

PPS03 triggers cancer-selective necroptosis in hepatocellular carcinoma via macropinocytosis-dependent ROS overload

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ABSTRACT

Hepatocellular carcinoma (HCC) may be curable when detected and at an early stage. However, recurrence and metastatic progression occur frequently following therapeutic intervention, leading to treatment-refractory disease. Such refractory HCCs are resistant to systemic therapies, facilitating metastatic dissemination and disease relapse, highlighting the need for alternative therapeutic strategies. To address the limited efficacy of available systemic therapies, we developed a new polyaspartic acid (PASP) based polymer-metal complex, PPS03, with well-defined physicochemical properties, including nanoscale size distribution and stable metal incorporation. We showed that PPS03 induced cancer-selective necroptosis through preferential macropinocytic uptake in patient-derived metastatic HCC models. Specifically, PPS03 promoted necroptotic cell death by stimulating an accumulation of the mitochondrial reactive oxygen species (ROS) via selective intratumoral elevation of hydrogen peroxide (H₂O₂). Mechanistically, PPS03 exploited differential macropinocytic activity, which is high in tumor cells but very low in normal hepatocytes. This activity enabled selective uptake of PPS03-bound selenomethionine and ferrous iron, amplifying H₂O₂-mediated mitochondrial ROS production within cancer cells. Consistent with this selectivity, PPS03 exhibited minimal cytotoxicity in normal THLE-2 hepatocytes, while it strongly suppressed the viability of HCC cells. Notably, in patient-derived cisplatin-resistant metastatic HCC models, PPS03 significantly inhibited tumor growth both *in vitro* and *in vivo* whereas cisplatin showed no significant efficacy. Furthermore, PPS03 reduced stem-like tumor features, as evidenced by decreased sphere formation and reduced CD133 expression, likely reflecting preferential elimination of stem-like tumor cells. Collectively, these findings present PPS03 as a polymer-metal complex that selectively induces necroptosis in refractory HCC with minimal off-target toxicity, providing a potential therapeutic strategy to address drug resistance in advanced disease.

1. Introduction

Hepatocellular carcinoma (HCC) is the predominant form of primary liver cancer, accounting for more than 90% of cases worldwide [1–3]. Despite recent advances in systemic therapies that have improved survival in advanced disease, treatment resistance remains a major clinical challenge [4–6]. Recurrent or metastatic progression frequently develops following therapeutic intervention, leading to refractory disease

and high mortality rates [7–9]. Although molecular heterogeneity has been associated with poor outcomes in metastatic or recurrent HCC, the mechanisms driving therapeutic resistance remain poorly understood, underscoring the need for more effective treatment strategies [10,11]. Patients with advanced-stage HCC characterized by vascular invasion or metastatic dissemination typically require systemic therapy, because local modalities such as monotherapy are insufficient to control chemoresistant tumors [12]. However, refractory HCC often persists despite

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substantial therapy-induced cellular stress, largely due to the survival of cancer stem cells (CSCs) [13,14]. CSCs display pronounced resistance to microenvironmental stress via epigenetic and metabolic reprogramming, allowing them survive under nutrient-deprived conditions and promote relapse and metastatic spread [15,16].

Macropinocytosis, a non-selective endocytic process that facilitates the uptake of extracellular fluid and nutrients, has emerged as a key metabolic adaptation in malignant cells [17]. In contrast, macropinocytic activity in normal cells is generally low and can be transiently induced only under specific physiological conditions, such as immune activation or particular nutrient demands [18,19]. Cancer cells, however, frequently display constitutive macropinocytosis to compensate for metabolic stress within the tumor microenvironment. Such differential dependency highlights macropinocytosis as a potential therapeutic vulnerability, whereby selective targeting of this pathway may impair malignant cells while avoiding normal hepatocytes. In this study, we sought to exploit differential macropinocytic activity as a strategy to develop a cancer-selective therapeutic approach for refractory HCC. Specifically, we investigated whether preferential macropinocytic uptake could be harnessed to deliver therapeutic agents capable of overcoming resistance in highly aggressive and drug-resistant HCC. Accordingly, we developed PPS03, a novel PASP-based polymer-metal complex composed of poly (aspartic acid) (PASP) conjugated with selenomethionine (SeMet) and coordinated with ferrous iron (Fe^{2+}), designed to induce cancer-selective necroptosis through reactive oxygen species (ROS) generation. In addition, we examined whether PPS03 can target stem-like tumor populations that contribute to therapeutic resistance.

2. Results and discussion

2.1. Clinicopathological features and differential molecular signatures between non-metastatic and metastatic HCC cells

We isolated HCC cells from patients who had received systemic anticancer therapy and subsequently developed metastatic disease. The cohort comprised 21 individuals (15 men and 6 women; [Supplementary Table 1](#)). To define transcriptional differences between non-metastatic and metastatic HCC cells, we performed RNA sequencing and qRT-PCR analyses in patient-derived metastatic HCC cells, with a non-metastatic patient-derived HCC cell line (YUMC-NM-H2) and established HCC cell lines serving as comparators. Compared with non-metastatic HCC cells, metastatic HCC cells exhibited markedly elevated expression of metastatic markers (*NDUFA4L2*, *EBF1*, *TYMP*, and *MET*; [Fig. 1A–C](#)), cancer stemness-associated genes (*PROM1*/*CD133*, *ALDH1A1*, *KRT17*, and *KRT19*; [Fig. 1A–D](#)), and epithelial-mesenchymal transition-related markers (*SNAIL1/2*, *ZEB1/2*, *WNT1/4*, and *TWIST1*; [Fig. 1A–E](#)), indicating a distinct aggressive molecular profile. To confirm the hepatocellular origin of patient-derived cells, expression of established HCC markers (GPC3 and HSP70) was assessed in normal hepatocytes (THLE-2), established HCC cell lines (Huh7, HepG2, and Hep3B), and patient-derived HCC cells. All patient-derived HCC cells exhibited HCC marker expression comparable to that of established HCC cell lines, whereas normal hepatocytes showed minimal or undetectable expression ([Fig. 1B](#)). Based on clinical relevance and molecular characteristics, four patient-derived HCC cell lines were selected for further analyses: one non-metastatic line (YUMC-NM-H2) and three metastatic lines (YUMC-M-H4, -H5, and -H6) ([Table 1](#); [Fig. 2](#)). RNA sequencing and qRT-PCR analyses demonstrated that metastatic HCC cells displayed significantly higher expression of metastatic

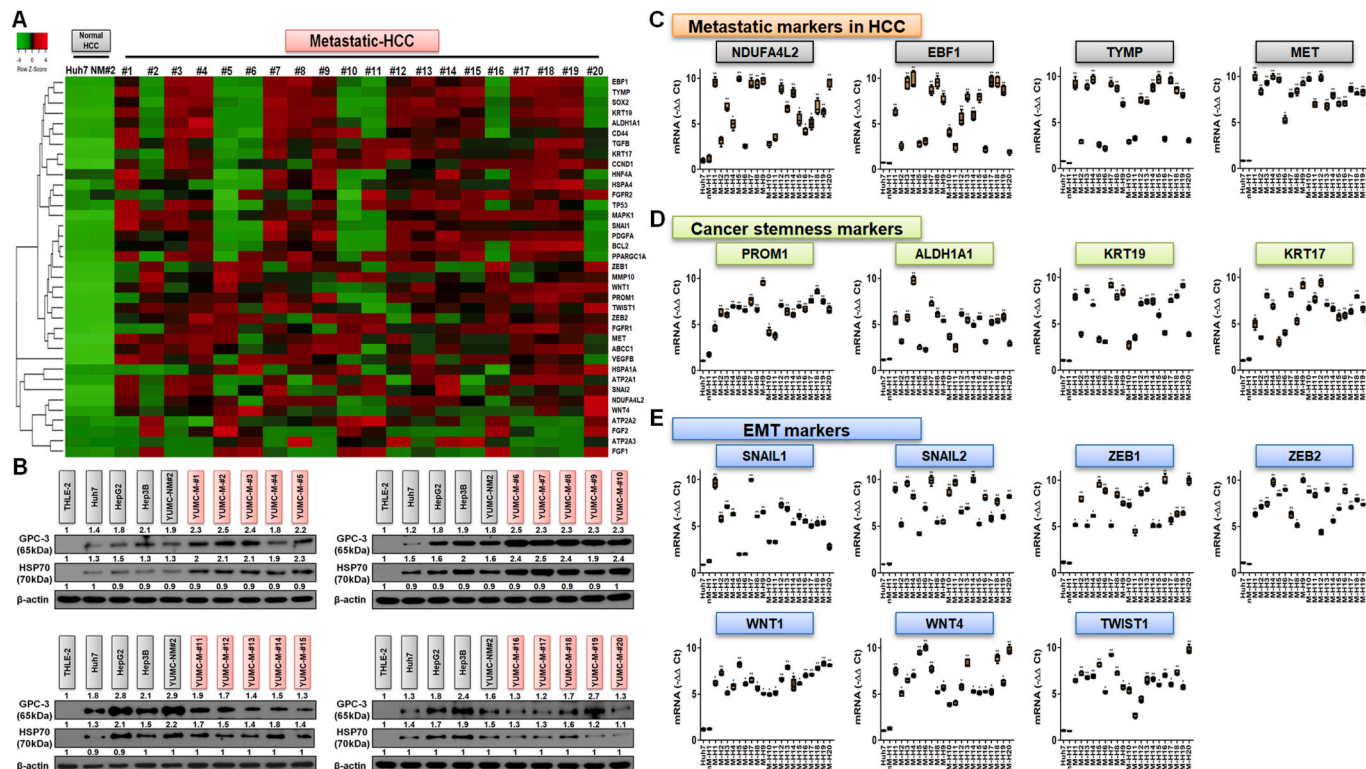


Fig. 1. Target gene expression profiling in patient-derived metastatic HCC. **A**, Hierarchical clustering shows differential gene expression. Gene expression profiles in patient-derived non-metastatic HCC ($n = 1$, male) and metastatic HCC cells ($n = 20$, male; 14, female; 6) compared to those of the established HCC cell line (Huh7). **B**, Immunoblot analysis shows the expression of HCC markers in the non-metastatic and metastatic HCC cells compared with that in normal liver cells. **C–E**, RNA expression variances are evaluated using qRT-PCR in non-metastatic and metastatic HCC cells compared with the established HCC cell line (Huh7). *, $p < 0.05$ and **, $p < 0.01$ versus Huh7. HCC, hepatocellular carcinoma.

Table 1
Clinical characteristics and pathological features of the patients from whom hepatocellular carcinoma cells were derived. Patient-derived HCC cells were isolated from the corresponding surgical or biopsy specimens.

	YUMC-NM-H2	YUMC-M-H4	YUMC-M-H5	YUMC-M-H6
Age at Diagnosis	65	36	69	60
Gender	Male	Male	Male	Male
Primary Disease Site	Liver	Liver	Liver	Liver
Stage	T2N0M0	T1N0M1	T1N1M1	T3N0M0
Primary Pathology	Hepatocellular carcinoma	Hepatocellular carcinoma(Metastasis after cisplatin treatment)	Hepatocellular carcinoma(Metastasis after cisplatin treatment)	Hepatocellular carcinoma(Metastasis after cisplatin treatment)
Classification of specimen used for culture	Fresh tumor	Fresh tumor	Fresh tumor	Fresh tumor
Obtained from	Severance Hospital, Seoul, Korea	Severance Hospital, Seoul, Korea	Severance Hospital, Seoul, Korea	Severance Hospital, Seoul, Korea

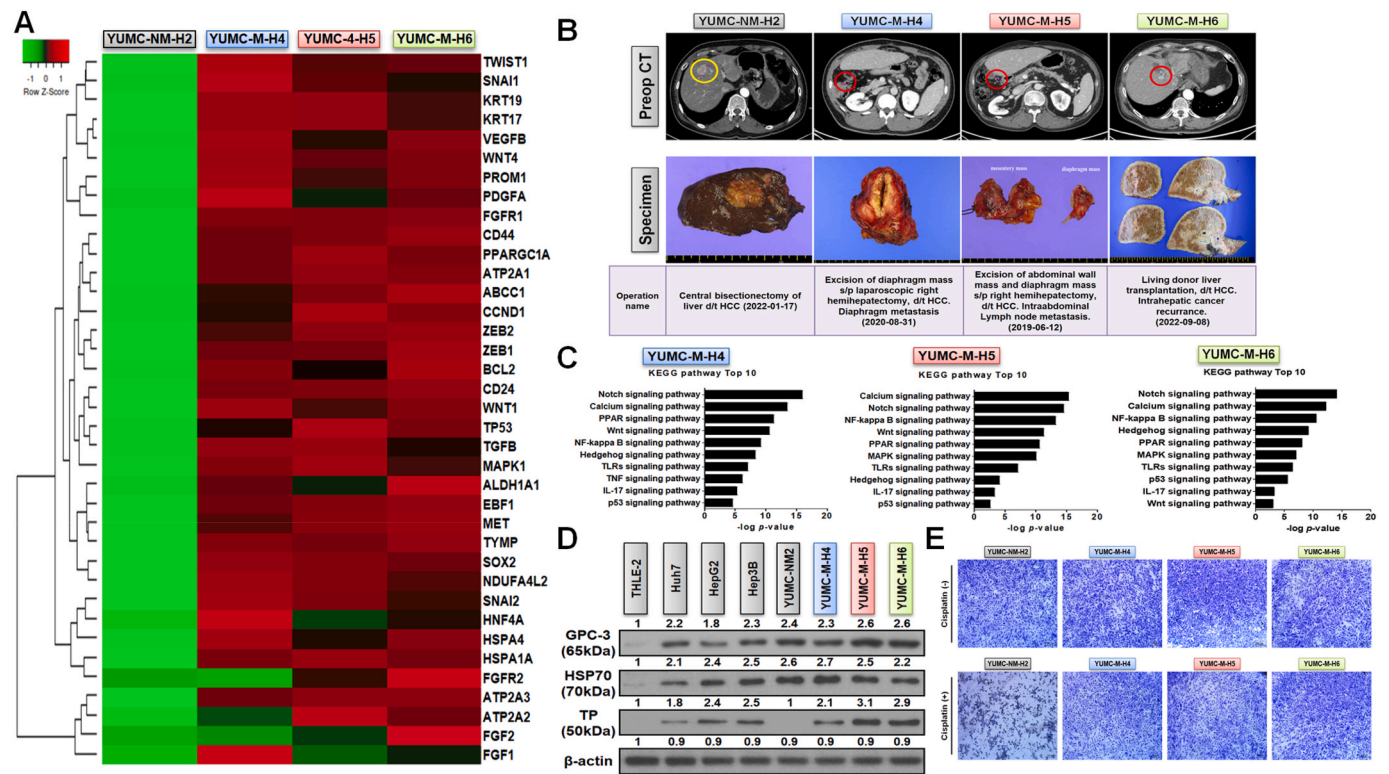


Fig. 2. Target gene expression and signaling pathway profiling based on HCC specimens used for the establishment of patient-derived cell lines. **A**, Hierarchical clustering demonstrates distinct gene expression patterns in metastatic HCC cells compared with patient-derived non-metastatic HCC cells. **B**, CT images and sectioned primary tumor specimens are presented for each case: a 65-year-old man with HCC, from whom the non-metastatic cell line YUMC-NM-H2 was established; a 36-year-old man with HCC, yielding the metastatic cell line YUMC-M-H4; a 69-year-old man with HCC, yielding YUMC-M-H5; and a 60-year-old man with HCC, yielding YUMC-M-H6. Yellow circles highlight primary tumors without evidence of metastasis or recurrence, whereas red circles denote metastatic lesions. **C**, Bar plot showing the top 10 pathways markedly upregulated in patient-derived metastatic HCC cells compared with their non-metastatic counterparts. **D**, Immunoblot analysis validating the expression of HCC and metastatic markers in patient-derived non-metastatic and metastatic HCC cells, compared to those in normal liver cells and established HCC cell lines. **E–I**, Gene expression differences between non-metastatic and metastatic HCC cells are assessed using qRT-PCR analysis. Each assay is performed at least in quintuplicate. * $p < 0.05$ and ** $p < 0.01$ versus non-metastatic HCC, YUMC-NM-H2. CT, computed tomography; HCC, hepatocellular carcinoma. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

markers (TYMP, EBF1, NDUFA4L2, and MET; Fig. 2A–F), the multidrug-resistance gene ABCC1 (Fig. 2G), cancer stemness-associated genes (PROM1/CD133, ALDH1A1, KRT17, KRT19, CD44, CD24, and SOX2; Fig. 2H), and EMT-related genes (SNAIL1, SNAIL2, WNT1, WNT4, ZEB1, ZEB2, and TWIST1; Fig. 2I). Pathway enrichment analysis revealed increased activity of signaling pathways associated with metabolic adaptation and cell survival, including NF κ B, PPAR, calcium, Notch, Hedgehog, and Wnt signaling, in metastatic HCC cells compared with non-metastatic cells (Fig. 2C). Consistent with their clinical origin, patient-derived metastatic HCC cells showed intrinsic resistance to cisplatin. Cisplatin treatment markedly reduced the viability of non-

metastatic HCC cells, whereas metastatic HCC cells showed minimal sensitivity (Fig. 2E).

2.2. PPS03 induces cancer-selective necroptosis through ROS overload in HCC cells

To assess the anticancer activity of PPS03, cell viability was examined in patient-derived non-metastatic HCC cells (YUMC-NM-H2), cisplatin-resistant metastatic HCC cells (YUMC-M-H4, -H5, and -H6), and normal hepatocytes (THLE-2). Cells were treated with increasing concentrations of PPS03 or cisplatin (0–400 μ g/mL or μ M), and dose-

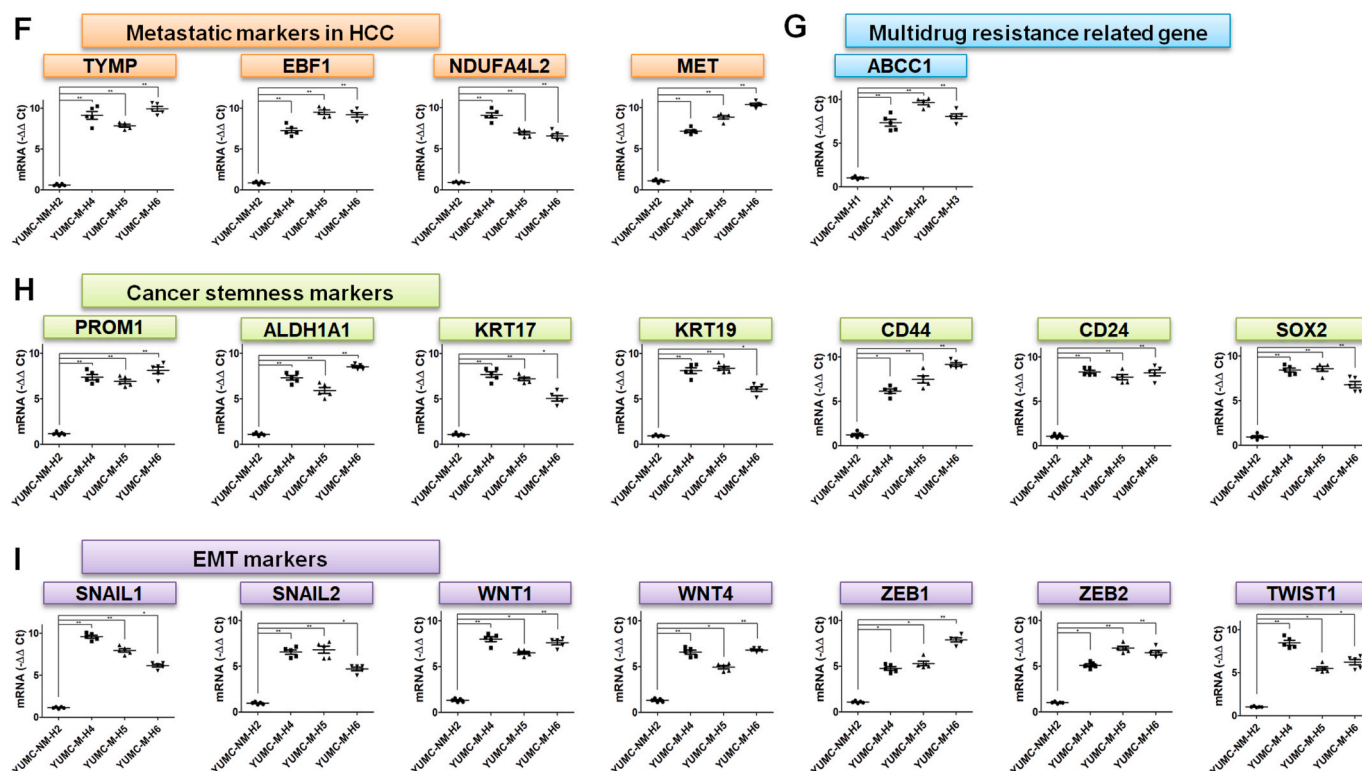


Fig. 2. (continued).

dependent cytotoxicity was evaluated (Fig. 3A–G). PPS03 and cisplatin both reduced viability in non-metastatic HCC cells in a dose-dependent manner (Fig. 3A). In contrast, cisplatin had minimal effects on metastatic HCC cells, whereas PPS03 markedly suppressed the viability of all metastatic HCC cell lines examined (Fig. 3B–D). Established HCC cell lines (Huh7, HepG2, and Hep3B) showed response patterns comparable to those of non-metastatic patient-derived HCC cells (Fig. S1A). To directly compare cytotoxic effects between cancer cells and normal hepatocytes, PPS03 and cisplatin were evaluated under identical conditions in THLE-2 cells. Cisplatin treatment resulted in a pronounced reduction in THLE-2 cell viability, whereas PPS03 exhibited minimal cytotoxicity even at the highest concentrations (Fig. 3E and F). Notably, under the same treatment conditions, PPS03 induced substantial cell death in both non-metastatic and metastatic HCC cells, with minimal effects on THLE-2 cells, indicating a clear differential sensitivity. IC₅₀ values for PPS03 and cisplatin across non-metastatic HCC cells, metastatic HCC cells, and normal hepatocytes are summarized in Table 2. Consistently, the IC₅₀ value of PPS03 was markedly higher in THLE-2 cells than in HCC cells, further supporting reduced cytotoxicity in normal hepatocytes. Furthermore, treatment with PPS03 at 25 µg/mL had negligible effects on THLE-2 cells but significantly reduced the viability of both non-metastatic and metastatic HCC cells (Fig. 3G), reinforcing the cancer-selective activity of PPS03.

Next, cell viability assays were performed using PPS03 in combination with the pan-caspase inhibitor z-VAD to determine whether PPS03-induced cytotoxicity is more closely associated with necroptosis than apoptosis. Co-treatment with z-VAD did not result in a significant change in cell viability compared with PPS03 treatment alone. The expression of necroptosis- and apoptosis-related markers was further examined by immunoblotting. PPS03 treatment markedly increased the levels of phosphorylated RIP1, RIP3, and MLKL compared with untreated controls (Fig. 3I). In contrast, the apoptosis-related marker caspase-3 showed no substantial change in response to PPS03 treatment (Fig. 3I). These findings suggest that PPS03 induces necroptosis rather than apoptosis. Consistent with this, PPS03 treatment led to a robust

accumulation of intracellular ROS in HCC cells. Notably, ROS induced by PPS03 was predominantly mitochondrial in origin and associated with increased hydrogen peroxide (H₂O₂) levels, rather than lipid peroxidation-associated ROS. Mitochondrial ROS levels were increased in both non-metastatic and metastatic HCC cells; however, metastatic cells exhibited a markedly greater increase following PPS03 exposure (Fig. 3J and K). To establish a causal relationship between ROS accumulation and necroptosis, rescue experiments were performed using the ROS scavenger N-acetylcysteine (NAC) and RIP1 inhibitor Necrostatin-1 (Nec-1) (Fig. 3L–Q). NAC pretreatment significantly reduced PPS03-induced ROS levels (Fig. 3L) and restored cell viability (Fig. 3M – P). Consistently, the increase in ROS induced by PPS03 was strongly attenuated by NAC co-treatment. In contrast, Nec-1 did not significantly affect PPS03-induced ROS levels (Fig. 3L), while both NAC and Nec-1 pretreatment significantly rescued PPS03-induced loss of cell viability (Fig. 3M – P). Importantly, NAC also attenuated the phosphorylation of RIP1, RIP3, and MLKL, indicating that ROS accumulation is required for necroptotic signaling. In parallel, Nec-1 treatment markedly restored cell viability and suppressed RIP1/RIP3/MLKL phosphorylation without altering ROS levels, suggesting that RIP1 functions downstream of ROS (Fig. 3Q). These findings strongly support that PPS03-induced cell death is mediated through a RIP1-dependent necroptotic pathway rather than alternative forms of regulated cell death.

Collectively, these findings provide direct evidence that ROS accumulation is required for PPS03-induced necroptosis and acts upstream of RIP1-dependent signaling.

2.3. Mechanistic basis of PPS03 uptake and ROS overload in HCC cells

To elucidate the mechanism underlying the cancer-selective cytotoxicity of PPS03, we next sought to determine how PPS03 is preferentially internalized by HCC cells. Given that PPS03 is a polymer–metal complex with macromolecular characteristics, we hypothesized that its cellular uptake may be mediated by macropinocytosis, a non-selective endocytic pathway known to facilitate the internalization of

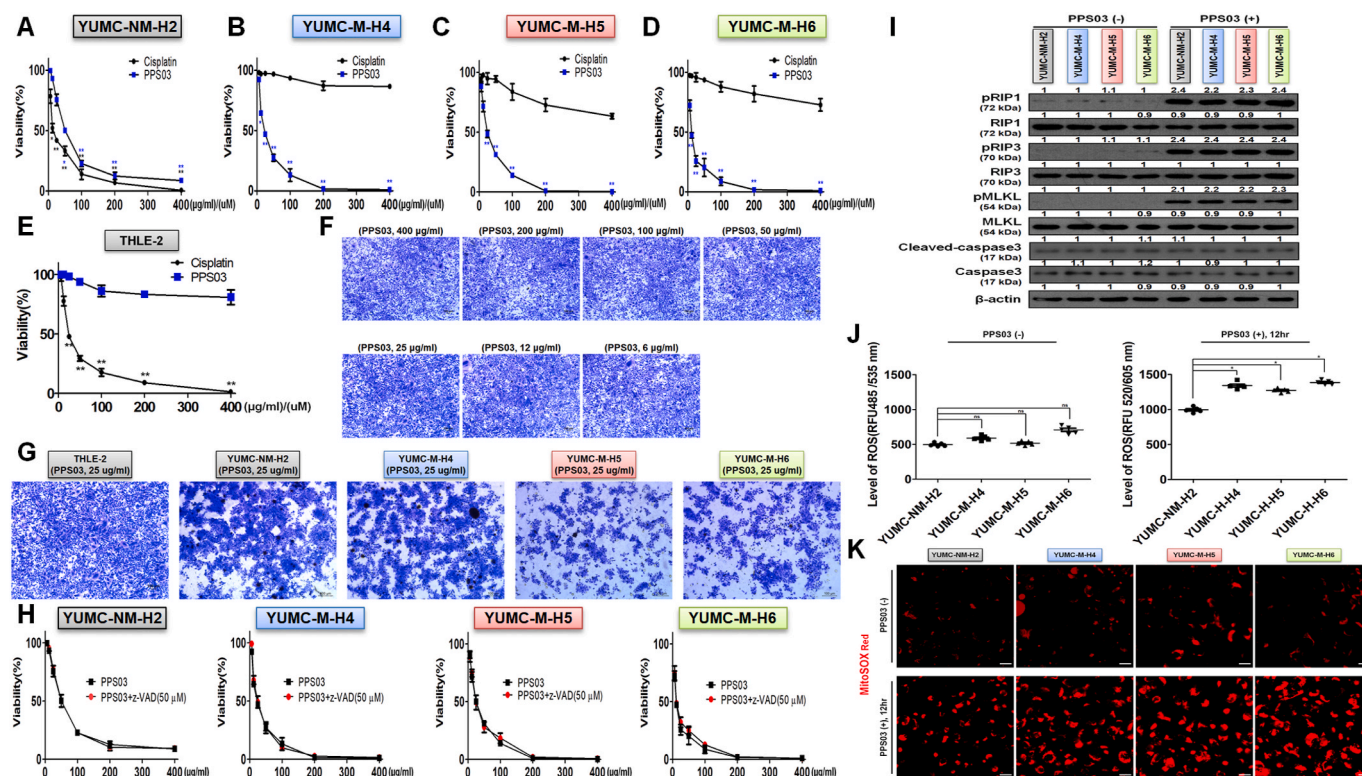


Fig. 3. PPS03 induces cancer-specific necroptosis in HCC by selectively exploiting ROS overload. **A–D**, Cell viability of patient-derived non-metastatic and metastatic HCC cells following cisplatin or PPS03 treatment. *cisplatin; *PPS03; * $p < 0.05$ and ** $p < 0.01$ versus controls (untreated cisplatin or PPS03 group, respectively). Each assay is performed at least in quintuplicate. **E and F**, Cell viability and cytomorphological changes in normal liver THLE-2 cells treated with cisplatin or PPS03. *cisplatin; *PPS03; * $p < 0.05$ and ** $p < 0.01$ versus controls (untreated cisplatin or PPS03 group, respectively). Quantitative data are presented as mean $\pm$ SEM from three independent experiments. **G**, Representative cytomorphology of normal liver cells, non-metastatic HCC, and metastatic HCC cells following PPS03 treatment (25 $\mu\text{g/mL}$). **H**, Cell viability of patient-derived non-metastatic and metastatic HCC cells following PPS03 or with z-VAD (apoptosis inhibitor) treatment. Quantitative data are presented as mean $\pm$ SEM from three independent experiments. **I**, Immunoblot analysis of necroptosis markers in non-metastatic and metastatic HCC cells in the presence or absence of PPS03. **J and K**, Intracellular or mitochondrial ROS levels and confocal imaging of non-metastatic and metastatic HCC cells with or without PPS03 (25 $\mu\text{g/mL}$) treatment. * $p < 0.05$ and ** $p < 0.01$ versus control (YUMC-NM-H2). Magnification, $\times 400$. Scale bar, 20 μm . Each assay is performed at least in quintuplicate. **L**, Intracellular ROS levels were measured following treatment with PPS03 in the presence or absence of NAC or Nec-1. * $p < 0.05$ and ** $p < 0.01$. **M–P**, Cell viability assays were performed in metastatic HCC cells treated with PPS03 with or without NAC or Nec-1 pretreatment. * $p < 0.05$ and ** $p < 0.01$ versus control (absence of PPS03). Quantitative data are presented as mean $\pm$ SEM from three independent experiments. **Q**, Immunoblot analysis of necroptosis-associated signaling proteins following PPS03 treatment in the presence or absence of NAC or Nec-1. Each assay is performed at least in quintuplicate. HCC, hepatocellular carcinoma; NAC, N-acetylcysteine; Nec-1, Necrostatin-1; ROS, reactive oxygen species.

extracellular fluid and high-molecular-weight substances. Notably, macropinocytosis is upregulated constitutively in many cancer cells to support metabolic demands under nutrient-deprived conditions, whereas its activity remains relatively low in normal hepatocytes. Therefore, we investigated whether PPS03 exploits macropinocytosis as a selective uptake process in HCC cells, to explain the mechanism underlying PPS03-induced cancer-selective necroptosis (Fig. 4). PPS03 is a PASP-based polymer–metal complex composed of polyaspartic acid chelated with SeMet and ferrous iron (Fe^{2+}) (Fig. 4A). The design of PPS03 as a PASP-based polymer–metal complex confers several advantages for macropinocytosis-mediated uptake. Its polymeric structure and hydrophilic properties facilitate efficient internalization via fluid-phase endocytosis. In addition, the incorporation of SeMet and ferrous iron enables intracellular redox reactions following uptake, leading to enhanced H_2O_2 production and subsequent mitochondrial ROS accumulation. These features collectively allow PPS03 to exploit elevated macropinocytic activity in cancer cells, achieving selective intracellular delivery and cytotoxicity. Physicochemical characterization revealed that PPS03 had a uniform nanoscale size distribution with a narrow polydispersity index (PDI), along with a stable surface charge under physiological conditions (Fig. S5A–B). The hydrodynamic diameter and PDI remained stable in relevant biological media, indicating robust

colloidal stability (Fig. S5C). In addition, inductively coupled plasma–optical emission spectrometry (ICP-OES) analysis confirmed the element content, loading efficiency, and final concentrations of SeMet and ferrous iron in the formulation (Fig. S5D). These physicochemical properties further support the stability and potential translational applicability of PPS03. Although detailed metal release kinetics were not extensively profiled, ICP-based compositional analysis and stability assessment under physiological condition indicate that PPS03 maintains its structural integrity prior to cellular uptake, while functional intracellular activation is supported by ROS-dependent cytotoxicity and SeMet-dependent activity. A schematic overview of PPS03-mediated intratumoral H_2O_2 elevation and ROS overaccumulation is shown in Fig. 4B. Intracellular uptake of PPS03 was examined using fluorescent isotope-tagged PPS03 under confocal microscopy. PPS03 exhibited minimal uptake in normal hepatocytes (THLE-2), whereas substantial internalization was observed in HCC cells (Fig. 4C, top panel). Notably, metastatic HCC cells had significantly higher levels of PPS03 uptake compared with non-metastatic HCC cells. To determine whether PPS03 internalization occurred via macropinocytosis, HCC cells were pre-treated with the macropinocytosis inhibitor 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) prior to PPS03 exposure. Pharmacological inhibition of macropinocytosis markedly reduced intracellular PPS03 uptake

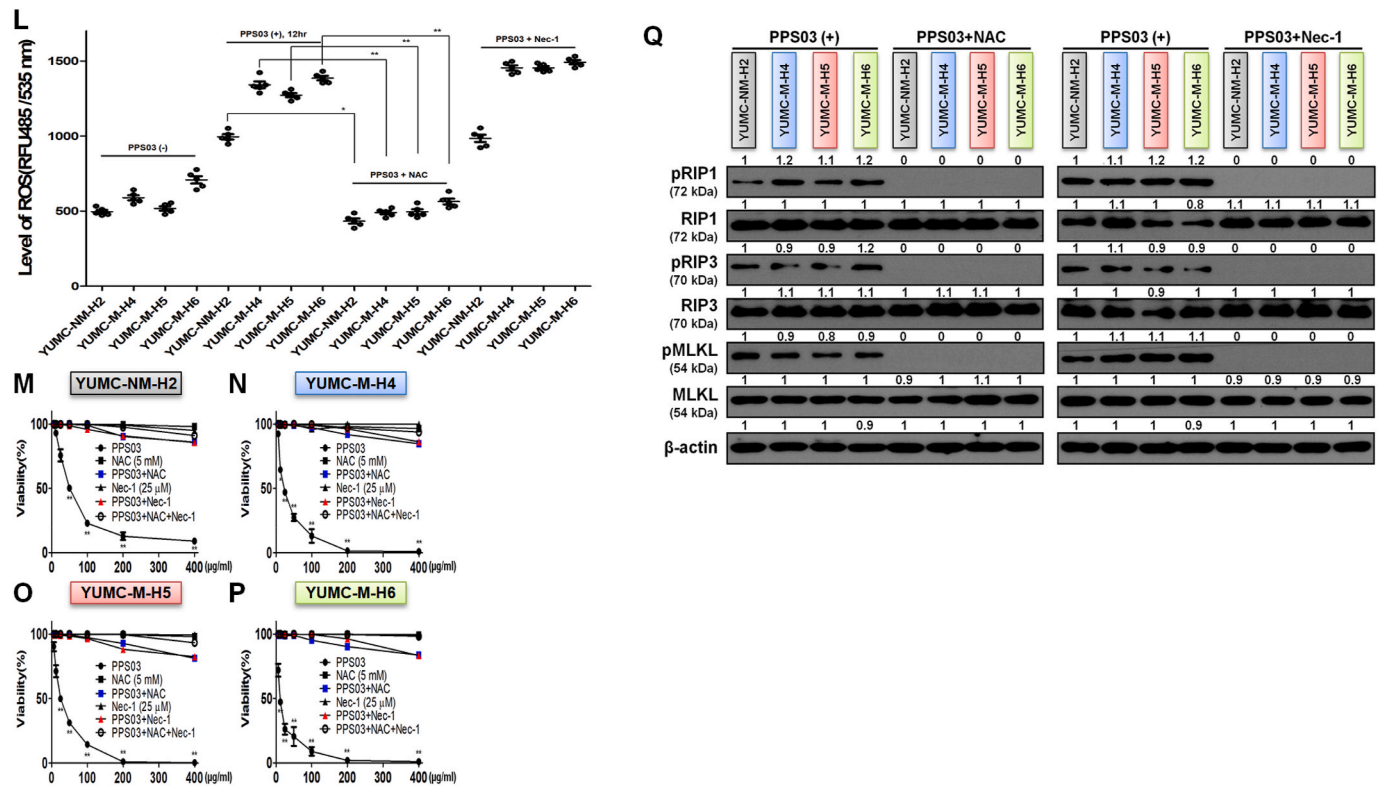


Fig. 3. (continued).

Table 2
IC₅₀ values of cisplatin and PPS03 in normal liver cells (THLE-2), non-metastatic HCC (YUMC-NM-H2), and metastatic HCC cells (YUMC-M-H4, -H5, and -H6). Data represent means from three independent MTT assays performed in triplicate. SEM, standard error of the mean; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC₅₀, half-maximal inhibitory concentration.

Cell Line	Histopathology	Animal	Cell proliferation IC ₅₀ (μM)	
			Cisplatin (uM)	PPS03 (μg/ml)
YUMC-NM-H2	Liver, HCC	Human	14.3 (± 0.2)	25.5 (± 0.1)
YUMC-M-H4	Liver, HCC	Human	-	20.4 (± 0.1)
YUMC-M-H5	Liver, HCC	Human	-	12.5 (± 0.3)
YUMC-M-H6	Liver, HCC	Human	-	12.2 (± 0.2)
THLE-2	Liver, normal	Human	25.4 (± 0.2)	-

(Fig. 4C, bottom panel) and significantly attenuated PPS03-induced cytotoxicity (Fig. 4D), indicating that macropinocytosis served as a principal route for PPS03 incorporation into HCC cells. To further validate macropinocytosis-mediated uptake, a dextran (a canonical marker of macropinocytosis-mediated fluid-phase uptake) uptake assay was performed using FITC-labeled dextran, a well-established marker of fluid-phase endocytosis. PPS03 treatment significantly increased dextran uptake in HCC cells, whereas pretreatment with the macropinocytosis inhibitor EIPA markedly reduced dextran internalization. Quantitative analysis of fluorescence intensity further confirmed that PPS03 significantly enhanced dextran uptake, which was effectively suppressed by EIPA co-treatment. Furthermore, the PPS03-induced increase in dextran uptake was effectively abolished by EIPA co-treatment. These findings provide strong functional evidence that PPS03 uptake is mediated, at least in part, by macropinocytosis (Fig. S2A). To assess the contribution of PPS03 components to its cytotoxic activity, a SeMet-depleted derivative of PPS03 [PPS03-S(-)] was generated (Fig. 4E). Dose-response analyses revealed that PPS03-S(-), which retains the polymer-Fe²⁺ backbone but lacks SeMet, exhibited substantially reduced cytotoxicity compared with PPS03 in HCC cells (Fig. 4F),

indicating that SeMet is a major determinant of PPS03-induced cell death.

2.4. PPS03 suppresses stem-like tumor cell properties in patient-derived HCC cells

Cancer stem-like cells are known to contribute to therapeutic resistance and metastatic progression in HCC. Given that patient-derived metastatic HCC cells exhibited elevated expression of stemness-associated markers (Fig. 2), we next investigated whether PPS03 affects stem-like tumor cell populations. To this end, sphere formation assays were performed under non-adherent, serum-free conditions. Control cells formed compact and well-defined tumor spheres, whereas under the PPS03 treatment, both the number and size of tumor spheres were markedly reduced, indicating impaired self-renewal capacity (Fig. S3A and B). Consistently, immunoblot analysis showed reduced CD133 levels following PPS03 treatment, supporting a decrease in stem-like tumor cell populations (Fig. S3C). These findings suggest that PPS03 exerts cytotoxic effects on bulk tumor cells and effectively suppresses stem-like tumor populations, rather than directly modulating stemness-related signaling pathways.

2.5. PPS03 preferentially suppresses metastatic HCC growth without inducing systemic toxicity in patient-derived xenograft models

To evaluate the in vivo relevance of PPS03-mediated cancer-selective cytotoxicity, we compared PPS03 with cisplatin in patient-derived HCC xenograft models. Dose-escalation studies (25–400 mg/kg) were first conducted in metastatic HCC xenografts (YUMC-M-H4, -H5, and -H6) to define the therapeutic window. Among the tested doses (25–400 mg/kg), 100 mg/kg consistently provided the optimal balance between antitumor efficacy and tolerability, showing robust tumor growth inhibition without significant body weight loss or observable systemic toxicity, and was therefore selected for subsequent in vivo experiments (Fig. S5A–C). Cisplatin (1.5 mg/kg) was administered

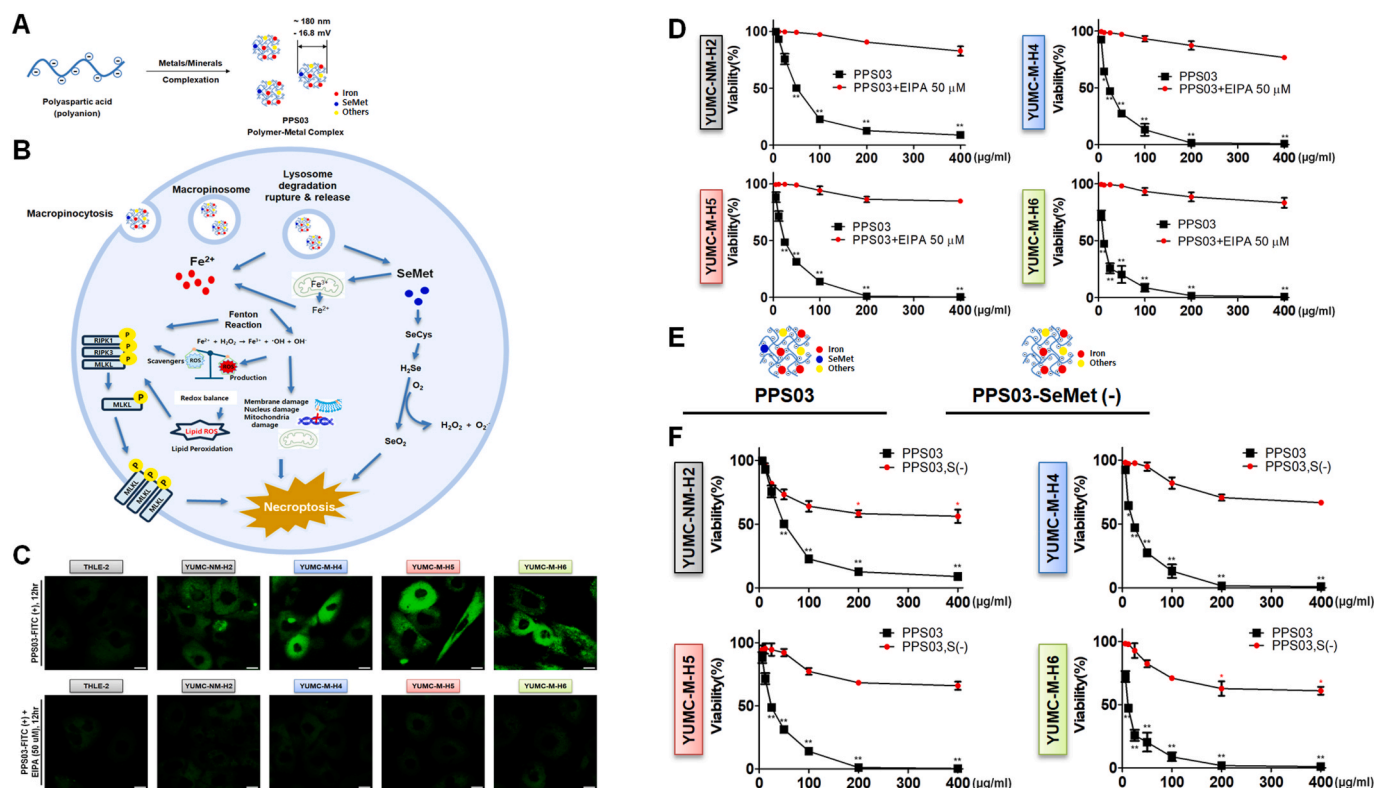


Fig. 4. PPS03 enhances cancer-selective necroptosis via ROS accumulation. **A**, PPS03 is a polymer-metal complex composed of polyaspartic acid chelated with SeMet and Fe²⁺. **B**, Schematic illustration depicting the enhancement of necroptosis driven by ROS overaccumulation through PPS03-mediated intratumoral H₂O₂ elevation. **C**, Confocal imaging of fluorescent isotope-tagged PPS03 uptake in normal liver cells, non-metastatic HCC cells, and metastatic HCC cells. Magnification, ×400. Scale bar, 20 μm. **D**, Viability of HCC cells treated with PPS03 alone or in combination with EIPA, a macropinosytosis inhibitor. **E**, Illustration of PPS03 and PPS03 without SeMet. **F**, Cell viability in non-metastatic and metastatic HCC cells treated with PPS03 or SeMet-depleted PPS03 in a dose-dependent manner. *PPS03; *PPS03 without SeMet; **p < 0.05 and ***p < 0.01 versus controls (untreated PPS03 or SeMet-depleted PPS03 group, respectively). Each assay is performed at least in quintuplicate. Fe²⁺, ferrous iron; HCC, hepatocellular carcinoma; ROS, reactive oxygen species; SeMet, selenomethionine.

based on commonly used dosing regimens in preclinical xenograft models to allow for comparative evaluation under clinically relevant conditions. Longitudinal tumor growth curves over 43 days and representative excised tumors at study termination are shown in Fig. S6A–F. In xenografts derived from non-metastatic HCC cells (YUMC-NM-H2), both cisplatin and PPS03 significantly reduced tumor growth compared to the controls (Fig. 5A, top). In contrast, in metastatic HCC xenografts (YUMC-M-H4, -H5, and -H6), cisplatin exerted minimal antitumor activity, whereas PPS03 induced marked tumor regression (Fig. 5B–D, top). Consistent with these findings, tumor weights were significantly reduced in response to both treatments in non-metastatic HCC xenografts, while only PPS03 significantly decreased tumor weight in metastatic models (Fig. 5A–D, middle panel). Body weight remained stable in PPS03-treated mice across both non-metastatic and metastatic models, whereas cisplatin treatment resulted in progressive body weight loss, consistent with systemic toxicity (Fig. 5A–D, bottom panel). Immunoblot analysis of tumor lysates revealed robust activation of necroptotic signaling, with increased phosphorylation of RIP1, RIP3, and MLKL in PPS03-treated tumors compared with that in controls (Fig. 5E). In contrast, the apoptosis-related marker caspase-3 showed no substantial change following PPS03 treatment (Fig. 5E). These results suggest that PPS03 selectively increased necroptotic cell death (bypassing apoptotic execution). To further assess systemic toxicity, survival was monitored in non-tumor-bearing mice. PPS03 treatment did not affect survival compared with that of untreated controls, whereas cisplatin significantly reduced survival during the observation period (Fig. 5F). Histological analyses demonstrated that PPS03 administration did not induce detectable hepatic injury in either non-metastatic or metastatic HCC xenograft models, whereas carbon

tetrachloride-treated controls exhibited hepatic damage (Fig. 5G). No evidence of cardiac toxicity was observed following PPS03 treatment (Fig. 5H). To further evaluate systemic toxicity, serum biochemical analyses were performed to assess liver function in tumor-bearing mice following treatment. Specifically, serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured in vehicle-, PPS03-, and cisplatin-treated groups. Cisplatin-treated mice exhibited a significant elevation in both AST and ALT levels compared with the vehicle group, indicating substantial hepatotoxicity. In contrast, PPS03-treated mice showed AST and ALT levels comparable to those observed in the vehicle group, suggesting minimal hepatic toxicity (Fig. S4A and B). These findings are consistent with the stable body weight profiles and histological analyses showing no detectable liver damage in PPS03-treated mice, further supporting the favorable in vivo safety profile of PPS03.

The clinical benefit of systemic anticancer therapies has been demonstrated across multiple malignancies, including in the neo-adjuvant and adjuvant settings [20–22]. However, no standardized perioperative systemic strategy for HCC has been established [23], and disease recurrence and metastatic progression remain the principal drivers of mortality [24]. A major contributor to this unmet clinical need is the emergence of metastatic HCC populations that acquire resistance to conventional cytotoxic agents through extensive epigenetic and metabolic reprogramming [25]. These adaptations enable tumor cells to attenuate endoplasmic reticulum stress, evade calcium-dependent apoptosis, and sustain survival under chemotherapeutic and metabolic stress [26–32]. Macropinosytosis has emerged as a key metabolic adaptation supporting the survival of highly malignant and drug-resistant cancer cells by facilitating the non-selective uptake of

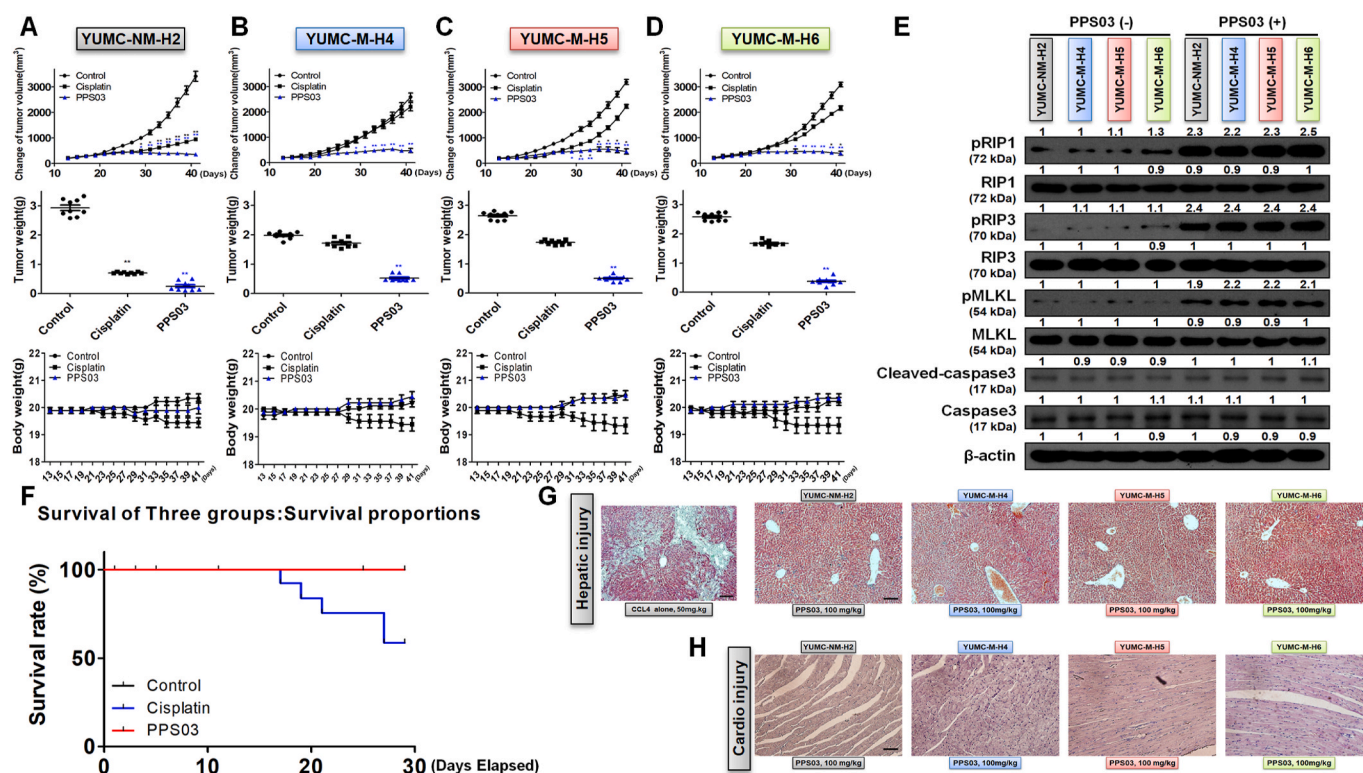


Fig. 5. PPS03 enhances cancer-selective necroptosis in xenograft models established from patient-derived non-metastatic and metastatic HCC cells. Based on the dose-dependent tumor-suppressive effects of PPS03, a fixed dose (100 mg/kg) is applied for subsequent treatment in both non-metastatic and metastatic HCC xenograft models. **A**, non-metastatic HCC cells (YUMC-NM-H2), **B**, **C**, and **D** metastatic HCC cells (YUMC-M-H4, -H5, and -H6). Changes in relative tumor volumes (top), resected tumor weights (middle), and whole-body weights (bottom) of patient-derived HCC cells (each group, $n = 10$). Tumor volumes are calculated in NOD/Shi-scid, IL-2R γ KOJic (NOG) mice. Animals are treated with a fixed dose of PPS03 (100 mg/kg). Data represent the mean $\pm$ standard error of the mean. * $p < 0.05$ and ** $p < 0.01$ versus control (untreated PPS03 group). **E**, Immunoblot analysis of necroptosis markers in non-metastatic and metastatic HCC tissue lysate in the presence or absence of PPS03. **F**, The change of death ratio measurement following treatment with PPS03 (100 mg/kg) in normal mice (not xenograft model) compared with the non-treated group (control). **G** and **H**, Histological evaluation of liver and heart tissues following the same treatments, assessed by Masson's trichrome staining. Nuclei appear black, cytoplasm and muscle fibers appear red, and extracellular matrix components appear blue. All sections are examined at $\times 200$ magnification (scale bar: 100 μ m). Each assay is performed in quintuplicate, and representative images are shown. HCC, hepatocellular carcinoma. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

extracellular nutrients and macromolecules [17,19,33–35]. Importantly, basal macropinocytic activity is low in normal hepatocytes and is induced only under specific physiological conditions, whereas in cancer cells, this pathway is constitutively activated in response to nutrient-deprived tumor microenvironments [18,19,36–38]. This differential dependency identifies macropinocytosis as a potential therapeutic vulnerability that may be selectively targeted in malignant cells [39–44].

In this study, we exploited this vulnerability to develop PPS03, a PASP-based polymer-metal complex that preferentially enters HCC cells via macropinocytosis and delivers Fe^{2+} and SeMet, thereby inducing excessive mitochondrial ROS accumulation. These findings further support that the incorporation of SeMet, rather than the polymer-iron backbone alone, is critical for PPS03-induced cytotoxicity, highlighting the functional importance of SeMet in driving ROS-mediated necroptotic signaling. While EIPA is not entirely specific, the combined use of pharmacological inhibition and dextran-based functional assays provides consistent evidence supporting macropinocytosis-mediated uptake. Mechanistically, PPS03 promotes H_2O_2 -dependent mitochondrial ROS overload, resulting in robust activation of the RIP1-RIP3-MLKL necroptotic pathway. ROS-based therapeutic strategies have been widely explored in HCC, primarily aiming at inducing oxidative stress in cancer cells beyond the tolerable threshold. Several anticancer approaches exploit elevated basal ROS levels in tumor cells to trigger cell death; however, their clinical applicability has been limited by insufficient selectivity and off-target toxicity in normal tissues. In this context,

PPS03 differs from conventional ROS-inducing agents by leveraging macropinocytosis-mediated selective uptake, thereby enabling tumor-specific ROS amplification while sparing normal hepatocytes. This selective mechanism may overcome a major limitation of ROS-based therapies and improve their translational potential in HCC. PPS03 exhibited pronounced cancer selectivity, sparing normal hepatocytes while effectively suppressing both non-metastatic and metastatic HCC cells, including cisplatin-resistant patient-derived models. Importantly, PPS03 did not induce elevation of serum AST or ALT levels, in contrast to cisplatin, further supporting its minimal hepatotoxicity and potential clinical applicability. These results are further supported by both biochemical and histological assessments demonstrating minimal systemic toxicity. These findings are particularly notable given the limited efficacy of conventional chemotherapeutics in refractory metastatic HCC and underscore the therapeutic potential of inducing necroptosis as an alternative to apoptosis-based strategies. Although immune cells are known to exhibit macropinocytic activity, PPS03 administration did not induce overt systemic or immune-related toxicities in non-tumor-bearing mice during a 29-day repeated-dose study. Furthermore, the use of patient-derived HCC cells obtained from individuals previously exposed to systemic therapy enhances the translational relevance of our findings. Notably, PPS03 significantly suppressed sphere-forming capacity and reduced CD133 expression, indicating effective targeting of stem-like tumor cell populations. Given that cancer stem-like cells are closely associated with therapeutic resistance and tumor recurrence, these findings further enhance the translational relevance of PPS03. By

targeting both bulk tumor cells and stem-like subpopulations, PPS03 may provide a therapeutic advantage in preventing disease relapse and overcoming drug resistance in advanced HCC.

Nevertheless, some limitations warrant consideration. Although ROS-dependent cell death mechanisms such as ferroptosis cannot be excluded, the observed activation of the RIP1–RIP3–MLKL axis and the robust rescue effects of Necrostatin-1 strongly support necroptosis as the predominant mechanism of cell death induced by PPS03. Further characterization of ROS species, including the potential contribution of lipid peroxidation, would provide additional insight into the precise mechanisms of PPS03-induced cell death. Future studies incorporating ferroptosis-specific assays and inhibitors may help to further distinguish between different ROS-dependent cell death pathways. The downstream signaling events linking ROS overload to necroptotic execution require further delineation, and comprehensive pharmacokinetic and pharmacodynamic profiling remain essential to support clinical translation. In addition, future studies should examine the interaction of PPS03 with the tumor immune microenvironment. Given that necroptosis is known to promote the release of damage-associated molecular patterns (DAMPs), PPS03-induced cell death may have the potential to modulate the tumor immune microenvironment. Although the present study focused on the direct cytotoxic and mechanistic effects of PPS03, its immunomodulatory impact remains to be fully elucidated. Future studies incorporating immunofluorescence-based analysis of tumor tissues to assess immune cell infiltration and activation will be important to further define the *in vivo* mechanisms of PPS03.

In summary, our findings provide mechanistic and preclinical evidence that macropinocytosis-dependent metabolic vulnerabilities can be exploited to selectively eliminate refractory HCC. By inducing ROS-driven necroptosis without causing the damage to normal hepatocytes, PPS03 represents a promising therapeutic candidate for patients with drug-resistant metastatic disease, for whom effective treatment options remain limited.

3. Conclusions

This study identifies PPS03, a novel PASP-based polymer–metal complex, as a therapeutic candidate that induces cancer-selective necroptosis through differential macropinocytic uptake. Capitalizing on the increased macropinocytic activity in malignant hepatocytes, PPS03 preferentially delivers SeMet and Fe²⁺ to tumors, amplifying H₂O₂-mediated mitochondrial ROS accumulation and promoting selective cytotoxicity. PPS03 exhibits minimal effects on normal hepatocytes while demonstrating robust antitumor activity in both non-metastatic and metastatic HCC models, including cisplatin-resistant cells. Collectively, these findings provide mechanistic and translational support for targeting macropinocytosis-dependent metabolic vulnerabilities in refractory HCC and suggest PPS03 as a potential therapeutic strategy for patients with limited treatment options.

4. Materials and methods

4.1. Patient characteristics

The patients recruited for this study were diagnosed with HCC and underwent liver resection between January 2017 and December 2024 at the Severance Hospital (Seoul, South Korea). The study protocol was approved by the Institutional Review Board (IRB) of Severance Hospital, Yonsei University College of Medicine (IRB protocol: 3-2022-0331). Cell samples were obtained from the patients at the Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. Further details of the protocol have been published previously [45,46].

4.1.1. Patient 1

YUMC-NM-H2 was a 65-year-old man who presented with a 2.5 cm multinodular-confluent HCC located in segment 4 of the liver. On

January 17, 2022, the patient underwent a central segmentectomy with hepatic parenchyma preservation under general anesthesia. The post-operative pathology confirmed hepatocellular carcinoma with clear resection margins. During routine follow-up examinations, including radiological studies and serum tumor markers (alpha-fetoprotein), no evidence of recurrence was observed.

4.1.2. Patient 2

YUMC-M-H4 was a 36-year-old man who was diagnosed with hepatocellular carcinoma with portal vein thrombosis in October 2018. He received intra-arterial floxuridine chemotherapy and intra-arterial fluorouracil plus cisplatin. On June 19, 2019, he underwent right hepatectomy under general anesthesia. After transarterial chemoembolization for recurrence in May 2020, pleural recurrence occurred. On August 31, 2020, he underwent excision of a diaphragmatic mass under general anesthesia, at which time tissue was obtained for the study.

4.1.3. Patient 3

YUMC-M-H5 was a 69-year-old man who underwent right hepatectomy under general anesthesia on February 9, 2018. Following diaphragm recurrence in 2019, he underwent wide excision of a right diaphragmatic metastasis on April 1, 2019, followed by sorafenib therapy. On March 15, 2020, recurrence in perihepatic lymph nodes was treated with excision of a perihepatic mass under general anesthesia; on March 16, 2020, he underwent wide excision of right diaphragmatic metastasis and omentectomy for peritoneal and diaphragm recurrence. On August 16, 2021, intrahepatic recurrence was treated with wedge resection of the liver. After three cycles of FP chemotherapy (fluorouracil plus cisplatin), following further hepatic and peritoneal recurrence, he underwent wedge resection of the liver, segmental resection of the colon, and excision of a retroperitoneal mass under general anesthesia on March 16, 2022, at which time tissue was obtained for research. The patient subsequently discontinued hospital visits and was lost to follow-up.

4.1.4. Patient 4

YUMC-M-H6 was a 60-year-old man whose tumor was initially considered unresectable; therefore, in December 2021, he received intra-arterial floxuridine chemotherapy and intra-arterial fluorouracil plus cisplatin. He subsequently underwent living-donor liver transplantation under general anesthesia on September 7, 2022. Post-operative culture specimens were obtained. The patient has since been followed regularly in the outpatient department.

4.2. Tissue specimens derived from all four patients

Tumor specimens were obtained from patients with histologically and biochemically confirmed recurrent or metastatic tumors who received treatment at Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. Fresh tumor tissues were collected at the time of surgical resection.

4.3. Ethical considerations

The study protocol was approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine (IRB No. 3-2022-0331). Owing to the retrospective and observational design of the study, the requirement for formal written informed consent was waived.

4.4. Tumor cell isolation and primary culture

Following excision, tumor specimens were placed in normal saline containing antifungal agents, penicillin–streptomycin solution (Gibco, WA, USA) and promptly transported to the laboratory. Adipose and adjacent non-tumorous tissues were removed, and the specimens were

washed with $1 \times$ Hank's Balanced Salt Solution. The cells were then resuspended in Roswell Park Memorial Institute (RPMI)-1640 medium (Hyclone, South Logan, UT, USA) supplemented with 20% fetal bovine serum (FBS) (Hyclone, South Logan, UT, USA) and 2% penicillin/streptomycin (Gibco, Grand Island, NY, USA). Additional details of the experimental procedures have been described previously [28,47,48].

4.5. Cell culture

The patient-derived tumor cells (PDTCs) and THLE-2, Huh-7, HepG2, and Hep3B cells were obtained from fresh tumors of patients or American Type Culture Collection (ATCC, Manassas, VA, USA). YUMC-NM-H2 (non-metastatic HCC) and YUMC-M-H4, -H5, and -H6 (metastatic HCC) cells were isolated from patients with HCC treated at the Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. HCC cells were cultured in RPMI-1640 medium containing 20% FBS. THLE-2 cells were cultured in BEGM (Cat#CC3170, BEGM Bullet Kit, Lonza, Basel, Switzerland) where gentamycin/amphotericin and epinephrine were discarded, and extra 5 ng/mL EGF, 70 ng/mL phosphoethanolamine, and 10% fetal bovine serum were added in addition to the supplied additives. Cells were authenticated using short tandem repeat profiling, karyotyping, and isoenzyme analysis. Mycoplasma contamination was evaluated using a Lookout Mycoplasma PCR Detection Kit (Sigma-Aldrich, St. Louis, MO, USA; MP0035).

4.6. mRNA sequencing and analysis

Whole RNA was isolated from PDTCs, YUMC-NM-H2 (non-metastatic HCC) and YUMC-M-H4, -H5, and -H6 (metastatic HCC), using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Paired-end sequencing reads were generated on the Illumina sequencing NovaSeqX platform. Prior to analysis, adapter sequences were removed and low-quality bases were trimmed using Trimmomatic v0.38. For total RNA enrichment in the current study, raw sequencing reads were preprocessed to remove low-quality reads and adapter sequences, and the processed reads were aligned to the *Homo sapiens* genome assembly (GRCh38) using HISAT v2.1.0 (HISAT2, RRID: SCR 015530) [49]. Further details of the protocol have been published previously [50].

4.7. Statistical analysis of the gene expression level

Relative gene abundance was quantified as read counts using StringTie. Statistical analyses were performed to identify differentially expressed genes (DEGs) based on prevalence estimates across samples. In the present study, raw p-values were used instead of FDR-adjusted p-values because FDR correction yielded an insufficient number of genes for downstream Gene Ontology (GO) and KEGG enrichment analyses. Hierarchical clustering of DEG sets was conducted using complete linkage and Euclidean distance as the similarity metric. Gene-enrichment, functional annotation, and pathway analyses of statistically significant genes were performed using the KEGG and GO databases. Further details of the protocol have been previously published [45,46].

4.8. Total RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from tumor cells using the RNeasy Mini Kit (Qiagen, Cat# 74106), following the manufacturer's instructions. Reverse transcription and amplification were performed with the One-Step RT-PCR Kit (Qiagen, Cat# 204243). Gene expression levels were normalized to α -tubulin as the internal control. Primer sequences are provided in [Supplementary Table 2](#).

4.9. Immunoblot analysis

Primary antibodies against GPC3 (1:500, Cat#174851, Abcam, Cambridge, UK), HSP70 (1:500, Cat#2787, Abcam), TP (1:500, Cat#226917, Abcam), pRIP1 (1:300, Cat# PA5-104645, Thermo Scientific, Waltham, MA, USA), RIP1 (1:400, Cat# PA5-20811, Thermo Scientific), pRIP3 (1:300, Cat#209384, Abcam), RIP3 (1:400, Cat#62344, Abcam), pMLKL (1:300, Cat# 187091, Abcam), MLKL (1:500, Cat# 184718, Abcam), CD133 (1:500, Cat# 19898, Abcam), caspase-3 (1:500, Cat# 9661, Cell Signaling), and β -actin (1:500, Cat#47778, Santa Cruz Biotechnology, CA, USA) were used. Equal amounts of protein were separated on an 8–10% sodium dodecyl sulfate-polyacrylamide gel, and the resolved proteins were electrotransferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Total lysate was isolated with a protein extraction solution (PRO-PREP, iNtRON Biotechnology, Seoul, Korea, #17081). The experiment was performed in quintuplicate. Further details of the protocol have been previously published [26,27].

4.10. Preparation of PPSO3

PASP-based polymer-metal complex, PPSO3, was produced by TNMC Corporation (Seoul, Korea), whereas its chemical structure and detailed synthetic procedures remain confidential under a pending patent. Briefly, SeMet-conjugated polyaspartic acid sodium salt (1.1 wt%) was dissolved in 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 7.0). Ferrous fumarate (0.125 eq; JUNSEI) was added to the solution, and the mixture was stirred at room temperature for 10 min, heated at 100 °C for 30 min, and subsequently cooled to ambient temperature. The crude product was purified by dialysis (MWCO 14 kDa) against deionized water for 24 h, followed by 0.2- μ m membrane filtration and lyophilization, yielding PPSO3 as a light-brown powder. The hydrodynamic diameter and zeta potential of PPSO3 were measured using dynamic light scattering (DLS; Litesizer DLS 500, Anton Paar). To assess colloidal stability, PPSO3 was incubated in physiological saline, and size distribution was monitored over time. Element content and composition were analyzed using inductively coupled plasma-optical emission spectrometry (ICP-OES). The loading efficiency and final concentrations of SeMet and ferrous iron in PPSO3 were quantitatively determined based on ICP measurements.

4.11. Cell viability assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates at a density of 6×10^3 cells per well and allowed to adhere overnight until they reached approximately 80% confluence. The assay was performed following standard procedures described previously [29,51,52]. Experiments were conducted in quintuplicate, and cell viability was expressed as a percentage relative to vehicle-treated controls and presented as the mean $\pm$ standard error of the mean.

4.12. Measurement of intracellular or mitochondrial reactive oxygen species

Intracellular (Abcam, Cambridge) or mitochondrial (Invitrogen) ROS levels were measured using a commercial detection kit. Cells seeded in 96-well plates were incubated with 2',7'-dichlorofluorescein diacetate (DCFDA), a cell-permeable fluorogenic probe. Following cellular uptake, DCFDA was deacetylated by intracellular esterases and subsequently oxidized by ROS to generate the fluorescent compound 2',7'-dichlorofluorescein. Fluorescence intensity was measured using a glass-bottom plate reader at the excitation and emission wavelengths of 495 and 529 nm, respectively. Fluorescence intensity was quantified and normalized to cell number. Mitochondrial ROS production was measured using Mito-SOX™ Red mitochondrial superoxide indicator

(Invitrogen). Cells were seeded on coverslips and washed with PBS, and then pre-incubated with 1 μ M Mito-SOX Red in PBS for 20 min at 37 °C in the dark. Representative images were obtained using a confocal microscope (LSM Meta 700; Zeiss, Oberkochen, Germany) and were analyzed using Zeiss LSM Image Browser software (version 4.2.0121).

4.13. Dextran uptake assay

To assess macropinocytosis activity, cells were incubated with fluorescein isothiocyanate (FITC)-labeled dextran (0.5–1 mg/mL) for 30 min at 37 °C. Where indicated, cells were pretreated with the macropinocytosis inhibitor 5-(N-ethyl-N-isopropyl)-amiloride (EIPA, 50 μ M) for 30 min prior to dextran exposure. After incubation, cells were washed extensively with cold PBS to remove extracellular dextran and subsequently fixed with 4% paraformaldehyde. Fluorescence intensity was analyzed using confocal microscopy or quantified using a fluorescence plate reader.

4.14. Cytomorphology and crystal violet assay

Cells were seeded in 6-well plates (6×10^4 cells/well) and incubated in RPMI 1640 supplemented with 10% FBS for 24 h. Cells were treated with cisplatin (IC₅₀: 14.3 μ M) or PPS03 (dose-dependent manner; 0, 6, 12, 25, 50, 100, 200, 400 μ g/mL) for 12 h. The morphology of the cells was examined under an inverted light microscope. Cell viability was evaluated using crystal violet staining assay. Cells were seeded in 6-well plates (6×10^4 cells/well) and incubated in BEGM (Cat#CC3170, Lonza) or RPMI 1640 supplemented with 10% or 20% FBS for 24 h. The treated cells were washed with PBS and then mixed with crystal violet solution and incubated within 10 min. The morphology of the cells was examined under an inverted light microscope. The experiment was performed in quintuplicate.

4.15. Sphere formation assay

Cells were seeded in ultra-low attachment plates StemFIT 3D dish 853 Hole, (Cat#: H853400, MicroFIT, Hanam-si, Republic of Korea) at a density of 5×10^3 cells per well in serum-free DMEM/F12 medium supplemented with B27, EGF (20 ng/mL), and bFGF (20 ng/mL). Cells were treated with PPS03 (10 μ g/mL) alone or with EIPA (50 μ M) at the indicated concentrations and cultured for 3 days. Tumor spheres larger than 50 μ m in diameter were counted under a microscope, and representative images were captured.

4.16. In vivo mouse xenograft model with patient-derived HCC cells

All animal experiments were conducted in accordance with institutional guidelines and approved by the Animal Experimentation Committee of Yonsei University (IACUC No. 2025-0067). Patient-derived HCC cells (4.5×10^6 cells per mouse) were subcutaneously implanted into the upper left flank of female NOD/Shi-scid, IL-2R γ KOJic mice. Mice were randomized into experimental groups (n = 10 per group) 13 days after implantation. Tumor dimensions were measured with calipers, and volumes were calculated using formula $L \times S^2/2$, where L and S represent the longest and shortest diameters. When tumors reached 100–200 mm³, treatment was initiated. Mice received cisplatin (1.5 mg/kg, i.p., three times weekly) and PPS03 (100 mg/kg, p.o., every other day). PPS03 was initially screened across multiple doses (25, 50, 100, 200, 400 mg/kg) to determine the optimal concentration; fixed doses of PPS03 were used for treatment. Tumor volumes were monitored every 2 days. All animals were maintained under specific pathogen-free conditions throughout the study. Tumor growth was closely monitored throughout the study. Animals were euthanized when tumor volume approached the ethical limit or when predefined humane endpoints were reached, in accordance with institutional guidelines. All procedures were performed in strict compliance with the Institutional Animal

Care and Use Committee (IACUC)-approved protocols to minimize animal distress.

4.17. PPS03 treatment alone does not affect normal cardiac function in mice

All procedures were conducted in accordance with the guidelines of the Animal Experiment Committee of Yonsei University. Six-week-old BALB/c nude mice, without tumor cell inoculation, were randomly assigned to receive cisplatin (1.5 mg/kg, i.p., three times per week) or PPS03 (100 mg/kg, p.o., every other day) for 29 days. Animals were housed under specific pathogen-free conditions throughout the study. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Yonsei University (IACUC No. 2025-0067).

4.18. Hematoxylin and Eosin and Masson trichrome staining to evaluate hepatic and cardiac injury

Hepatic and cardiac tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 5 μ m. Sections were stained with Masson's trichrome or hematoxylin and eosin (H&E), according to the manufacturers' protocols. Histopathological assessment of hepatic and cardiac injury was performed based on cellular and structural alterations visualized by these stains. Masson's trichrome staining delineated nuclei in dark red, muscle fibers and cytoplasm in red, and extracellular matrix components in blue.

4.19. Serum biochemical analysis

To assess systemic toxicity, blood samples were collected from mice at the end of the treatment period. Serum was obtained by centrifugation at 3000 rpm for 10 min and stored at –80 °C until analysis. Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using a commercial biochemical assay kit according to the manufacturer's instructions. All measurements were performed in accordance with standard protocols, and values were expressed as mean $\pm$ SEM.

CRediT authorship contribution statement

Kyung Hwa Choi: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Writing – original draft, Writing – review & editing. **Juhee Park:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software. **Hyemee Yoon:** Data curation, Formal analysis, Resources, Software. **Keunwan Park:** Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation. **Cheolgoo Hur:** Conceptualization, Data curation, Formal analysis, Methodology. **Seok-Mo Kim:** Data curation, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Ki Cheong Park:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Jin Hong Lim:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biomaterials.2026.124323>.

Data availability

No data was used for the research described in the article.

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