispersed in deionized water, and the iron concentration was quantified by inductively coupled plasma analysis.

To conjugate poly-L-lysine to the $\text{NH}_2\text{-Fe}_3\text{O}_4$ MNPs, 400 μL of 55% (w/w) glutaraldehyde was mixed with 100 μL of 0.1 mM MNP suspension and stirred for 4 h at room temperature. Then, 5 mL of 1% (w/v) low molecular weight poly-L-lysine (molecular weight 1,000–5,000) was added, followed by stirring for 30 min at room temperature. The mixture was centrifuged at 14,000 rpm for 10 min, and the resulting poly-L-lysine-modified MNPs (PL-MNPs) were washed twice with deionized water. The final PL-MNPs were resuspended in deionized water and stored until use. For plasmid DNA absorption, 500 ng/ μL plasmid DNA was incubated with 0.22 μM MNPs or PL-MNPs. Subsequently, deionized water and rhodamine 6G (R6G) were added to the mixture to achieve a final R6G concentration of 10 μM . The volume ratio of plasmid DNA (500 ng/ μL):MNPs or PL-MNPs (0.22 μM):deionized water was 2:1:7. The solution was stirred at room temperature for at least 30 min and then incubated at 4°C for 16 h with constant stirring to ensure stable DNA absorption onto the nanoparticles.

Physical Characterization of Plasmid DNA-Loaded MNPs, PL-MNPs, and Her-PL-MNPs

The hydrodynamic size of three batches of plasmid DNA (*pBIRC5/Emp*, *pBIRC5/As-BIRC5*, and *pBIRC5/dN-BIRC5*)-loaded MNPs, PL-MNPs, and Her-PL-MNPs was measured using dynamic light scattering (DLS) with a DelsaNano C instrument (Beckman Coulter). The zeta potential of the three batches of plasmid DNA-loaded MNPs, PL-MNPs, and Her-PL-MNPs was determined using an ELSZ-2000 zeta potential analyzer (Photal Otsuka). The surface morphology and shape of the nanoparticles were examined using scanning electron microscopy (SEM) with a JSM-7001F instrument (JEOL), operated at an accelerating voltage of 10 kV and a working distance of 9 mm. For SEM analysis, nanoparticle suspensions were drop-cast onto copper stubs and air-dried at room temperature. To obtain nanoscale in-situ information on particle size and elemental composition, liquid transmission electron microscopy (liquid TEM) and energy-dispersive X-ray spectroscopy (EDS) were performed by Liquid View Technology (services provided by Liquid View Technology).^{33,34} Plasmid DNA-loaded PL-MNPs were loaded into microcapsules fabricated using microelectromechanical systems and sealed to prevent solution leakage. Imaging was conducted on a JEM-2100F transmission electron microscope (JEOL) at an accelerating voltage of 200 kV.

In vitro Plasmid DNA Release Assay

The release profile of plasmid DNA from plasmid-loaded PL-MNPs was assessed under various pH conditions. Briefly, 15 μg of plasmid DNA-loaded PL-MNPs were suspended in phosphate-buffered saline (PBS) at pH 4.0, 5.0, 6.0, or 7.4 and incubated for 1, 3, 6, 9, 12, or 24 h at room temperature. After incubation, the PL-MNPs were separated from the supernatant using a permanent magnet, and the supernatant was transferred to a new microcentrifuge tube. The concentration of released plasmid DNA in the supernatant was measured spectrophotometrically with a spectrophotometer (MaestroGen, Inc). The total amount of plasmid DNA released (μg) was calculated according to

the equation: Amount of plasmid DNA released (μg)=concentration of plasmid DNA ($\mu\text{g}/\mu\text{L}$) $\times$ 1,000 μL . The release percentage was determined using the formula:

$$\% \text{ of releasing} = \frac{\text{Amount of plasmid DNA releasing } (\mu \text{ g})}{\text{Total amount of plasmid DNA loaded } (\mu \text{ g})} \times 100$$

DNase I Protection Assay

One microgram of plasmid DNA or plasmid DNA-loaded PL-MNPs was incubated with 1 unit of DNase I at 37°C for 15, 30, 60, 120 and 180 min in a total volume of 10 μL . The enzymatic digestion was inactivated by heating the samples at 75°C for 10 min. The integrity of the plasmid DNA was then evaluated through electrophoresis on a 0.8% agarose gel at 75 V.

LDH Cytotoxicity Assay

Cell cytotoxicity was evaluated using an LDH release assay. MIA PaCa-2, KB, and KB-TAX50 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated overnight. The following day, cells were treated with endocytosis inhibitors, including cytochalasin D (10 μM), wortmannin (50 nM), colchicine (10 μM), monodansylcadaverine (MDC, 200 μM), or chloroquine (125 μM), for 24 h. Cytotoxicity was measured using the CytoScan™ LDH Cytotoxicity Assay kit (G Biosciences, cat. no. 786–210) according to the manufacturer's instructions. Briefly, following treatment, cells were incubated with lysis buffer at 37 °C in a humidified 5% CO_2 atmosphere for 45 min. The supernatant was then collected after centrifugation. Subsequently, the supernatant was mixed with the Substrate Mix solution and incubated at room temperature for 30 min. The reaction was terminated by adding the stop solution, and the absorbance was measured at 490 nm using a microplate reader.

RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was extracted from cultured cells using TRIzol™ reagent (Thermo Fisher Scientific, cat. no. 15596018) following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg of RNA with the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, cat. no. K1632) according to the provided protocol. Quantitative real-time PCR was performed with Fast SYBR Green Master Mix (Applied Biosystems, cat. no. 4385612) on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). The relative mRNA levels of *BIRC5* were determined using gene-specific primers: human *BIRC5* forward 5'-CTGCCTGGCAGCCCTTT-3', and reverse 5'-CCTCCAAGAAGGGCCAGTTC-3'; human *ACTA1* forward 5'-GGCGGCACCACCATGTACCCT-3', and reverse 5'-AGGGGCCGGAAGTCTGTCATACT-3'. Data analysis was conducted using the comparative threshold cycle ($2^{-\Delta\Delta\text{Ct}}$) method, where $\Delta\text{Ct} = \text{Ct}_{\text{target gene}} - \text{Ct}_{\text{ACTA1}}$ and $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{treatment}} - \Delta\text{Ct}_{\text{control}}$. All experiments were performed in at least three biological replicates.

In situ Proximity Ligation Analysis (PLA)

In situ PLA was performed to visualize protein-protein interactions in MIA PaCa-2 cells. Cells were seeded onto cover slides and allowed to adhere overnight. The next day, cells were treated with $1 \times \text{IC}_{50}$ concentrations of PL-MNP-p*BIRC5*/Emp and PL-MNP-p*BIRC5*/dN-BIRC5 for 72 h. After treatment, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Fixed cells were permeabilized with PBS containing 1% Triton X-100 for 30 min, then blocked in Blocking Solution at 37°C for 1 h. Cells were incubated overnight at 4°C with primary antibodies targeting the proteins of interest. The next day, cells were washed twice with washing buffer and incubated with PLA probes diluted 1:5 in antibody diluent for 1 h at 37°C. Subsequently, the ligation solution was added, and the cells were incubated at 37°C for 30 min, followed by incubation with the amplification solution at 37°C for 100 min. Finally, cells were mounted with Duolink in situ mounting medium containing DAPI for 10 min at room temperature. Fluorescence images were acquired using a confocal microscope (FV1000, Olympus).

Cell Cycle Analysis by Flow Cytometry

Cells were treated with $1 \times IC_{50}$ concentrations of PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, or PL-MNP-pBIRC5/dN-BIRC5 for 72 h. After treatment, cells were harvested, washed with PBS, and fixed in ice-cold 70% ethanol at 4°C for 16 h. Fixed cells were washed again with PBS and subsequently stained with a propidium iodide (PI) solution containing RNase A for 30 min at room temperature, following standard protocols. A minimum of 10,000 PI-stained cells per sample were analyzed using a CytoFLEX flow cytometer (Beckman Coulter; excitation 488 nm, emission 535 nm). Cell cycle distribution was determined using CytoExpert software (Beckman Coulter). All experiments were performed independently at least three times.

Preparation of Herceptin[®] (Trastuzumab)-Conjugated PL-MNPs

Trastuzumab-conjugated PL-MNPs were prepared through carbodiimide-mediated coupling with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS). Briefly, Trastuzumab was incubated with EDC and NHS at a molar ratio of 1:20:50 (antibody: EDC: NHS) for 15 min at room temperature. Next, $10 \times$ PBS was added to adjust the pH to 7.4. PL-MNPs were then added to the reaction mixture and incubated for 2 h at room temperature. To quench the reaction, glycine was added, and the mixture was incubated for an additional 30 min at room temperature. PL-MNPs were separated using a magnetic separator, and the supernatant was discarded. The particles were washed and resuspended with double-distilled water (d_2H_2O) before use. Trastuzumab was obtained from the pharmacy of National Cheng Kung University Hospital (Tainan, Taiwan) and manufactured by Genentech.

In vivo Drug Potency Evaluation

All animal experiments were carried out following protocols approved by the Institutional Animal Care and Use Committee (IACUC) of National Cheng Kung University. The zebrafish xenograft model was used to assess the anticancer effectiveness of PL-MNP-pBIRC5/As-BIRC5 and PL-MNP-pBIRC5/dN-BIRC5 against MIA PaCa-2, KB, KB-TAX50, and SK-BR-3 cancer cell lines. Wild-type zebrafish embryos (strain AB) were obtained from the Laboratory Animal Center, College of Medicine, National Cheng Kung University. Human cancer cells were labeled with PKH67 fluorescent dye for visualization under a fluorescence microscope (Leica, Germany). At 48 h post-fertilization (hpf), zebrafish embryos were anesthetized with tricaine and transplanted with fluorescently labeled cancer cells into the yolk sac. For the MIA PaCa-2, KB, and KB-TAX50 cell lines, 500 cells were transplanted per embryo. After confirming the transplantation of fluorescence-labeled cells into embryos with a fluorescence microscope, the embryos were randomly assigned to experimental groups. To ensure sufficient statistical power based on previous studies, each group consisted of 24 embryos. At 24 h post-transplantation, embryos received an injection of PBS or the indicated concentrations of PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, or PL-MNP-pBIRC5/dN-BIRC5 into the yolk sac. For SK-BR-3 cells, 750 cells were transplanted per embryo, followed by drug or PBS injection 1 h post-transplantation. After drug administration, embryos were kept at 35°C for 48 h. Tumor size and body length were measured every 12 h using fluorescence microscopy. At the end of experiments (120 hpf), zebrafish embryos were anesthetized with tricaine until unresponsive and subsequently euthanized using 1% bleach, in accordance with IACUC guidelines and standard zebrafish euthanasia protocols. The sizes of tumors and swim bladders in the control and treated embryos were quantified by analyzing bright-field images with ImageJ. The fluorescence signal area was also measured with ImageJ.

In vivo Hepatotoxicity Evaluation

The zebrafish hepatotoxicity assay was performed by the Taiwan Zebrafish Core Facility at the National Health Research Institute. In brief, 48 h post-fertilization (hpf) transgenic zebrafish embryos (fabp10a:mCherry) were exposed to the specified concentrations of PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, and PL-MNP-pBIRC5/dN-BIRC5 ($n = 24$ per group) for 48 h. Liver size was then evaluated using fluorescence microscopy.

Statistical Analysis

All experiments were performed thrice unless otherwise specified. Data are presented as the mean $\pm$ standard deviation (SD). For multiple-group comparisons, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was applied. For the in vivo drug potency evaluation studies involving multiple groups and factors, two-way ANOVA with Tukey's post hoc test was used. Statistical analyses were performed using GraphPad Prism version 10 (GraphPad Software, Inc., USA). A p-value < 0.05 was considered statistically significant.

Results

Liposomal Delivery of pBIRC5/dN-BIRC5 Induces Cytotoxicity in BIRC5-Expressing Cancer Cells

Previously, we constructed a plasmid DNA, pBIRC5/As-BIRC5, in which the BIRC5 promoter (pBIRC5) drives the expression of a full-length antisense BIRC5 (As-BIRC5) sequence (Figure 1A). We demonstrated that activation of the BIRC5 promoter on the plasmid DNA correlates with endogenous BIRC5 transcription levels across cancer cell types, supporting its cancer type-specific regulation. Liposomal delivery (*ie.*, transfection) of this plasmid effectively down-regulates BIRC5 protein expression and induces apoptosis in various cancer cells.¹⁸ In the current study, we generated an additional plasmid, pBIRC5/dN-BIRC5, which encodes a pBIRC5-driven “BIRC5 T34A-C84A dominant-negative mutant protein”-expressing sequence (dN-BIRC5) (Figure 1A).

The BIRC5 protein, which contains a single Baculovirus IAP Repeat (BIR) domain, forms homodimers in cells.³⁵ It interacts with HSP90 via its BIR domain, thereby preventing BIRC5 degradation.³⁶ Disrupting the BIR domain with the C84A mutation promotes proteasome-mediated degradation of BIRC5.³⁷ Therefore, prior to assessing the function of pBIRC5/dN-BIRC5, we examined whether the dN-BIRC5 protein (*ie.*, the pBIRC5/dN-BIRC5 expression product) can bind to wild-type (WT) BIRC5, triggering its degradation via heterodimerization. Protein–protein interaction modeling showed that the BIR domain-disrupted (T34A-C84A) dN-BIRC5 protein binds to WT-BIRC5, with prediction quality scores of iPTM = 0.79 and pTM = 0.81 (AlphaFold; alphafoldserver.com) (Figure 1B). To experimentally confirm the interaction, we performed ectopic expression of tagged proteins (dN-BIRC5 and WT-BIRC5) followed by fluorescence resonance energy transfer (FRET) analysis. FRET results showed that ectopically expressed mGFP-tagged dN-BIRC5 physically interacts with mRFP-tagged WT-BIRC5 in transfected MIA PaCa-2 pancreatic cancer cells, confirming the binding capability of dN-BIRC5 to WT-BIRC5 (Figure 1C).

The cellular and molecular effects of pBIRC5/dN-BIRC5 were next evaluated. Similar to liposomal delivery of pBIRC5/As-BIRC5 (the positive control), delivery of pBIRC5/dN-BIRC5 reduced viability in BIRC5-expressing (*BIRC5*⁺) MIA PaCa-2 cells but had no effect on BIRC5-non-expressing (*BIRC5*[−]) HMEC-1 endothelial and WI-38 VA-13 subline 2RA fibroblast cells (Figure 1D). BIRC5 interacts with and stabilizes BIRC4 (XIAP, another member of the IAP family),³⁸ thereby preventing apoptosis and promoting DNA repair.³⁹ Similar to liposomal delivery of pBIRC5/As-BIRC5, delivery of pBIRC5/dN-BIRC5 reduced BIRC4 expression, increased γ -H2AX levels, and induced PARP cleavage in *BIRC5*⁺ MIA PaCa-2 cells (Figure 1E).

Physicochemical Characterization of pBIRC5/As-BIRC5 and pBIRC5/dN-BIRC5 Loaded Nanoparticles

Poly-L-lysine is a biocompatible, biodegradable polypeptide widely used to enhance nucleic acid delivery. To improve the therapeutic potential of pBIRC5/dN-BIRC5, we synthesized a series of amino (NH₂)-modified iron oxide-based magnetic nanoparticles (MNPs) loaded with either pBIRC5/As-BIRC5 (positive control) or pBIRC5/dN-BIRC5. Additionally, poly-L-lysine-modified amino (NH₂)-modified iron oxide-based magnetic nanoparticles (PL-MNPs) carrying the same plasmids were prepared (Figure S4A).

Dynamic light scattering (DLS) analysis showed that the hydrodynamic diameters of MNP-pBIRC5/Emp (containing the empty pBIRC5 plasmid), MNP-pBIRC5/As-BIRC5, and MNP-pBIRC5/dN-BIRC5 were 106 nm, 94 nm, and 110 nm, respectively (Table 2). The mean zeta (ζ) potentials for these nanoparticles were −1.7 mV, 1.8 mV, and 0.67 mV, respectively (Table 2). Surface polymer modification is known to improve encapsulation efficiency and loading capacity

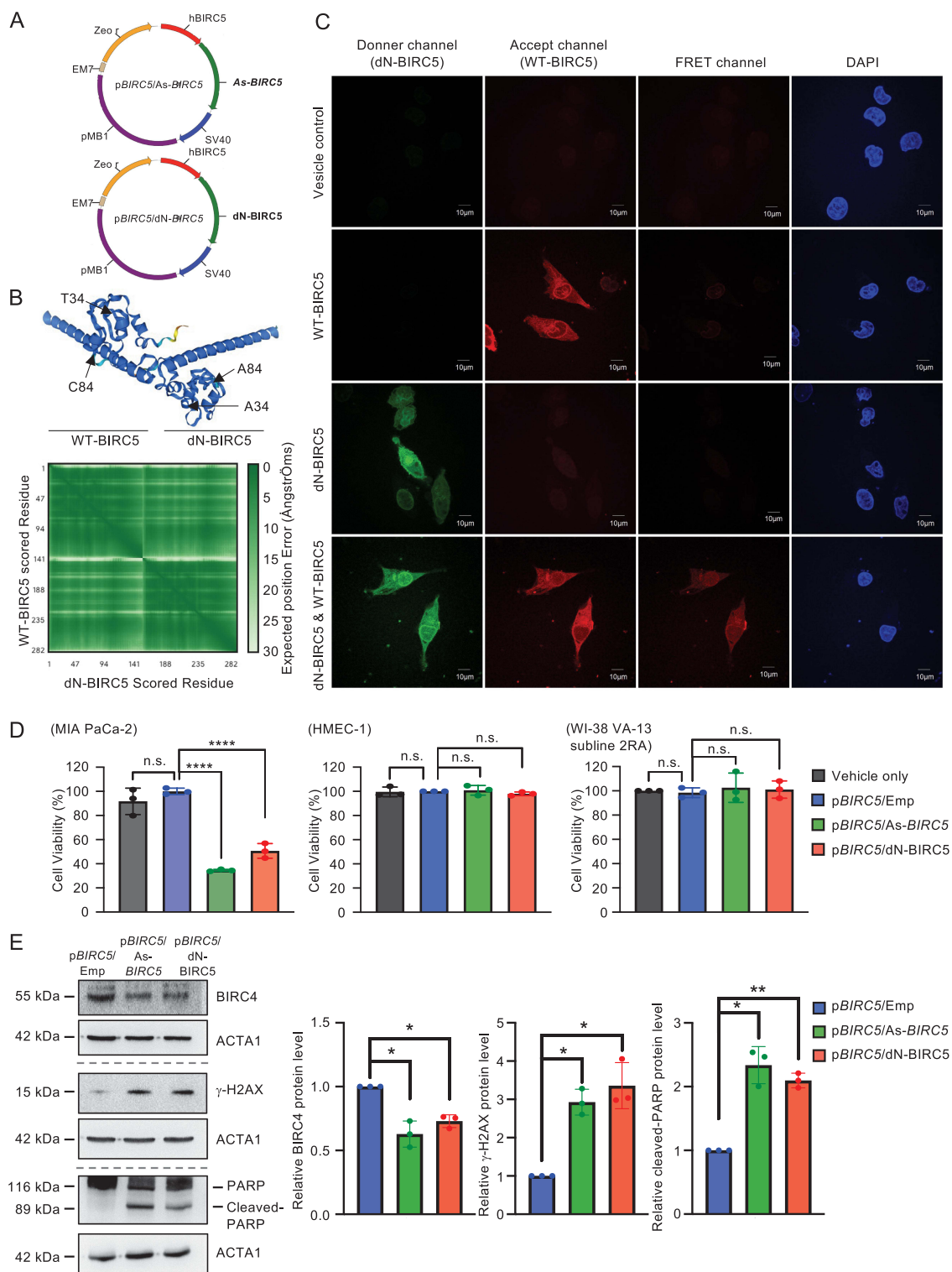


Figure 1 Transfection with pBIRC5/As-BIRC5 and pBIRC5/dN-BIRC5 decreases viability in BIRC5-expressing cancer cells. **(A)** Schematic maps of the pDRIVE-hBIRC5/As-BIRC5 (pBIRC5/As-BIRC5) and pDRIVE-hBIRC5/dN-BIRC5 (pBIRC5/dN-BIRC5) plasmids. **(B)** Predicted three-dimensional protein-protein interaction between WT-BIRC5 and dN-BIRC5, generated using AlphaFold (<https://alphafoldserver.com>). **(C)** MIA PaCa-2 cells were co-transfected with pCMV6-AC-mGFP-dN-BIRC5 and pCMV6-AC-mRFP-WT-BIRC5 plasmids for 48 h, followed by confocal fluorescence imaging. Scale bars: 10 μm. **(D)** Cell viability of MIA PaCa-2, HMEC-1, and WI-38 VA13 subline 2RA cells transfected with pBIRC5/Emp, pBIRC5/As-BIRC5, or pBIRC5/dN-BIRC5 for 96 h, assessed using the MTT assay. The vehicle group refers to cells treated with plasmid DNA-free liposomal transfection reagent. Data represent at least three independent experiments. **(E)** Expression levels of BIRC4, γ-H2AX, and PARP proteins in pBIRC5/As-BIRC5- or pBIRC5/dN-BIRC5-transfected MIA PaCa-2 cells, determined by Western blot post 48-h transfection. ACTA1 served as a loading control. Data represent at least three independent experiments. The bar graphs show quantified band intensities from the mean ± SD. The statistical analysis was performed using one-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ vs. pBIRC5/Emp. **Abbreviation:** ns, not significant.

Table 2 Physical Characterization of pBIRC5 Plasmid DNA–Adsorbed MNPs and PL-MNPs. The Size and ζ Potential of pBIRC5 Plasmid DNA–Adsorbed MNPs and PL-MNPs Were Measured Using DLS. Encapsulation Efficiency Was Calculated as (Total pBIRC5 Plasmid DNA Added – free, Non-Entrapped pBIRC5 Plasmid DNA) ÷ total pBIRC5 Plasmid DNA Added. Loading Capacity Was Calculated as Total Entrapped pBIRC5 Plasmid DNA ÷ total Nanoparticle Weight

Formulation of Nanoparticle	Size (nm)	Zeta Potential (mV)	Polydispersity Index (PDI)	Encapsulation Efficiency (%)	Loading Capacity (%)
MNP Series nanoparticle					
MNP	17.43±10.7	0.7±0.25	0.339±0.051	–	–
MNP-pBIRC5/Emp	105.6±6.4	–1.7±0.20	0.267±0.009	1.5±0.2	16±2.20
MNP-pBIRC5/As-BIRC5	94.15±27.5	1.8±0.06	0.343±0.078	2±0.8	22±8.78
MNP-pBIRC5/dN-BIRC5	109.9±6.4	0.67±0.26	0.403±0.062	1.8±0.6	20±6.59
Poly-L-lysine-MNP series nanoparticle					
PL-MNP	306.6±9.0	4.23±2.95	0.272±0.003	–	–
PL-MNP-pBIRC5/Emp	389.6±22.6	–47.58±6.91	0.296±0.019	53±6.3	74±8.81
PL-MNP-pBIRC5/As-BIRC5	387.8±16.8	–37.08±5.76	0.199±0.011	52±4.6	73±6.43
PL-MNP-pBIRC5/dN-BIRC5	393.9±9.9	–27.11±0.74	0.297±0.097	58±2.8	81±3.92
Her-PL-MNP- pBIRC5/Emp	465.9±35.5	–94.6±8.62	0.416±0.029	55±2.4	75±5.86
Her-PL-MNP-pBIRC5/As-BIRC5	478.5±33.1	–100±11.46	0.441±0.085	58±3.2	77±9.21
Her-PL-MNP-pBIRC5/dN-BIRC5	451.2±30.4	–92.5±7.46	0.427±0.062	57±1.4	82±2.56

of nanoparticles.⁴⁰ Consistent with this observation, poly-L-lysine surface modification increased nanoparticle size, producing PL-MNPs with hydrodynamic diameters of approximately 390 nm (Table 2, Figure S4B). DLS results indicated that PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, and PL-MNP-pBIRC5/dN-BIRC5 remained well dispersed in aqueous solution.

The chemical composition of the “core region” (the central part) of PL-MNPs was analyzed using liquid transmission electron microscopy (liquid TEM) and energy dispersive X-ray spectroscopy (EDS). Since poly-L-lysine carries a positive charge and DNA is negatively charged, liquid TEM showed that the diameter of the iron oxide magnetic core (the core region) was approximately 5.8 nm across all PL-MNP formulations (Figure 2A). EDS mapping further revealed the close proximity of phosphorus (P), which represents the phosphate groups of the DNA backbone, and iron (Fe) signals in the nanoparticle cores of the PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, and PL-MNP-pBIRC5/dN-BIRC5 groups, confirming successful plasmid DNA loading under optimized conditions (Figure 2A). The poly-L-lysine surface modification greatly enhanced encapsulation efficiency and loading capacity, increasing them from about 2% to 54% and from 19% to 76%, respectively (Table 2). The mean ζ potentials of PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, and PL-MNP-pBIRC5/dN-BIRC5 were –47.58 mV, –37.08 mV, and –27.11 mV, respectively, indicating strong colloidal stability and low aggregation tendency in aqueous media (Table 2). Scanning electron microscopy (SEM) images further showed that PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, and PL-MNP-pBIRC5/dN-BIRC5 nanoparticles exhibited predominantly spherical morphology under dehydrated conditions (Figure 2B).

The DNA-degrading enzyme DNase I is abundant in the bloodstream.⁴¹ To evaluate the protective ability of PL-MNPs against DNase I-mediated enzymatic degradation, PL-MNP-pBIRC5/As-BIRC5 and PL-MNP-pBIRC5/dN-BIRC5 were incubated with DNase I, and the integrity of the encapsulated plasmid DNA was assessed. Results showed that naked (unformulated) pBIRC5/As-BIRC5 and pBIRC5/dN-BIRC5 plasmid DNA were completely degraded within 15 min of DNase I exposure, whereas plasmid DNA loaded within PL-MNPs remained intact after 120 min of DNase I treatment (Figure 2C). PL-MNPs also provided sustained protection in DNase I-containing culture medium for up to 180 min, further demonstrating their ability to shield plasmid DNA under enzymatically challenging conditions (Figure 2D). In addition, the hydrodynamic diameters of PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, and PL-MNP-pBIRC5/dN-BIRC5 remained stable in serum-containing cell culture medium (Figure S4C), further supporting their stability under physiologically relevant conditions.

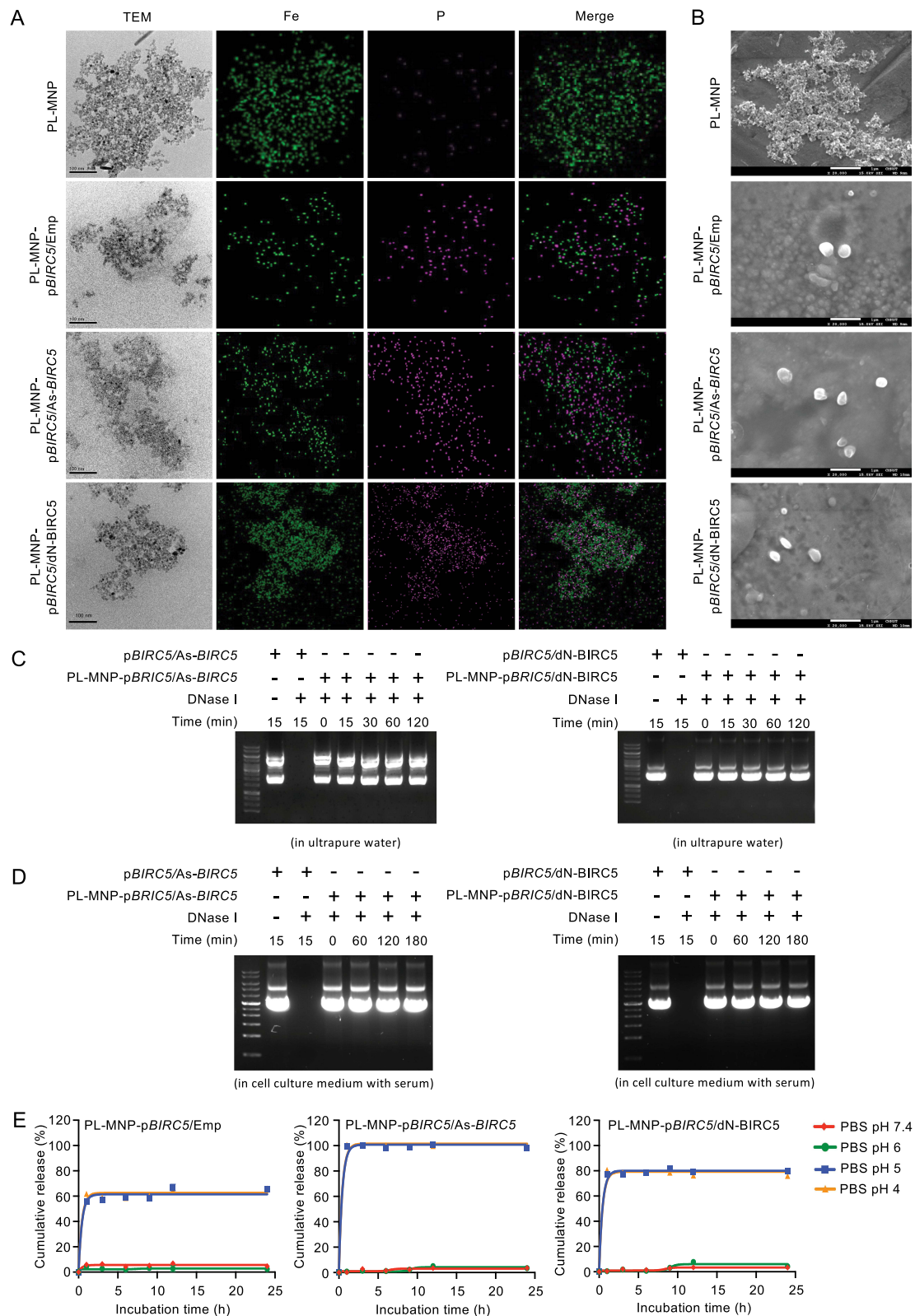


Figure 2 Physical and chemical characterization of PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, and PL-MNP-pBIRC5/dN-BIRC5. **(A)** Elemental distribution of PL-MNPs, PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, and PL-MNP-pBIRC5/dN-BIRC5 analyzed by liquid TEM and EDS. Fe indicates iron; P indicates phosphorus. Scale bars: 100 nm. **(B)** Morphology of PL-MNPs and plasmid DNA-loaded PL-MNPs observed by SEM. Scale bars: 1 μ m. **(C)** DNase I protection assay assessing the integrity of 1 μ g of pBIRC5/As-BIRC5, pBIRC5/dN-BIRC5, PL-MNP-pBIRC5/As-BIRC5, and PL-MNP-pBIRC5/dN-BIRC5 incubated with or without DNase I at 37°C for various durations. **(D)** DNase I protection assay assessing the integrity of 1 μ g of pBIRC5/As-BIRC5, pBIRC5/dN-BIRC5, PL-MNP-pBIRC5/As-BIRC5, and PL-MNP-pBIRC5/dN-BIRC5 incubated in cell culture medium (RPMI with 10% FBS and 1% PSG) with or without DNase I at 37°C for various durations. **(E)** Plasmid DNA release profiles from PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, and PL-MNP-pBIRC5/dN-BIRC5 after incubation in PBS at different pH values for the indicated durations. DNA release was quantified by spectrometry.

Following cellular endocytosis and lysosomal entrapment, nanoparticles release their payloads in response to the acidic environment (pH 4.5–5.0).⁴² To determine whether plasmid DNA is effectively released from the nanoparticles under acidic conditions,⁴³ PL-MNP-p*BIRC5*/Emp, PL-MNP-p*BIRC5*/As-*BIRC5*, and PL-MNP-p*BIRC5*/dN-*BIRC5* were incubated in PBS at varying pH values (4.0 to 7.4) for up to 24 h at 37°C. The percentage of plasmid DNA released was measured over time. Less than 10% of the loaded plasmid DNA was released at neutral and near-neutral pH levels of 6.0 and 7.4 (Figure 2E). In contrast, approximately 80–98% of plasmid DNA was released from PL-MNP-p*BIRC5*/As-*BIRC5* and PL-MNP-p*BIRC5*/dN-*BIRC5* at acidic pH levels of 4.0 and 5.0 (Figure 2E). These results suggest that PL-MNP-p*BIRC5*/As-*BIRC5* and PL-MNP-p*BIRC5*/dN-*BIRC5* exhibit pH-responsive release behavior consistent with nanodrug-like properties, supporting their potential for further biological and pharmacological testing.

Biofunctional Characterization of PL-MNP-p*BIRC5*/dN-*BIRC5* in vitro

Cells internalize nanoparticles mainly through endocytosis, which in mammalian systems includes five main pathways: receptor-mediated endocytosis, phagocytosis, pinocytosis, caveolae-mediated endocytosis, and clathrin-mediated endocytosis.⁴⁴ To investigate the mechanisms by which PL-MNP-p*BIRC5*/Emp, PL-MNP-p*BIRC5*/As-*BIRC5* and PL-MNP-p*BIRC5*/dN-*BIRC5* are internalized by cancer cells, MIA PaCa-2 cells were treated with R6G-labeled nanoparticles and then co-incubated with various endocytosis inhibitors, cytochalasin D (phagocytosis inhibitor), wortmannin (macropinocytosis inhibitor), colchicine (pinocytosis inhibitor), monodansylcadaverine (MDC, clathrin-mediated endocytosis inhibitor), and chloroquine (CQ, clathrin-mediated endocytosis inhibitor), at a non-toxic concentrations (Figure S5). Fluorescent microscopic and spectrophotometric analyses showed that cells treated with MDC and CQ displayed reduced R6G fluorescence signals compared to controls, indicating that these nanoparticles enter cancer cells partly via clathrin-mediated endocytosis (Figure 3A and B).

To determine the intercellular activation of plasmid DNA carried by PL-MNPs in *BIRC5*⁺ cancer cells, an R6G-labeled pCMV6-AC-GFP plasmid DNA-loaded nanoparticle (PL-MNP-pCMV6-AC-GFP) was synthesized using the same formulation as PL-MNP-p*BIRC5*/As-*BIRC5* and PL-MNP-p*BIRC5*/dN-*BIRC5*. GFP expression was then assessed in *BIRC5*⁺ MIA PaCa-2 cells treated with PL-MNP-pCMV6-AC-GFP. Fluorescence and confocal microscopy showed red (R6G) and green (GFP) signals in MIA PaCa-2 cells 48 hours post-treatment, confirming successful nanoparticle endocytosis and demonstrating intracellular plasmid activation (Figure 3C and D).

Next, to verify that cancer cells can intracellularly activate PL-MNP-p*BIRC5*/dN-*BIRC5*, *BIRC5*⁺ MIA PaCa-2 cells (Figure 4A) were treated with the PL-MNPs. As shown in Figure 2E, PL-MNP-p*BIRC5*/As-*BIRC5* and PL-MNP-p*BIRC5*/dN-*BIRC5* release their loaded plasmid DNA under acidic conditions. Co-incubation with the lysosomotropic agent, bafilomycin-A1 (BAF), which inhibits lysosomal acidification, partially attenuated the reduction in cell viability induced by PL-MNP-p*BIRC5*/As-*BIRC5* and PL-MNP-p*BIRC5*/dN-*BIRC5* in MIA PaCa-2 cells (Figure 4B), supporting the plasmid DNA cargo is released in part via lysosomal escape⁴⁵ and the requirement of endo-lysosomal acidification for intracellular activation of these nanodrugs. At the molecular level, *BIRC5* interacts with CASP3 (caspase-3) to inhibit its pro-apoptotic activity by forming the *BIRC5*-CASP3 protein complex.⁴⁶ In situ proximity ligation assay results demonstrated that treatments with PL-MNP-p*BIRC5*/As-*BIRC5* (positive control) and PL-MNP-p*BIRC5*/dN-*BIRC5* reduced the abundance of the *BIRC5*-CASP3 complex in MIA PaCa-2 cells (Figure 4C). Moreover, similar to PL-MNP-p*BIRC5*/As-*BIRC5*, PL-MNP-p*BIRC5*/dN-*BIRC5* induced γ -H2AX expression, indicating DNA damage (Figure 4D).

The effects of PL-MNP-p*BIRC5*/dN-*BIRC5* on cell viability were tested in vitro using *BIRC5*⁺ cancer cells, including MIA PaCa-2, NTUB1, NTU0.017 (NTUB1-derived ABCB1-expressing cells) (Figure 4E), and SK-OV-3 (with and without IFN γ -stimulation) cells, as well as *BIRC5*^{low} non-cancerous cells, including MCF10A, HMEC-1 endothelial, WI-38 VA-13 subline 2RA fibroblast, HaCaT keratinocyte, and THP-1 monocyte cells to assess the off-target toxicity. The IFN γ -treated SK-OV-3 cells are widely used as a model to study immune checkpoint therapy resistance, as IFN γ stimulation elevates kynurenine levels by upregulating IDO1. The secretion of kynurenine from cancer cells into the tumor microenvironment led to CD8 T cell exhaustion.⁴⁷ The results showed the negative control nanoparticle, PL-MNP-p*BIRC5*/Emp (up to 30 ng/ μ L), had minimal effect on cell viability across all tested cell lines (Figure 4F). In contrast, but similar to PL-MNP-p*BIRC5*/As-*BIRC5*, PL-MNP-p*BIRC5*/dN-*BIRC5* significantly reduced the viability of MIA PaCa-2, NTUB1, NTU0.017, SK-OV-3, and IFN γ -treated IDO-expressing SK-OV-3 cells (Figure S6A) in a dose-dependent

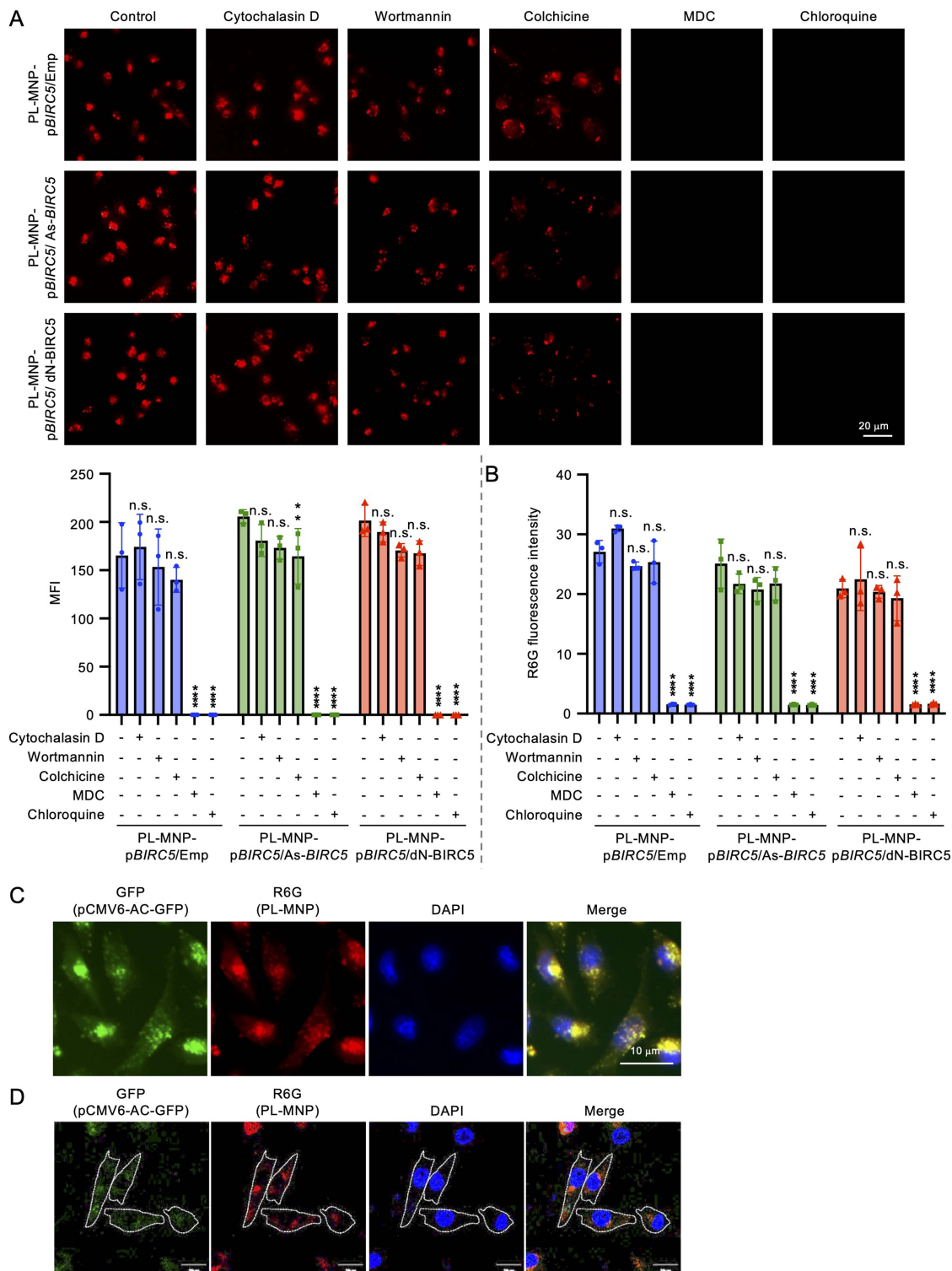


Figure 3 Clathrin-mediated endocytosis regulates plasmid DNA-loaded PL-MNP uptake in cancer cells. **(A and B)** MIA PaCa-2 cells were pretreated with endocytosis inhibitors, including 10 μ M cytochalasin D, 50 nM wortmannin, 10 μ M colchicine, 200 μ M monodansylcadaverine (MDC), or 125 μ M chloroquine, for 30 min, followed by treatment with 10 ng/ μ L PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, or PL-MNP-pBIRC5/dN-BIRC5 for 24 h. **(A)** Uptake efficiency was assessed by fluorescence microscopy, and the fluorescent intensity was quantified using ImageJ. Scale bars: 20 μ m. **(B)** MIA PaCa-2 cells were lysed with Cellytic™ M reagent, and fluorescence intensity was measured using a SpectraMax ID3 microplate reader. **(C)** MIA PaCa-2 cells were treated with 10 ng/ μ L PL-MNP-pCMV6-AC-GFP for 48 h. Fluorescence microscopy images show GFP protein expression (green), PL-MNPs (red), and nuclei counterstained with DAPI (blue). Scale bars: 10 μ m. **(D)** Confocal microscopy images of MIA PaCa-2 cells treated with 10 ng/ μ L PL-MNP-pCMV6-AC-GFP for 48 h, showing GFP expression (green), PL-MNPs (red), and DAPI-stained nuclei (blue). Scale bars: 20 μ m. Data represent at least three independent experiments. Bar graphs indicate the mean $\pm$ SD. The statistical analysis was performed using one-way ANOVA. ** $P < 0.01$, and *** $P < 0.0001$ vs. untreated control (no inhibitor). **Abbreviation:** ns, not significant.

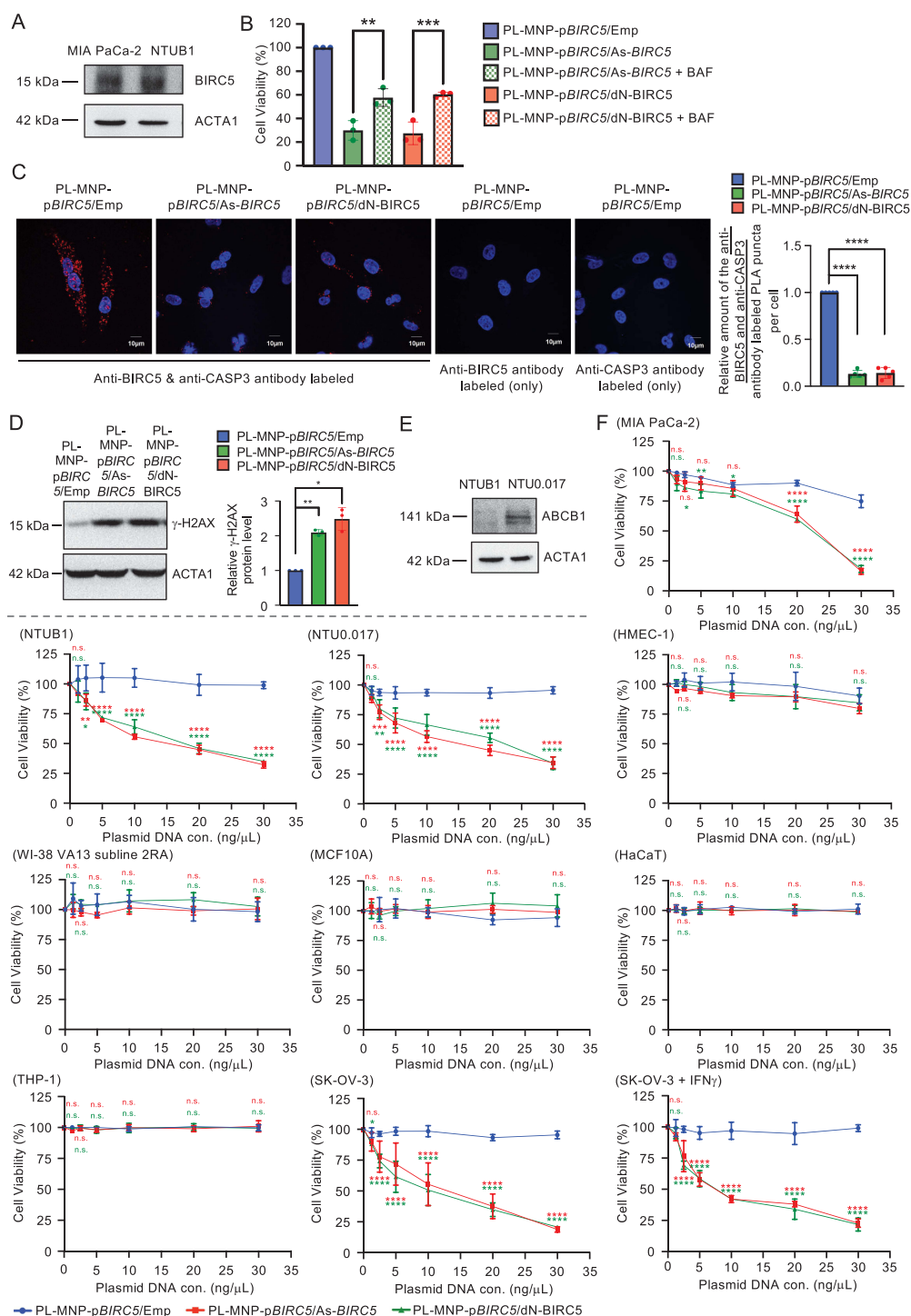


Figure 4 Anti-cancer potency of PL-MNP-pBIRC5/As-BIRC5 and PL-MNP-pBIRC5/dN-BIRC5 in BIRC5-Expressing cells. **(A)** Western blot analysis of BIRC5 expression in MIA PaCa-2 and NTUB1 cells. ACTA1 served as a loading control to verify equal protein loading. **(B)** MIA PaCa-2 cells were co-treated with lysosomotropic agent 3 nM BAF and IC_{50} concentrations of PL-MNP-pBIRC5/As-BIRC5 or PL-MNP-pBIRC5/dN-BIRC5 for 96 h. Cell viability was determined using the MTT assay. **(C)** MIA PaCa-2 cells were treated with PL-MNP-pBIRC5/Emp or PL-MNP-pBIRC5/dN-BIRC5 at their respective IC_{50} concentrations for 48 h. BIRC5-CASP3 protein-protein interactions were detected by in situ PLA using anti-BIRC5 and anti-CASP3 antibodies. Scale bars: 10 μ m. **(D)** γ -H2AX expression in MIA PaCa-2 cells treated with IC_{50} concentrations of PL-MNP-pBIRC5/As-BIRC5 or PL-MNP-pBIRC5/dN-BIRC5 for 48 h, assessed by Western blotting. ACTA1 served as a loading control. Data represent at least three independent experiments. The bar graph represents the quantified band intensities from independent experiments, with γ -H2AX expression levels normalized to the corresponding ACTA1 band intensity for each sample. **(E)** Western blot analysis of ABCB1 expression in NTUB1 and NTU0.017 cells. ACTA1 served as a loading control to verify equal protein loading. **(F)** Cell viability of MIA PaCa-2, NTUB1, NTU0.017, SK-OV-3 (with or without 25 ng/mL IFN γ), MCF10A, HMEC-1, WI-38 VA13 subline 2RA, HaCaT, and THP1 cells treated with PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, or PL-MNP-pBIRC5/dN-BIRC5 for 96 h, measured by the MTT assay and WST assay (for THP-1 cells). Data represent at least three independent experiments. Bar and curve graphs indicate the mean $\pm$ SD. Statistical analyses were performed using one-way ANOVA for panels (B–D), and two-way ANOVA for panel (F). * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 vs. PL-MNP-pBIRC5/Emp or non-BAF-treated group; n.s., not significant. Statistical symbols are color-coded to match the corresponding experimental groups in the figure.

manner, while exerting minimal effects on MCF10A, WI-38 VA-13 subline 2RA, HaCaT, and THP-1 cells (Figure 4F). Minimal effects were observed on HMEC-1 cells, supporting the targeting specificity of PL-MNP-pBIRC5/dN-BIRC5 for *BIRC5*⁺ cancer cells (Figure 4F). Collectively, these findings suggest that PL-MNP-pBIRC5/dN-BIRC5 represent promising anticancer agents, and, mechanistically, the plasmid DNA cargo is released in part via lysosomal escape.⁴⁵

PL-MNP-pBIRC5/dN-BIRC5 Exhibits Antitumor Effects in vivo Independent of ABCB1 Expression in Cancer Cells

The antitumor efficacy of PL-MNP-pBIRC5/dN-BIRC5 was evaluated using a zebrafish xenograft model, a common approach for preclinical evaluation of anticancer drugs.^{48–50} PKH67-labeled (green fluorescent) MIA PaCa-2 cancer cells were microinjected into the yolk sacs of zebrafish embryos. Similar to treatment with PL-MNP-pBIRC5/As-BIRC5, PL-MNP-pBIRC5/dN-BIRC5 significantly decreased the size and fluorescence area of MIA PaCa-2 xenograft tumors (Figure 5A and B). Both nanoparticle formulations were well tolerated at the tested concentrations, with no significant adverse effects on zebrafish body length, survival, or swim bladder integrity, which is a surrogate marker for lung toxicity (Figure 5C–E). Since the zebrafish model is also frequently used to evaluate drug-induced hepatotoxicity,^{51,52} liver size was measured to evaluate potential hepatotoxic effects. The results showed that neither PL-MNP-pBIRC5/As-BIRC5 nor PL-MNP-pBIRC5/dN-BIRC5 had a negative impact on liver size at any tested concentration (Figure 5F).

The expression of ABCB1 contributes to multidrug resistance in tumors.^{53,54} To assess whether ABCB1 expression affects the efficacy of PL-MNP-pBIRC5/dN-BIRC5, the antitumor activity of PL-MNP-pBIRC5/dN-BIRC5 was tested in human ABCB1-non-expressing *BIRC5*⁺ KB cancer cells and in the KB-derived, ABCB1-expressing, multidrug-resistant *BIRC5*⁺ KB-TAX50 cancer cells (Figure S6B and S6C).³² We previously showed that, compared to KB cells, KB-TAX50 cells exhibit higher ABCB1 expression at both the transcriptional and protein levels, and show resistance to Paclitaxel (an anti-mitotic agent), Vincristine (another anti-mitotic agent), and YM155 (a small molecule that suppresses *BIRC5* gene expression).^{28,55} In this study, no significant difference was observed in the potency of PL-MNP-pBIRC5/As-BIRC5 and PL-MNP-pBIRC5/dN-BIRC5 between KB and KB-TAX50 cells in vitro (Figure 6A). Cell viability assays showed IC₅₀ of 8.8 and 8.3 ng/μL for PL-MNP-pBIRC5/As-BIRC5 and 7.6 and 6.3 ng/μL for PL-MNP-pBIRC5/dN-BIRC5 in KB and KB-TAX50 cells, respectively. KB-TAX50 cells displayed higher endogenous BIRC5 expression compared to KB cells (Figure S6B). Consistent with this, cell cycle analysis revealed that BIRC5-targeting PL-MNPs caused a greater percentage of sub-G1 (dead) cells in KB-TAX50 than in KB cells 72 h post-treatment (Figure S7A). At the molecular level, Western blot analysis confirmed that both PL-MNP-pBIRC5/As-BIRC5 and PL-MNP-pBIRC5/dN-BIRC5 induced PARP and CASP3 cleavage, markers of apoptosis, in both cell lines (Figure 6B, S7B, and S7C). For in vivo testing, PKH67-labeled (green fluorescent) KB and KB-TAX50 cells were microinjected into the yolk sacs of zebrafish embryos. Consistent with the effect of PL-MNP-pBIRC5/As-BIRC5, PL-MNP-pBIRC5/dN-BIRC5 treatment significantly reduced tumor size and relative fluorescence area in both xenograft models (Figure 6C and D). Furthermore, PL-MNP-pBIRC5/As-BIRC5 and PL-MNP-pBIRC5/dN-BIRC5 were well tolerated at the tested concentrations, with no significant effects on body length, survival, or swim bladder integrity in zebrafish bearing KB or KB-TAX50 xenografts (Figure 6E–G). Collectively, these results suggest that the antitumor potency of PL-MNP-pBIRC5/dN-BIRC5 is not affected by the expression of the multidrug efflux pump ABCB1 in cancer cells.

Antibody Conjugation Restores the Potency of PL-MNP-pBIRC5/As-BIRC5 and PL-MNP-pBIRC5/dN-BIRC5 in Clathrin-Mutated Cancer Cells

Inhibition of clathrin significantly decreased the internalization of PL-MNP-pBIRC5/As-BIRC5 and PL-MNP-pBIRC5/dN-BIRC5 in MIA PaCa-2 cells. Likewise, pharmacological inhibition of clathrin using MDC and CQ reduced the uptake of these PL-MNPs in both KB and KB-TAX50 cells (Figure S8A and S8B) without inducing significant cytotoxicity (Figure S5). These results support that the internalization of PL-MNP-pBIRC5/As-BIRC5 and PL-MNP-pBIRC5/dN-BIRC5 into cancer cells occurs, at least in part, through clathrin-mediated endocytosis. However, some cancer cells exhibit altered clathrin organization and function. Human SK-BR-3 breast cancer cells, which display low clathrin levels and high expression of ERBB2 (HER2) and BIRC5, are commonly used as a model to study ERBB2-

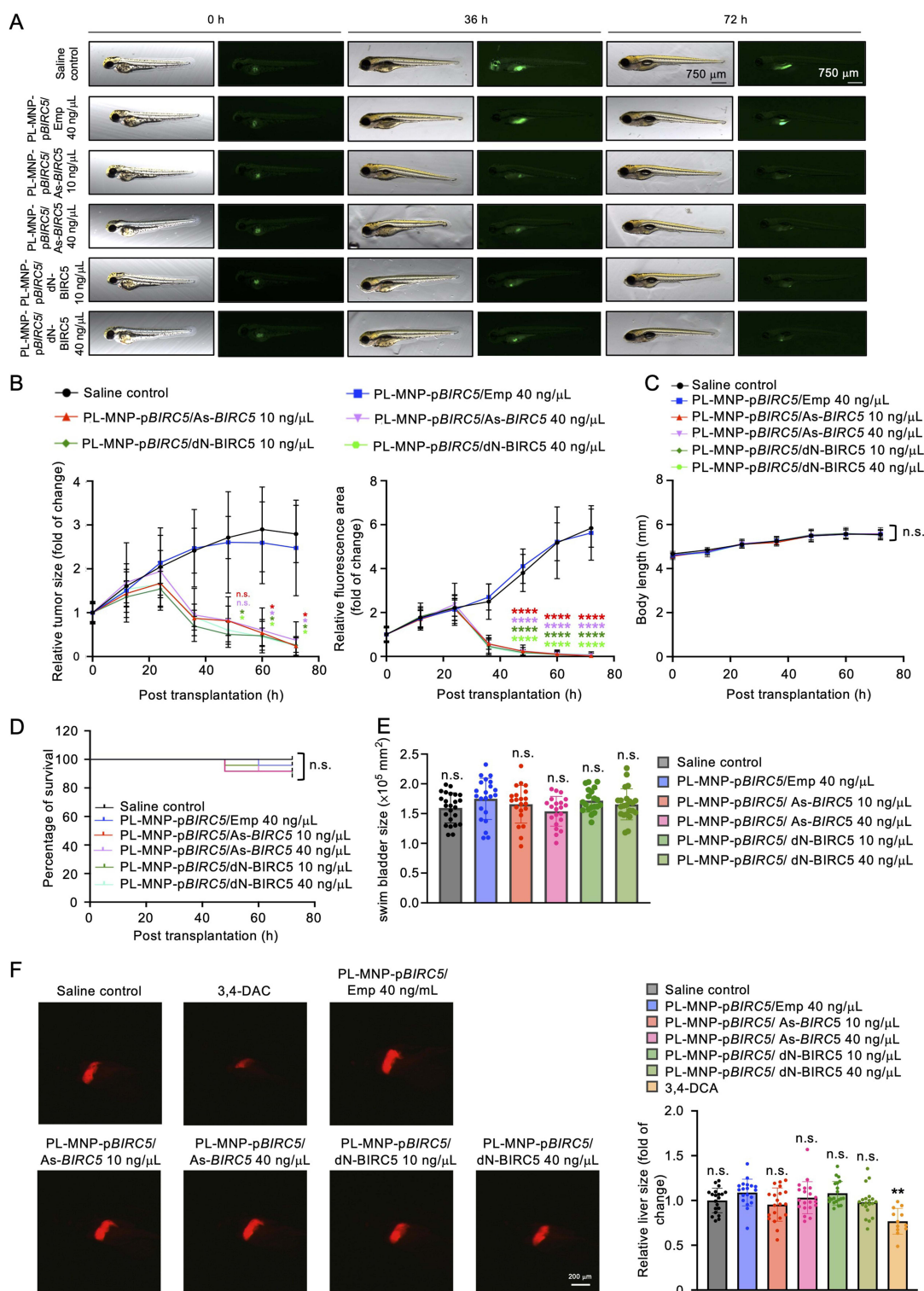


Figure 5 Anti-cancer effects of PL-MNP-pBIRC5/As-BIRC5 and PL-MNP-pBIRC5/dN-BIRC5 in MIA PaCa-2 cell-bearing xenograft zebrafish models. PKH67-labeled MIA PaCa-2 cancer cells were transplanted into 48 h post-fertilization (hpf) zebrafish embryos (N = 24 per group). At 24 h post-transplantation, embryos were treated with saline or the indicated concentrations of PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, or PL-MNP-pBIRC5/dN-BIRC5 for 48 h. (A and B) Tumor size, (C) body length, and (D) percentage survival were measured by immunofluorescence microscopy every 12 h. (E) The swim bladder size was quantified using immunofluorescence microscopy at the end of the experiment. (F) Seventy-two hpf transgenic zebrafish embryos (fabp10a:mCherry) were treated with saline, 3,4-dichloroaniline (3,4-DAC; 1 mg/L), PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, or PL-MNP-pBIRC5/dN-BIRC5 for 48 h. Liver size was assessed by immunofluorescence microscopy 48 h post-treatment. Scale bars: 750 μm. Bar and curve graphs indicate the mean $\pm$ SD. Statistical analyses were performed using two-way ANOVA for panels (B and C), simple survival analysis (Kaplan-Meier) for panel (D), and one-way ANOVA for panels (E and F). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ vs. PL-MNP-pBIRC5/Emp; n.s., not significant. Statistical symbols are color-coded to match the corresponding experimental groups in the figure.

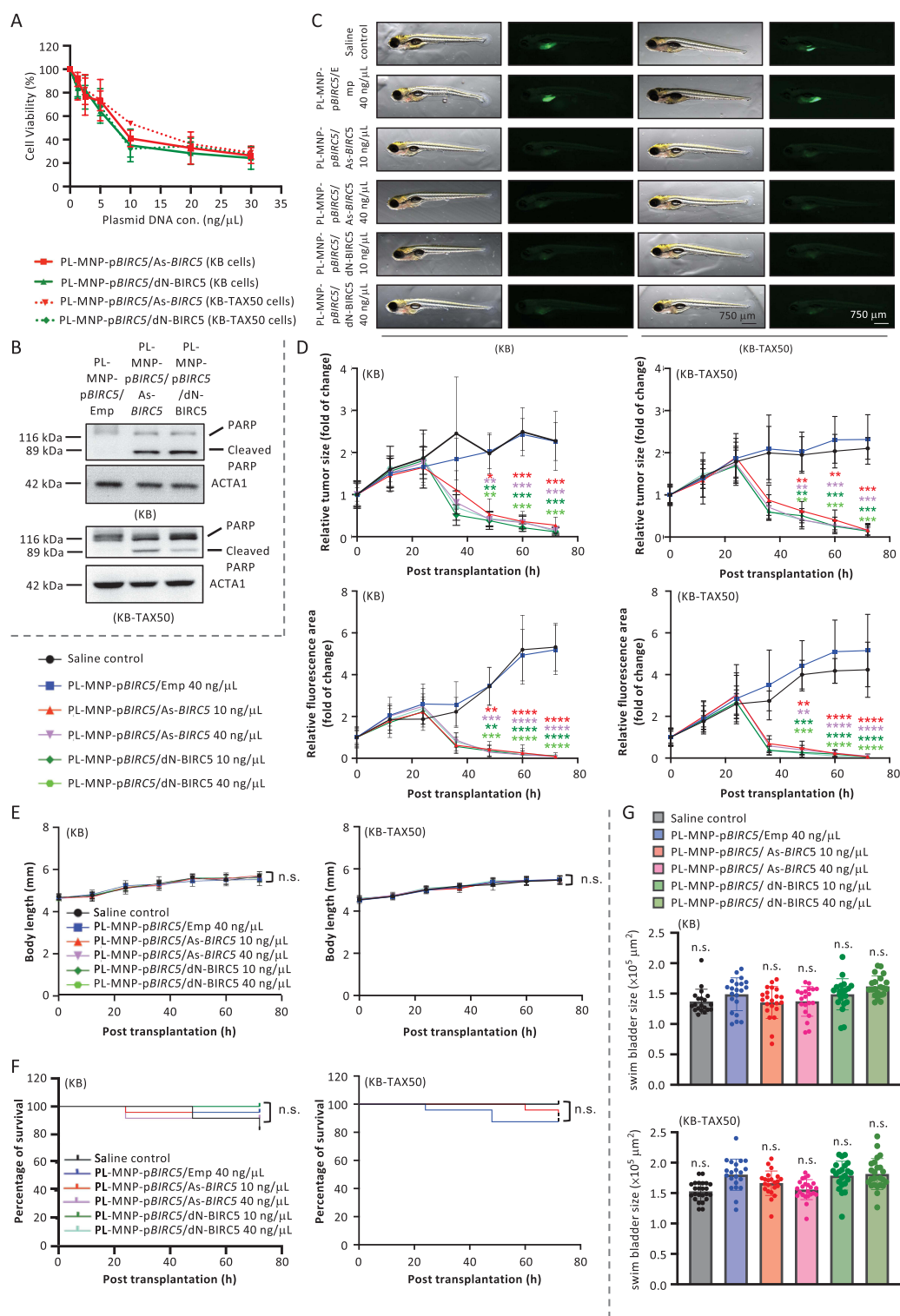


Figure 6 PL-MNP-pBIRC5/As-BIRC5 and PL-MNP-pBIRC5/dN-BIRC5 exhibit anticancer activity against ABCB1-expressing cancer cells in vitro and in vivo. **(A)** KB and KB-TAX50 cells were treated with PL-MNP-pBIRC5/As-BIRC5 or PL-MNP-pBIRC5/dN-BIRC5 for 96 h. Cell viability was assessed by the MTT assay. Data represent at least three independent experiments. **(B)** PARP expression in KB and KB-TAX50 cells treated with PL-MNP-pBIRC5/As-BIRC5 or PL-MNP-pBIRC5/dN-BIRC5 for 48 h, determined by Western blotting. ACTA1 served as a loading control. Data represent at least three independent experiments. In the zebrafish xenograft models, PKH67-labeled KB and KB-TAX50 cells were transplanted into 48 hpf zebrafish embryos ($N = 24$ per group). At 24 h post-transplantation, embryos were treated with saline or the indicated concentrations of PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, or PL-MNP-pBIRC5/dN-BIRC5 for 48 h. **(C and D)** Tumor size, **(E)** body length, and **(F)** percentage survival were measured every 12 h via immunofluorescence microscopy. Scale bars: 750 μm. **(G)** Swim bladder size was quantified at the end of the experiment using immunofluorescence microscopy. Bar and curve graphs indicate the mean $\pm$ SD. Statistical analyses were performed using two-way ANOVA for panels **(A)**, **(D)**, and **(E)**, simple survival analysis (Kaplan-Meier) for panel **(F)**, and one-way ANOVA for panel **(G)**. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. PL-MNP-pBIRC5/Emp; n.s., not significant. Statistical symbols are color-coded to match the corresponding experimental groups in the figure.

enriched breast cancer (Figure S6D).^{56–58} SK-BR-3 cells have a clathrin gene mutation that impairs clathrin-mediated endocytosis.^{59,60} In this context, and similar to PL-MNP-p*BIRC5*/As-*BIRC5*, PL-MNP-p*BIRC5*/dN-*BIRC5* did not affect the viability of SK-BR-3 cells in vitro, further suggesting that the efficacy of these PL-MNP constructs depends partly on cellular clathrin levels and the integrity of clathrin-mediated endocytosis (Figure 7A).

The feasibility of using a straightforward (direct) antibody conjugation method targeting cancer cell surface receptors to improve the efficacy of PL-MNP-p*BIRC5*/dN-*BIRC5* in clathrin-deficient and mutated cancer cells was investigated. The monoclonal anti-ERBB2 antibody, Trastuzumab, is widely used as an anticancer agent for ERBB2-enriched breast cancer by blocking the survival-promoting ERBB2 signaling pathway in tumor cells.⁶¹ EDC/NHS-mediated surface conjugation of Trastuzumab was performed on PL-MNP-p*BIRC5*/Emp, PL-MNP-p*BIRC5*/As-*BIRC5*, and PL-MNP-p*BIRC5*/dN-*BIRC5*. The efficiency of antibody conjugation to PL-MNPs was confirmed using HRP-conjugated goat anti-mouse IgG. The result showed successful antibody attachment under the established conjugation conditions, with a loading capacity of 91.2%±3.4% (Figure 7B). DLS analysis indicated that the hydrodynamic diameter of Trastuzumab-conjugated PL-MNPs was approximately 480 nm (Table 2). Trastuzumab conjugation significantly increased the cellular uptake of Her-PL-MNP-p*BIRC5*/Emp in ERBB2^{High} CLTCL1^{Low} SK-BR-3 cells in vitro (Figure 7C). No significant uptake differences were observed between Her-PL-MNP-p*BIRC5*/Emp and PL-MNP-p*BIRC5*/Emp in clathrin-expressing MCF7 and MDA-MB-231 cells (Figure 7D), indicating that clathrin-mediated endocytosis represents a major uptake mechanism for PL-MNP-p*BIRC5*/Emp. The Trastuzumab-conjugated Her-PL-MNP-p*BIRC5*/Emp caused a notable reduction in SK-BR-3 cell viability at concentrations above 30 ng/μL (Figure 7E). Her-PL-MNP-p*BIRC5*/As-*BIRC5* and Her-PL-MNP-p*BIRC5*/dN-*BIRC5* showed greater inhibition of cell viability in SK-BR-3 cells compared to Her-PL-MNP-p*BIRC5*/Emp (Figure 7E). In contrast, the Trastuzumab-unconjugated PL-MNP-p*BIRC5*/Emp, PL-MNP-p*BIRC5*/As-*BIRC5*, and PL-MNP-p*BIRC5*/dN-*BIRC5* did not significantly affect SK-BR-3 cell viability in vitro (Figure 7E).

We next assessed the antitumor effectiveness of Trastuzumab-conjugated Her-PL-MNP-p*BIRC5*/dN-*BIRC5* in clathrin-deficient/mutated tumors in vivo. PKH67-labeled SK-BR-3 cancer cells were microinjected into the yolk sacs of zebrafish embryos to establish xenograft tumors. Both PL-MNP-p*BIRC5*/Emp and Her-PL-MNP-p*BIRC5*/Emp had no significant effects on the growth or survival of the SK-BR-3 xenografts (Figure 7F and G). In contrast, similar to the effect of Her-PL-MNP-p*BIRC5*/As-*BIRC5*, treatment with Her-PL-MNP-p*BIRC5*/dN-*BIRC5* significantly reduced the size and fluorescence area of the xenograft tumors in zebrafish. Both Trastuzumab-conjugated formulations were well tolerated at the tested doses, with no significant effects on zebrafish body length and survival, indicating no severe toxicity in this model (Figure 7H and I).

Discussion

BIRC5 is highly expressed in many tumor types, where its upregulation promotes mitosis, inhibits apoptosis, and contributes to drug resistance and metastasis. As a result, *BIRC5* has long been considered a promising molecular target for cancer therapy; however, only a limited number of *BIRC5*-targeting agents have been developed over the past two decades. In this study, we engineered two *BIRC5*-targeting nanodrugs using PL-MNPs as delivery vehicles to target *BIRC5*-expressing cancer cells with an antisense *BIRC5* mRNA and a dominant-negative *BIRC5* protein-expressing plasmid DNA. We demonstrated their cell-specific targeting and anti-cancer effects both in vitro and in vivo. We also showed that enhanced nanodrug uptake in clathrin-deficient and mutated tumors, such as the SK-BR-3 breast cancer cell line, can be achieved by a straightforward cell surface receptor-targeting antibody conjugation strategy using agents like Trastuzumab.

Over the past three decades, several *BIRC5*-targeting anticancer agents have been developed; however, none have advanced to Phase III clinical trials. Among these, YM155 is perhaps the most well-known *BIRC5* expression suppressant to have reached phase I/II clinical trials. Mechanistically, YM155 disrupts the interaction between the transcription factor specificity protein 1 (SP1) and the *BIRC5* core promoter region, resulting in transcriptional downregulation of *BIRC5*.^{62,63} However, recent studies have questioned the target specificity of YM155, showing that it also causes direct DNA damage and affects molecules beyond *BIRC5*, suggesting that *BIRC5* downregulation may not be its primary anti-cancer mechanism.^{64–66} For example, YM155 binds not only to genomic DNA but also to mitochondrial DNA, leading to decreased oxidative phosphorylation and increased mitochondrial permeability.¹⁶ YM155 is a substrate

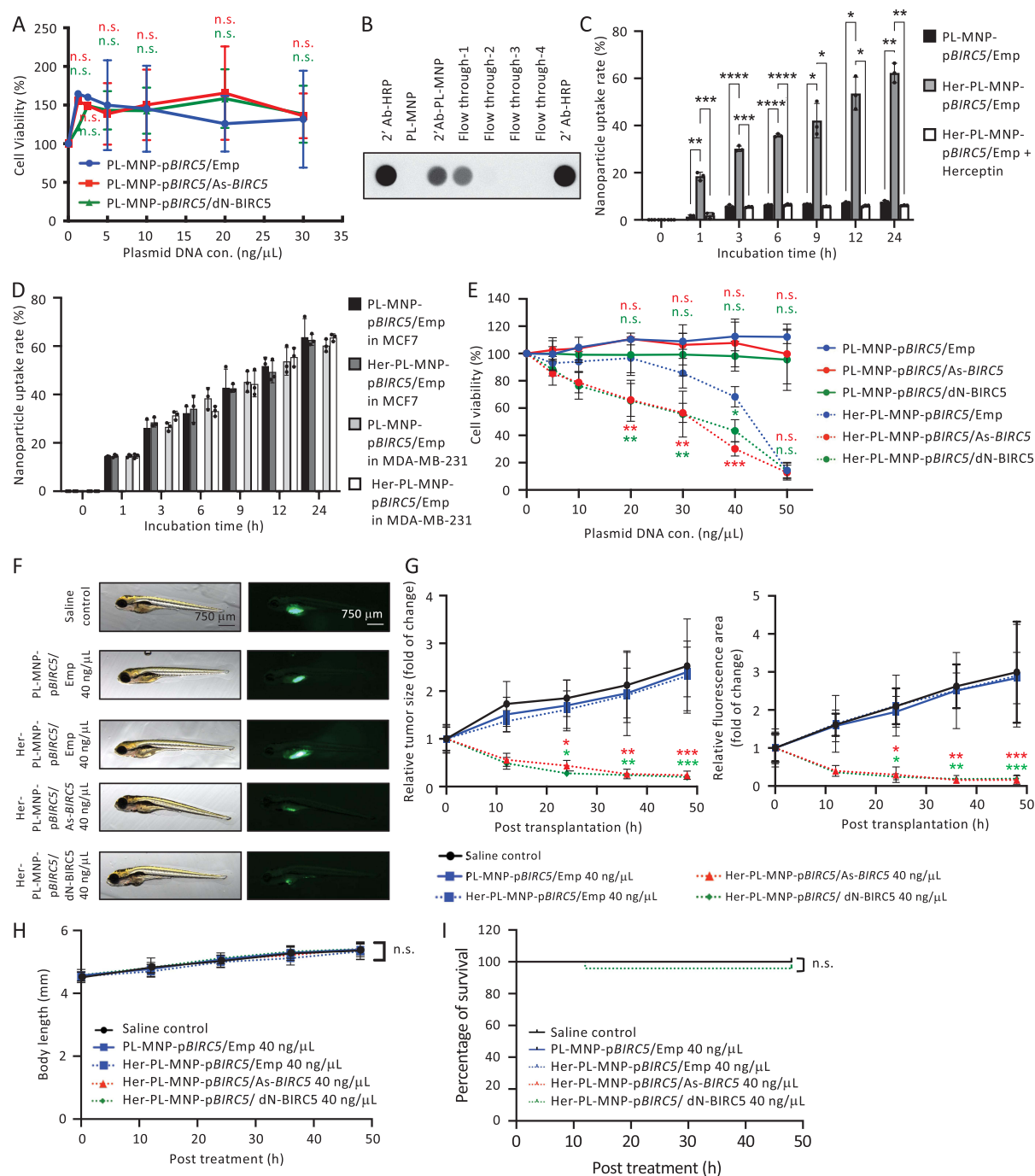


Figure 7 Trastuzumab conjugation enhances the anticancer potency of PL-MNP-pBIRC5/As-BIRC5 and PL-MNP-pBIRC5/dN-BIRC5 in vitro and in vivo. **(A)** SK-BR-3 cells were treated with PL-MNP-pBIRC5/Emp, PL-MNP-pBIRC5/As-BIRC5, or PL-MNP-pBIRC5/dN-BIRC5 for 96 h. Cell viability was assessed using the MTT assay. Data represent at least three independent experiments. **(B)** HRP-conjugated goat anti-mouse IgG antibodies were conjugated on PL-MNPs. The PL-MNPs, antibodies-conjugated PL-MNPs, and flow-through fractions were incubated with ECL substrate, and luminescent signals were detected using a FUJII LAS-100. **(C)** SK-BR-3 cells were co-treated with 10 μ g/mL R6G-labelled PL-MNP-pBIRC5/Emp or Her-PL-MNP-pBIRC5/Emp and with or without 20 μ g/mL Trastuzumab for 1, 3, 6, 9, 12, or 24 h. Cells were lysed with Cellytic™ M reagent, and fluorescence intensity was measured using a SpectraMax ID3 microplate reader. Data represent at least three independent experiments. **(D)** MCF7 and MDA-MB-231 cells were treated with 10 μ g/mL R6G-labelled PL-MNP-pBIRC5/Emp or Her-PL-MNP-pBIRC5/Emp for 1, 3, 6, 9, 12, or 24 h. Cells were lysed with Cellytic™ M reagent, and fluorescence intensity was measured using a SpectraMax ID3 microplate reader. Data represent at least three independent experiments. **(E)** SK-BR-3 cells were treated with PL-MNP-pBIRC5/As-BIRC5, PL-MNP-pBIRC5/dN-BIRC5, Her-PL-MNP-pBIRC5/As-BIRC5, or Her-PL-MNP-pBIRC5/dN-BIRC5 for 96 h. Cell viability was determined by the MTT assay. Data represent at least three independent experiments. In the zebrafish xenograft models, PKH67-labeled SK-BR-3 cells were transplanted into 48 hpf zebrafish embryos (N = 24 per group). Embryos were then treated with saline or the indicated concentrations of PL-MNP-pBIRC5/Emp, Her-PL-MNP-pBIRC5/Emp, Her-PL-MNP-pBIRC5/As-BIRC5, or Her-PL-MNP-pBIRC5/dN-BIRC5 for 48 h. **(F and G)** Tumor size, **(H)** body length, and **(I)** percentage survival were measured every 12 h using immunofluorescence microscopy. Scale bars: 750 μ m. Bar and curve graphs indicate the mean $\pm$ SD. Statistical analyses were performed using two-way ANOVA for panels (A), (E), (G), and (H), one-way ANOVA for panel (C) and (D), and simple survival analysis (Kaplan-Meier) for panel (I). * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 vs. PL-MNP-pBIRC5/Emp or Her-PL-MNP-pBIRC5/Emp; n.s., not significant. Statistical symbols are color-coded to match the corresponding experimental groups in the figure.

of the multidrug efflux pump ABCB1, and ABCB1 overexpression confers resistance to YM155 in cancer cells.^{67,68} In addition to YM155, antisense *BIRC5* oligonucleotides LY2181308 (18-mer) and SPC3042 (16-mer) have been investigated as potential therapeutic agents. Although these oligonucleotides target *BIRC5* in cancer cells, off-target effects and severe adverse reactions have been reported, leading to the discontinuation of their clinical development.^{12,15,17,69,70} For instance, SPC3042 has been shown to lower *BCL2* mRNA expression in human PC3 prostate cancer cells, indicating its limited target specificity.¹⁵ Preclinical studies have developed *BIRC5* siRNA-loaded nanoparticles, demonstrating their anti-cancer efficacy and tumor-targeting potential.^{71,72} Nevertheless, the clinical application of siRNA-based therapeutics is limited by inherent challenges, including endonuclease-mediated degradation, which collectively reduce their stability and bioavailability. The anti-cancer potential of purified, cell-penetrating recombinant dominant-negative BIRC5 proteins has also been studied. Our previous work showed that the cell-penetrating peptide poly-arginine (R9)-fused dominant-negative human BIRC5 C84A protein, expressed and purified from bacteria, induces apoptosis in prostate cancer cells in vitro.⁷³ The liposomal delivery of a purified histidine-tagged, dominant-negative human BIRC5 T34A-C84A protein induced apoptotic effects in MDA-MB-231 and MIA PaCa-2 cancer cells in vitro.¹⁹ However, the clinical use of recombinant dominant-negative BIRC5 proteins has been limited by low protein yields, purification difficulties, and instability.

PL-MNP-p*BIRC5*/As-*BIRC5* and PL-MNP-p*BIRC5*/dN-BIRC5 have three key pharmacological advantages that set them apart from previously developed BIRC5-targeting agents: (i) these nanodrugs are designed to be activated mainly in tumor tissues rather than in differentiated normal cells, reflecting the different expression pattern of the *BIRC5* gene, which is highly expressed in various cancers but absent or low in normal differentiated tissues; (ii) their anti-cancer potency is not affected by ABCB1 expression, avoiding a common resistance mechanism; and (iii) their cellular uptake efficiency and target cell specificity can be improved through a simple antibody conjugation method. Theoretically, DNA-based *BIRC5*-targeting methods may overcome the inherent instability of siRNA-based treatments in clinical use. In principle, the “full-length” antisense *BIRC5* mRNA produced by PL-MNP-p*BIRC5*/As-*BIRC5* provides greater target specificity for endogenous wild-type *BIRC5* mRNA in *BIRC5*⁺ cancer cells compared to shorter oligonucleotides like the 18-mer LY2181308 and 16-mer SPC3042. The intracellular expression of the dominant-negative BIRC5 T34A-C84A protein from PL-MNP-p*BIRC5*/dN-BIRC5 overcomes challenges associated with recombinant protein production, purification difficulties, and potential proteolytic degradation in systemic circulation.

The enhanced permeability and retention (EPR) effect is widely recognized as a key mechanism underlying drug accumulation and penetration at tumor sites. Nanoparticles with hydrodynamic diameters ranging from 100 to 400 nm, particularly those under 300 nm, are generally considered optimal for passive tumor targeting. Although the sizes of PL-MNP-p*BIRC5*/As-*BIRC5* and PL-MNP-p*BIRC5*/dN-BIRC5 exceed 300 nm (387.8 nm and 393.9 nm, respectively), potentially limiting their ability to penetrate solid tumors, several studies have shown that nanoparticles larger than 300 nm can still exhibit potent anticancer effects.^{32,74} For example, Guo et al reported that PEG-based nanoparticles loaded with microRNA *miR-122*, with a diameter around 451 nm, effectively targeted hepatocellular carcinoma cells both in vitro and in vivo.⁷⁴ Similarly, hyaluronic acid-based nanoparticles measuring 200–400 nm, loaded with wild-type *TP53* (*p53*) and *miR-125b* co-expressing plasmid DNA, induced tumor cell death in a mouse lung cancer model.⁷⁵ Chemotherapeutic drug-loaded iron oxide mesoporous magnetic microparticles with an average size of 765 nm penetrated deeply into tumor cell layers of dissected breast tumor tissues under conditions that mimic in vivo oxygen, nutrient, and energy gradients.⁷⁶ It is also noteworthy that nanoparticles with hydrodynamic diameters between 300 and 600 nm have been reported to mediate higher DNA transfection and gene expression efficiencies than those with diameters below 100 nm.^{77,78} In this study, although aggregation morphologies of PL-MNP-p*BIRC5*/Emp, PL-MNP-p*BIRC5*/As-*BIRC5*, and PL-MNP-p*BIRC5*/dN-BIRC5 were observed in liquid TEM (showing the “core” of the nanoparticles) and SEM (showing the “entire” nanoparticles), such aggregation is likely an artifact of water evaporation during sample preparation. DLS analysis in aqueous solution confirmed that these PL-MNP formulations are well-dispersed, supporting their suitability for biological applications. Taken together, these findings suggest that despite their relatively large size, PL-MNP formulations may still penetrate the tumor effectively and achieve effective tumor targeting. Although the EPR effect is widely cited as a mechanism for tumor accumulation of nanoparticles, recent clinical and translational work has highlighted significant variability in EPR across human tumor types, stromal density,

and patient populations.^{79,80} Therefore, while our PL-MNP size (~390 nm) is compatible with reported effective nanoparticle sizes in some models, the extent to which EPR contributes to tumor accumulation in human cancers remains uncertain and will require validation in more clinically relevant models and imaging modalities.

Cancer cells internalize nanoparticles via different endocytic pathways, depending on the nanoparticle size. Nanoparticles larger than 150 nm in diameter are mainly taken up through clathrin-mediated endocytosis, whereas particles between 5 and 10 μm undergo phagocytosis.^{81,82} In this study, cancer cells internalized PL-MNP-pBIRC5/As-BIRC5 and PL-MNP-pBIRC5/dN-BIRC5 partially through clathrin-dependent endocytosis, as indicated by lower uptake in clathrin-inhibited cancer cells and reduced anti-proliferative effects in clathrin-mutant SK-BR-3 cells. Conjugation with Trastuzumab enhanced the anti-proliferative effects of PL-MNP-pBIRC5/As-BIRC5 and PL-MNP-pBIRC5/dN-BIRC5 in SK-BR-3 cells both in vitro and in vivo. These results indicate that surface modification strategies, such as attaching cell surface protein-specific antibodies, can significantly enhance the cellular uptake efficiency and potentially increase the target specificity of these therapeutic nanoparticles (Figure 8). Of note, the pharmacokinetics and detailed

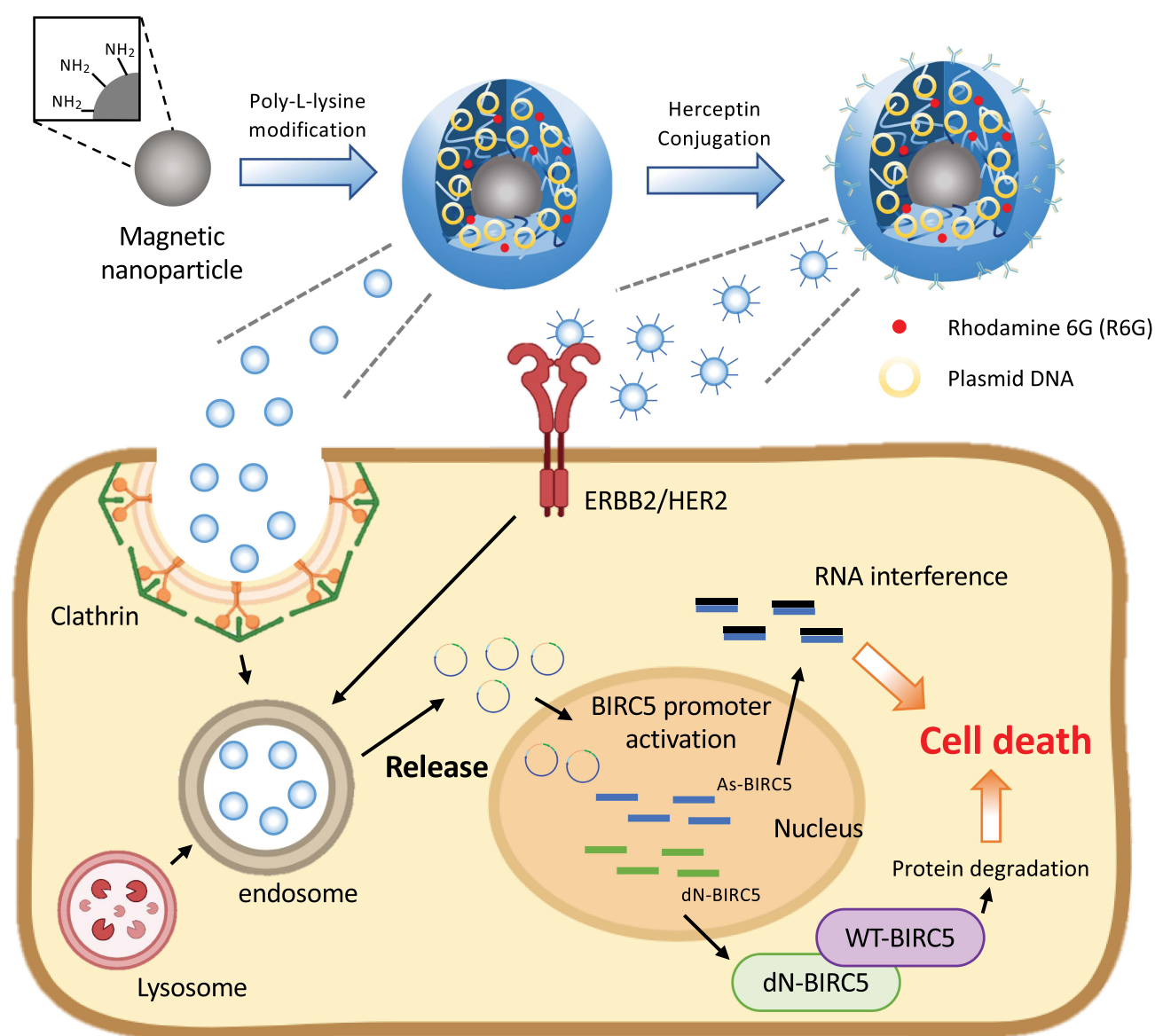


Figure 8 Structure and function of plasmid-adsorbed PL-MNPs and Her-PL-MNPs. Summary of the molecular and cellular effects of PL-MNP-pBIRC5/As-BIRC5, PL-MNP-pBIRC5/dN-BIRC5, Her-PL-MNP-pBIRC5/As-BIRC5, and Her-PL-MNP-pBIRC5/dN-BIRC5 in BIRC5-positive cancer cells.

biodistribution of these nanoparticles will need to be evaluated in mouse xenograft models to fully assess their in vivo performance.

The reticuloendothelial system (RES) acts as a major obstacle to systemic nanoparticle delivery because macrophages in the liver and spleen effectively identify and eliminate circulating nanomaterials.⁸³ Nanoparticles with hydrodynamic diameters over 200 nm are especially prone to quick RES-mediated clearance.⁸⁴ In this study, the plasmid-loaded PL-MNPs exceeded 200 nm in diameter, indicating a high probability of sequestration after systemic administration. Surface charge also critically influences nanoparticle biodistribution. Near-neutral zeta potentials (−10 to +10 mV) are generally associated with prolonged circulation due to reduced protein adsorption and macrophage recognition, whereas highly positive surfaces promote opsonization and clearance.⁸⁵ Although PL-MNPs displayed a strongly negative zeta potential (less than −30 mV), which may partially mitigate nonspecific protein interactions, particle size is likely the dominant factor promoting rapid RES sequestration. Therefore, detailed pharmacokinetic and biodistribution studies are required to quantify RES uptake and to guide optimization of physicochemical properties to improve systemic performance. The potential long-term toxicity of iron oxide-based nanocarriers also warrants consideration, as accumulation in reticuloendothelial organs, increased lysosomal burden, and iron-mediated oxidative stress may pose risks, particularly under repeated dosing. Since these aspects were not assessed in this study, future research should incorporate chronic exposure models, histopathological analysis, and evaluation of iron load and oxidative stress to facilitate clinical translation.

The plasmid DNA release profiles of PL-MNP-*pBIRC5*/As-*BIRC5*, and PL-MNP-*pBIRC5*/dN-*BIRC5* showed a pH-dependent pattern, with significantly higher release efficiencies under acidic conditions compared to near-neutral conditions. This pH sensitivity is due to electrostatic interactions that control plasmid DNA retention by the PL-MNPs. The cationic nature of PL enhances pH responsiveness by absorbing protons in acidic environments. PL mainly binds plasmid DNA via electrostatic interactions, condensing the DNA into a compact structure that protects it and facilitates delivery.^{86,87} At lower pH levels, increased proton concentrations compete with the plasmid DNA for binding sites on the PL, weakening the electrostatic forces and enabling more efficient DNA release.⁸⁸ Conversely, at pH 6 and above, the reduced proton concentration results in stronger electrostatic binding, leading to limited plasmid release. Although previous studies have underscored the role of electrostatic interactions in low pH-triggered plasmid DNA release, the present study did not further explore the buffering capacity and proton-sponge effect of PL. Future work addressing these aspects could better elucidate the mechanisms of pH-responsive DNA release.

Conclusion

We demonstrate that two *BIRC5* pathway-targeting nanodrugs, PL-MNP-*pBIRC5*/As-*BIRC5* and PL-MNP-*pBIRC5*/dN-*BIRC5*, have been successfully developed and exert significant anti-cancer activity both in vitro and in vivo. Importantly, their efficacy is maintained regardless of ABCB1 expression, a protein commonly associated with multidrug resistance, and IDO1 level, which is associated with reduced responsiveness to immune-based therapy in cancer cells. These nanodrugs represent promising therapeutic candidates for managing various malignancies, particularly in patients who develop ABCB1-related drug resistance after prolonged treatment regimens. Their ability to overcome such resistance mechanisms highlights their potential to improve outcomes in difficult-to-treat cancers and warrants further development and clinical evaluation.

The current study highlights the potential of *BIRC5*-targeted nanotherapeutics in a vertebrate preclinical setting; however, clinical translation will require validation in mammalian models, including rodent pharmacokinetic, biodistribution, and chronic-toxicity studies, before robust predictions can be made. Although the *BIRC5* promoter drives tumor-associated transcriptional activity, promoter leakiness and heterogeneity in *BIRC5* expression across tumor types may limit targeting specificity and efficacy. Moreover, plasmid DNA itself may trigger innate immune responses via TLR9,⁸⁹ despite our tumor-targeted design minimizing systemic exposure. These aspects were not directly examined in this study. Future research should assess cytokine profiles, splenocyte activation, and immune cell infiltration after systemic dosing, both to confirm safety and support clinical translation. While some of the proposed benefits are supported by our data, others remain theoretical or model-dependent and require further testing across different tumor types and in higher mammalian models.

Use of Artificial Intelligence Tools

The AI-assisted tool Grammarly Pro was used for preliminary language checking. The manuscript was subsequently reviewed and refined by all authors to ensure accuracy, originality, and integrity of the content. All authors take full responsibility for the final version of the manuscript.

Abbreviations

ABCB1/MDR1/P-gp, ATP binding cassette subfamily B member 1; As-*BIRC5*, full-length antisense *BIRC5*; BIR, baculovirus IAP repeat; BIRC4/XIAP, baculoviral IAP repeat-containing protein 4; *BIRC5*⁺, *BIRC5* gene endogenously-expressing; CQ, chloroquine; DLS, dynamic light scattering; dN-BIRC5, dominant-negative BIRC5 protein with the T34A and C84A mutation; EDC/NHS, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide; EDS, energy dispersive X-ray spectroscopy; ERBB2/HER2, v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2; EPR, enhanced permeability and retention; IAP, inhibitor of apoptosis protein; IDO1, indoleamine 2,3-dioxygenase 1; MDC, monodancylcadaverin; MNP, iron oxide magnetic nanoparticle; p*BIRC5*, *BIRC5* promoter; PL-MNP, poly-L-lysine-modified amino (NH₂)-modified iron oxide-based magnetic nanoparticles; PLA, in situ proximity ligation analysis; R6G, rhodamine 6G; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from Dr. Chun Hei Antonio Cheung on reasonable request.

Ethics Approval and Consent to Participate

The animal protocol was approved (approval no. 109273) by the Institutional Animal Care and Use Committee (IACUC) of National Cheng Kung University (Tainan, Taiwan).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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