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Mitochondria-targeted nanoparticles overcome chemoresistance via downregulating BACH1/CD47 axis in ovarian carcinoma

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ABSTRACT

The platinum-based chemotherapy is a routine strategy for the treatment of ovarian cancer, while it is prone to chemoresistance in clinical, which hinders the treatment. Therefore, it is urgently needed to elucidate the underlying mechanism of drug resistance and form the appropriate strategy. The sequencing results showed that cisplatin (DDP) resistant ovarian cancer overexpressed BTB and CNC homology 1 (BACH1), and up-regulated the “don't eat me” signal CD47. We identified that hemin, a BACH1 inhibitor, could effectively down-regulate BACH1 and simultaneously inhibit CD47. Moreover, hemin has a synergistic effect with DDP. We designed a pH-responsive nanoparticle (H/D@FA-CaP-NPs) in which folic acid (FA) ensured targeting of ovarian cancer cells, while hemin inhibited BACH1 as well as down-regulated CD47, achieving the promotion of apoptosis of tumor cells and inducing phagocytosis of tumors by macrophages. Moreover, hemin has a synergistic effect with DDP to promote apoptosis of tumor cells. Structurally, hemin and DDP was encapsulated within hydrophobic 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) to form a tight core, and hydrophilic polyethylene glycol 2000 (PEG2000) and calcium phosphate (CaP) formed the outside shell, and FA was modified on the surface of nanoparticles. In terms of function, (a) FA enhanced the active targeting of nanoparticles to tumors; (b) NPs targeted mitochondria to induce reactive oxygen species (ROS) production; (c) hemin encapsulated in nanoparticles could specifically target BACH1, thereby down regulating CD47; (d) hemin had a synergistic effect with DDP, thus augmenting the chemotherapy. Altogether, mitochondria-targeted nanoparticles H/D@FA-CaP-NPs promoted tumor apoptosis and mobilized phagocytosis to treat tumor, providing a novel scheme for clinical treatment of cisplatin-resistant ovarian carcinoma.

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Ovarian cancer is one of the deadliest gynecological malignancies [1], lacks of effective targeted therapies, has high relapse risk, and ranks among the top five causes of death in women [2]. Clinical data show that most ovarian cancer patients are initially sensitive to surgery and paclitaxel/platinum-based chemotherapy, while 80% of patients gradually develop chemotherapy resistance [3]. Patients with chemoresistance usually have poor prognosis [4]. The lack of approved targets and effective treatment strategies for ovarian cancer hinders the treatment and drives our search for new therapeutic targets.

The mechanism of the platinum-based chemotherapy is to damage the DNA of cancer cells, thus inducing apoptosis. Early study finds that inhibiting the function of BTB and CNC homology 1 (BACH1) can restore tumor sensitivity to DNA damage-based chemotherapy drugs [5]. BACH1 protein is involved in the process of DNA damage response and repair pathway [6]. These works suggest that BACH1 inhibitor may become clinical candidate for enhancing the efficacy of platinum-based chemotherapy. Early work reveals that BACH1 is a transcription factor which highly express in tumors compared to adjacent tissues [7]. Research show that BACH1 promotes the transcription of genes associated with tumor cells migration and metastasis [8,9], and BACH1 overexpression is associated with tumor metastasis and poor prognosis in patients with ovarian cancer [10]. Lee *et al.* find that BACH1 is associated with poor outcomes in cancer, suppressing BACH1 can restore the

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sensitivity to chemotherapy [11]. These studies suggest that BACH1 may be an oncogene and is associated with drug resistance.

Both BACH1 and CD47 are upregulated in patients with carcinoma [12] which suggest that BACH1 is associated with CD47. When CD47 binds to its receptor, it provides “don't eat me” signal on macrophages, thereby inhibiting the process of phagocytosis [13]. Downregulating CD47 *via* using anti-CD47 antibodies are being actively tested in clinical trials [14]. CD47 is associated with poor prognosis in ovarian cancer and has been identified as a tumor antigen in human ovarian cancer [15,16]. These results support that CD47 can be an effective target for treating the ovarian cancer.

In the present study, we found that BACH1 was upregulated in cisplatin-resistant ovarian cancer cells and positively correlated with CD47. Heme effectively inhibits BACH1, but it is unstable and easily be oxidized [11,17]. It is possible to downregulate BACH1 with small interfering RNA (siRNA), however, siRNA drugs are excessively expensive to produce and not easily stored [18]. Hemin, as the inhibitor of BACH1, can be easily stored and protected from degradation, as well as accessible. Hemin could inhibit the expression of BACH1, thereby induce apoptosis of ovarian cancer cells. In addition, our research found that BACH1 modulated CD47 and inhibiting BACH1 down-regulated CD47, thereby blocking the “don't eat me” signal from macrophages and promoting macrophage phagocytosis of ovarian cancer cells.

Tumor cells can efflux the drug resulting in the occurrence of drug resistance [19]. Researchers utilizes *in situ* biomineralization of cisplatin to promote the entry of platinum drugs into tumor cells for anticancer effects [20,21]. Using nanomaterials to deliver drugs can enhance the efficiency of drug delivery while reducing the side effects of the drug [22–25]. Thus, we used the nanosystem 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)-2000] (DSPE-PEG2000-NH₂) to encapsulate hemin and cisplatin (DDP). Studies have shown that folate receptor α (FOLR1) is highly expressed on the surface of ovarian cancer [26,27], therefore, we added the FOLR1-specific ligand folic acid (FA) as a target head and modified it on the DSPE-PEG2000-NH₂ material, which increased the ovarian cancer targeting of this nanomaterial. We generated nanoparticle H/D@FA-CaP-NPs, in which hemin and DDP were encapsulated by hydrophobic DSPE to form a compact core, and hydrophilic PEG2000 and calcium phosphate (CaP) formed the outer shell, and FA was trimmed on the surface as a target. DSPE can stabilize drugs in nanomaterials [28]; PEG2000 ensure the stability of drugs in the blood circulation [29]. The adsorption of calcium and phosphorus on the surface of nanoparticles can preserve the drug packed inside the nanoparticles and contribute to maintain the drug circulating in the body without being degraded and destroyed [30]; besides, the calcium-phosphorus system has acid sensitivity and can release drugs in the acidic tumor niche [31]; the nanoparticles have enhanced permeability and retention (EPR) effect and can be specifically enriched to the tumor site [32]; FA could assist target ovarian cancer.

Paclitaxel/platinum-based chemotherapy is the main strategy of the current ovarian cancer treatment, which can help patients realize substantial clinical remission. However, the majority of ovarian cancer patients inevitably become insensitive to chemotherapy, experience cancer recurrence, and eventually die of cancer [33–35]. To help ovarian cancer patients who are most likely to benefit from platinum-based chemotherapy, and to enable early drug intervention in patients with poor prognosis, we need to elucidate the molecular mechanisms of platinum-based drug resistance. We developed the cisplatin-resistant ovarian cancer cells. Cytotoxicity assay validated our successful construction of cisplatin-resistant ovarian cancer cells which named SKOV3DR (resistance index for cisplatin: 3.67) (Fig. 1a), and the parental cells were named as SKOV3DP. We sequenced the established drug-resistant cell lines

and the results showed that BACH1 and CD47 were highly expressed in drug-resistant cells (Fig. 1b). The Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis indicated an up-regulation of the platinum resistance pathway (Fig. 1c), suggesting that we successfully established a cisplatin-resistant ovarian cancer model. The previous studies hinted that BACH1 influenced metabolism, which affected the flow of ions. The gene ontology (GO) enrichment analysis showed an upregulation of ion and protein binding capacity in drug-resistant ovarian cancer (Fig. S1 in Supporting information).

The Kaplan–Meier Plotter database (<http://kmpplot.com/analysis/index.php?p=service&cancer=ovar>) supported that overexpression BACH1 predicted poor survival of patients with ovarian cancer (Fig. 1d), which indicated that BACH1 could be an oncogene in ovarian cancer. Furthermore, we found that CD47 was associated with poor survival by analyzing the Kaplan–Meier Plotter database (Fig. 1e), which suggested that CD47 could be an effective treatment target for ovarian cancer. In order to test the relation between BACH1 and CD47, TIMER database (<http://timer.comp-genomics.org/>) was used, and the results showed BACH1 and CD47 had positive correlation in patients with ovarian carcinoma (Fig. 1f). The above results indicated that both BACH1 and CD47 were associated with poor survival of ovarian cancer patients and were positively correlated.

We found that BACH1 and CD47 were highly expressed in DDP-resistant ovarian cancer cells SKOV3DR compared with parental ovarian cancer cells SKOV3DP (Figs. 2a and b). Hemin is the selective inhibitor of BACH1 (Fig. 2c). Cytotoxicity assay showed that hemin could effectively repress the cell survival of SKOV3DR (Fig. 2d). Studies have found that BACH1 protein is involved in the process of DNA damage response and repair pathway [6]. Related molecular inhibitors of BACH1 have been shown to be potent chemotherapeutics and promote susceptibility of tumor to DNA damage-based therapeutic agents [5]. The main target of DDP is DNA, which will damage the normal structure of DNA [36,37]. The above studies suggested a possible synergistic effect between hemin and DDP. To validate that hemin could work synergistically with DDP, we conducted drug synergy test. Synergism was detected using CompuSyn software (Ver.1.0), in which the Chou-Talay method was followed (combination index (CI) < 1 indicates synergism) [38]. The synergistic index of SKOV3DR was less than 1 when fraction affected (Fa) value was 0.5 (Fig. 2e), which suggested that hemin had a synergistic effect with DDP. Studies have found that both BACH1 and CD47 are upregulated in cancer [12], suggesting a link between BACH1 and CD47. Cancer cells could avoid the clearance by macrophages through the overexpression the “don't eat me” signals, such as CD47 [13]. Early study found that CD47 was relatively high in tumors compared with normal tissues, and CD47 overexpression promotes the growth and motility of ovarian cancer cells [39]. High expression of CD47 is associated with chemotherapy resistance in ovarian clear cell carcinoma [40]. In order to study the correlation between BACH1 and CD47, we used siRNA to interfere with the expression of BACH1. Results confirmed that downregulating BACH1 could repress CD47 (Figs. 2f and g). The confocal microscope showed that expression of BACH1 and CD47 were downregulated after treating with hemin in SKOV3DR cells (Fig. 2h), these data further supported that BACH1 was positive correlation with CD47. Following inhibition of BACH1 with siRNA, it obviously boosted the phagocytosis of tumor cells by macrophages (Fig. 2i and Fig. S2 in Supporting information).

Using nanomaterials to deliver drugs can enhance the efficiency of drug delivery while reducing the toxic side effects of the drug [22,23]. Thus, we used the DSPE-PEG2000-NH₂ to encapsulate hemin and DDP. Studies have shown that FOLR1 is highly expressed on the surface of ovarian cancer [26,27], therefore, we added the FA as a target head and modified it on the

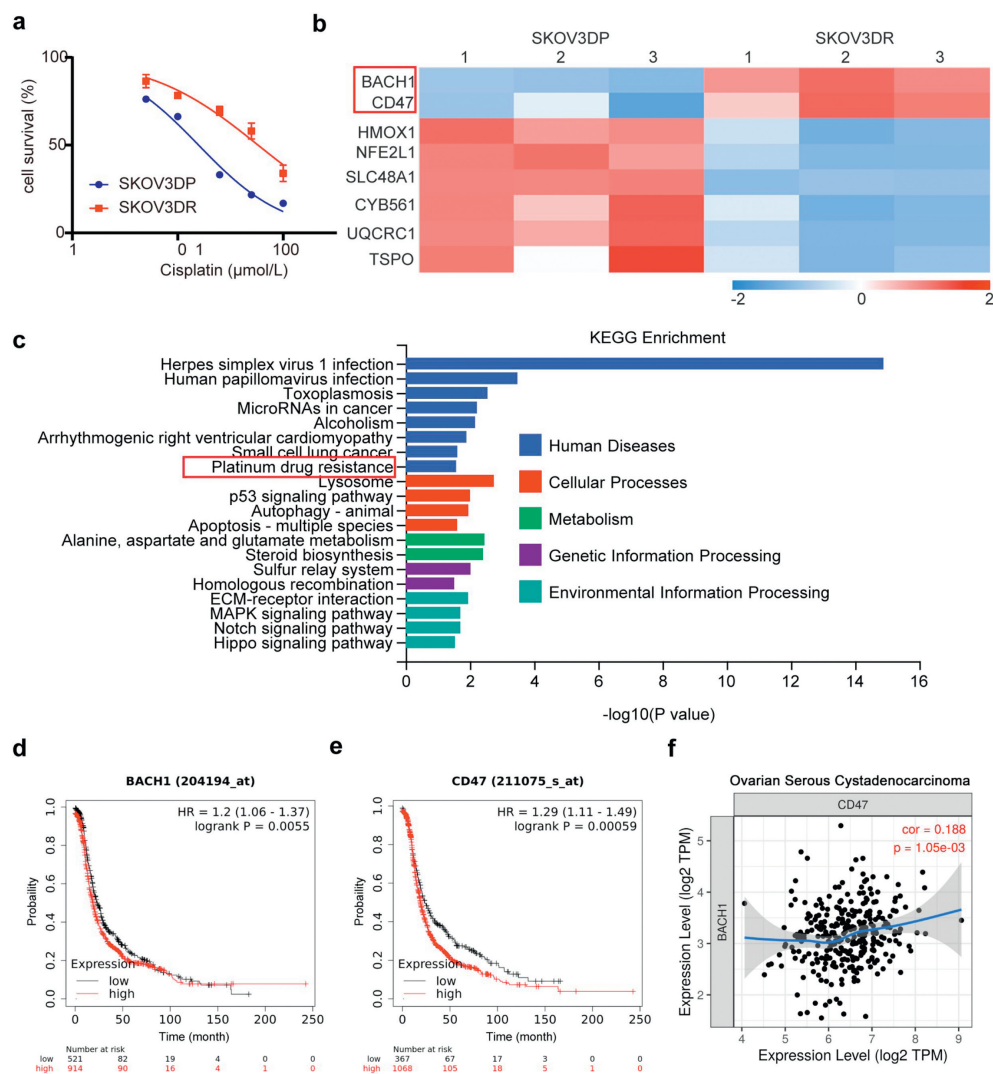


Fig. 1. BACH1 and CD47 were upregulated in the cisplatin-resistant ovarian cancer cells. (a) Cytotoxicity assay showed the survival of SKOV3DP and SKOV3DR cells after treating with different concentrations of cisplatin for 3 days. (b) Sequencing results to analyze the differentially expressed genes of SKOV3DP and SKOV3DR. (c) KEGG pathway analysis of SKOV3DP and SKOV3DR. (d) The results of Kaplan–Meier Plotter database analysis showed the relationship between ovarian cancer patient survival and BACH1 expression. (e) The results of Kaplan–Meier Plotter database analysis showed the survival rate of ovarian cancer patients and CD47 expression. (f) TIMER database predicted association of BACH1 and CD47 in ovarian cancer. COR indicates correlation. The P -value less than 0.05 is considered significant.

DSPE-PEG2000-NH₂ (Fig. 3a), which increased the tumor targeting ability. The NMR results revealed that the FA was linked with DSPE-PEG200-NH₂ (Fig. S3 in Supporting information). The nanoparticles (NPs) were formed through using the biomimetalization method. The hydrophobic liposome DSPE encapsulated the hydrophilic drugs hemin and DDP to form the core, the PEG2000 added the hydrophilicity of DSPE, then the Ca²⁺ and PO₄³⁻ absorbed into the porous shell (Fig. 3b), and Fig. S4 (Supporting information) revealed the calcium phosphate layer on the NPs. Both DSPE and PEG2000 are approved by FDA to use in human [41,42]. DSPE can stabilize drugs in nanomaterials [28], PEG2000 ensure the stability of drugs in the blood circulation [29]. The calcium-phosphorus system has acid sensitivity and can release drugs in the acidic tumor niche [31,43]. We used the zeta size analyzer to measure the size of NPs in aqueous solution (Fig. 3c). TEM results showed that H/D@FA–CaP–NPs had a spherical appearance and were dispersed in water solution (Fig. 3d). We measured the stability of H/D@FA–CaP–NPs through incubating the NPs in 5% serum solution (pH 7.4) at 37 °C incubator. The results showed the size and PDI of NPs had no change for 7 days (Fig. 3e), suggesting the H/D@FA–CaP–NPs could keep stable in the circula-

tion. The zeta potential of NPs in aqueous solution (pH 7.4) was -9.01 ± 0.126 mV, while in acidic solution, the zeta potential of NPs became $+6.05 \pm 0.083$ mV. These results supported Ca²⁺ and PO₄³⁻ as pH sensors that could regulate hemin and DDP release in the acidic tumor niche. Furthermore, the encapsulation efficiency (EE) values of hemin and DDP were 71.85% and 70.66%, respectively.

An important prerequisite for nanoparticles to function in cancer cells is that intracellular nanomedicines can escape from lysosomes and then be released into the cytoplasm or nucleus. Fig. 3f showed that most free drugs were trapped in lysosomes, whereas most nanomedicines successfully escaped from lysosomes. In addition, the fluorescence density of RB < RB@FA–CaP–NPs indicated that NPs greatly improved the efficiency of drug uptake by cells (Fig. 3f). Therefore, NPs could stably deliver drugs into cells and escape the degradation of drugs by lysosomes.

Apoptosis experiments showed that H/D@FA–CaP–NPs could effectively induce more apoptosis compared with the free hemin group, and blank nanoparticles had no side effects on cells (Figs. 4a and b). In addition, calcium and PI staining tests also showed that nanoparticles H/D@FA–CaP–NPs could significantly promote the apoptosis of SKOV3DR cells (Fig. S5 in Supporting informa-

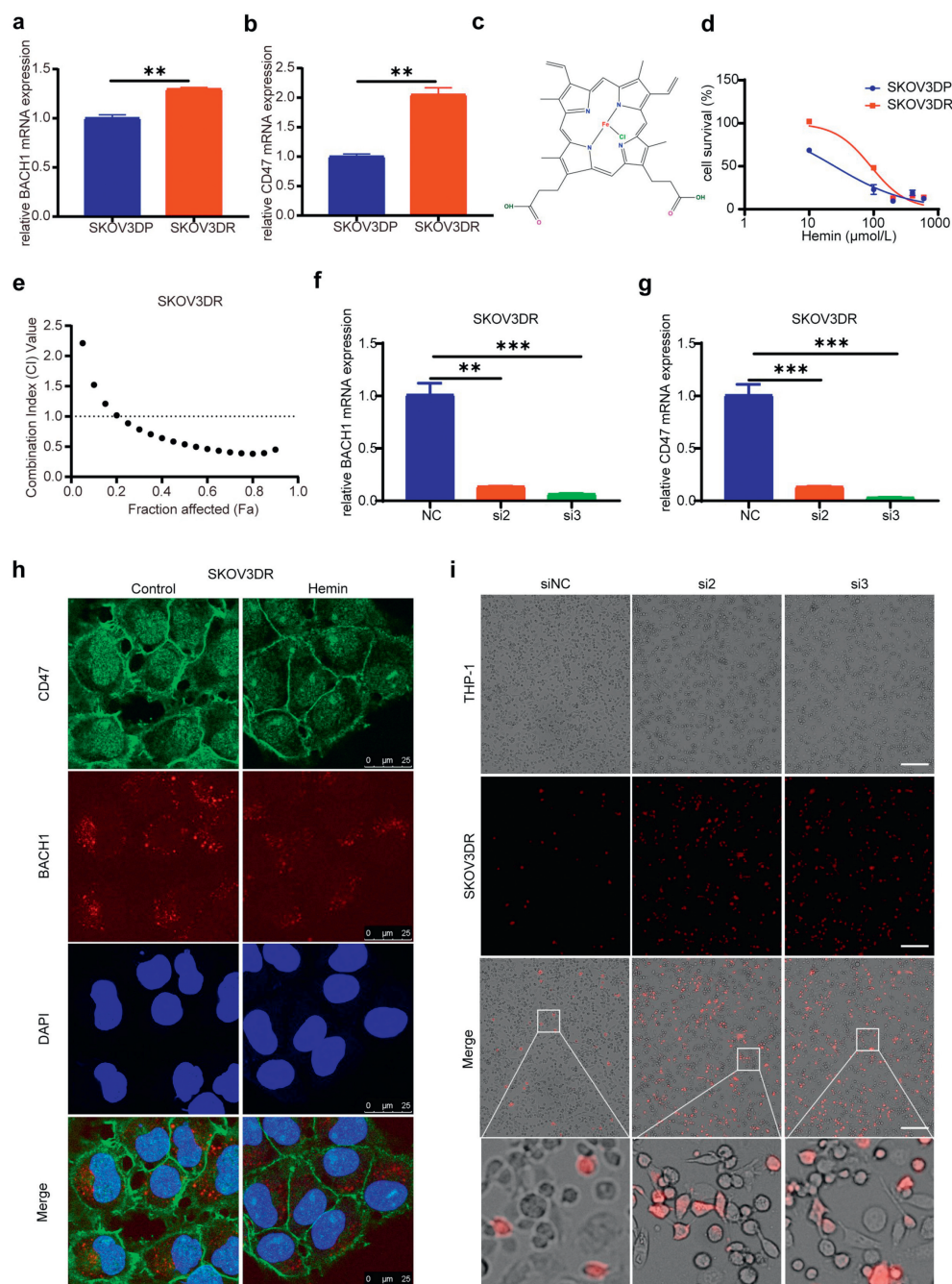


Fig. 2. BACH1 and CD47 were overexpressed in SKOV3DR cells, and the inhibition of BACH1 could induce the downregulation of CD47 and promote apoptosis. (a, b) The result of reverse transcription polymerase chain reaction (RT-PCR) assay showed the BACH1 and CD47 expression in SKOV3DP and SKOV3DR cells. Mean \pm standard error of mean (SEM), $n = 3$. (c) Chemical structure of hemin. (d) Cytotoxicity assay indicated the survival of SKOV3DP and SKOV3DR cells following treatment with different concentrations of hemin for 3 days. (e) CompuSyn software was used to detect the synergistic effect of hemin and DDP on SKOV3DR cells, and Chou-Talay method was used ($CI < 1$ indicated synergistic effect). (f, g) The result of RT-PCR assay showed the BACH1 and CD47 expression in SKOV3DR cells after treatment with siBACH1. Mean \pm SEM, $n = 3$. (h) The result of confocal microscope assay showed the protein level of BACH1 and CD47 in SKOV3DR cells after treating with certain drugs. 4',6-Diamidino-2'-phenylindole (DAPI) stains nucleus. Scale bar: 25 μ m. (i) Macrophages THP-1 phagocytosed SKOV3DR of cisplatin-resistant ovarian cancer cells in different treatment groups, THP-1 was the bright field and SKOV3DR was labeled with Dil in advance (40 \times). ** $P < 0.01$, *** $P < 0.001$.

tion). Fig. S6 (Supporting information) showed that nanomedicine H/D@FA-CaP-NPs could cause more cytotoxicity than free hemin. These results further supported that NPs could greatly enhance the killing efficiency compared to free drugs. Fig. 4c confirmed that H/D@FA-CaP-NPs promoted apoptosis of SKOV3DR cells by downregulating BACH1 and CD47. Down-regulating CD47 means blocking the "don't eat me" signal of macrophages and promoting macrophages to target and phagocytize cancer cells. Fig. 4d and Fig. S7 (Supporting information) showed that H/D@FA-CaP-

NPs could greatly increase the phagocytosis of SKOV3DR cells by macrophages. These results indicated that H/D@FA-CaP-NPs promoted apoptosis and induced macrophage phagocytosis of DDP-resistant ovarian cancer cells by downregulating BACH1/CD47.

Studies have previously covered that lipid-based nanoparticles could target mitochondria [44], including DSPE-PEG2000 [45,46]. Thus, we administered RB@FA-CaP-NPs nanoparticles to ovarian cancer cells and observed the localization of the nanoparticles on tumor. The nanoparticle did not target mitochondria at 1h,

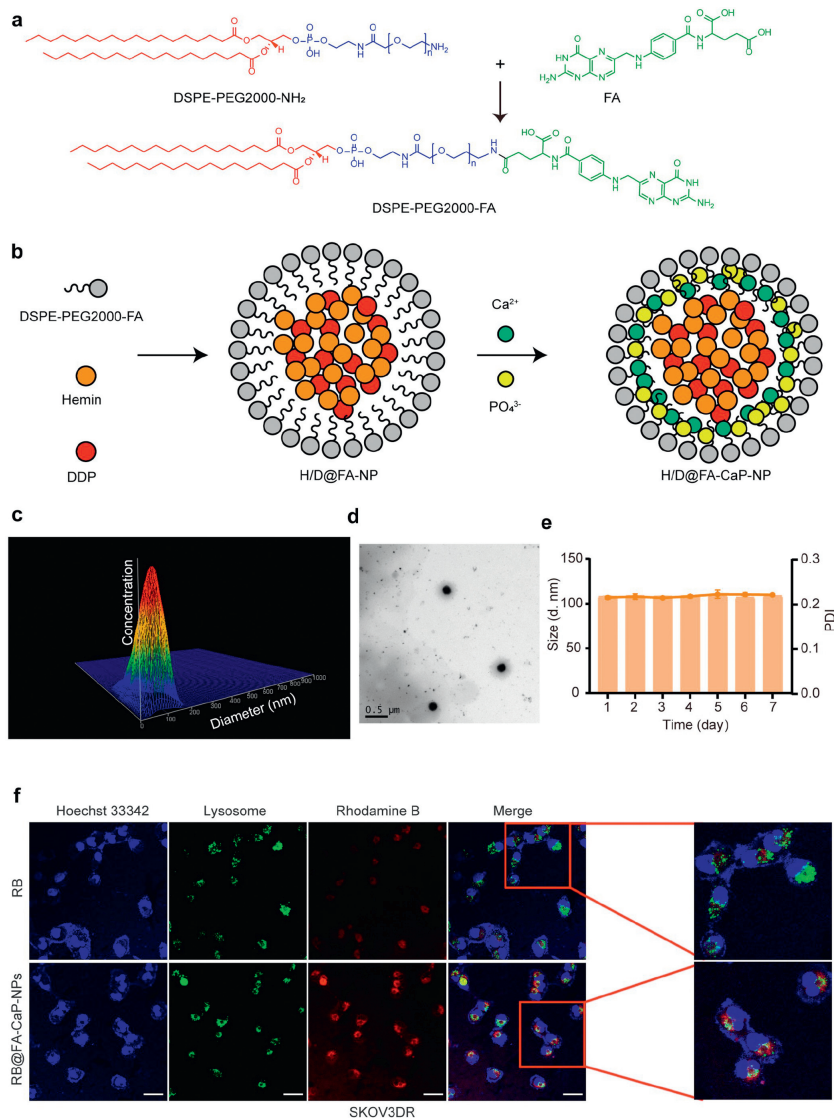


Fig. 3. The formation process and characterization of NPs. (a) Synthesis process of DSPE-PEG2000-FA. (b) The formation process of H/D@FA-CaP-NPs. Organic FA-DSPE-PEG2000 wrapped hemin and DDP to form the core, and then Ca^{2+} and PO_4^{3-} were absorbed into the porous shell. (c) The size of the nanoparticles in aqueous solution was 108 ± 0.625 nm, and the PDI was 0.225 ± 0.006 . (d) The result of transmission electron microscope (TEM) showed that H/D@FA-CaP-NPs have a spherical structure. Scale bar: $0.5 \mu\text{m}$. (e) The changes of the size and PDI of H/D@FA-CaP-NPs for 7 days. (f) SKOV3DR cells were incubated with free RB or RB@FA-CaP-NPs for 4 h. Blue represents the nucleus; green represents lysosomes; red represents RB or RB@FA-CaP-NPs; the overlap of green and red indicates that free RB fail to escape from lysosomes, while the separation of green and red indicates that the nanoparticles successfully escaped from lysosomes. Scale bar: $25 \mu\text{m}$.

whereas at 4 h, the nanoparticle obviously targeted mitochondria, and the overlap of green fluorescence signal and red fluorescence signal were observed. The findings revealed that the nanoparticle could target mitochondria (Fig. 4e). The mitochondria are the main generators of reactive oxygen species (ROS) [47], and ROS induce apoptosis [48]. We treated cells with nanodrugs and the highest ROS production was seen in the H/D@FA-CaP-NPs group and in both resistant and sensitive cells (Fig. 4f and Fig. S8 in Supporting information). The results demonstrated that H/D@FA-CaP-NPs could target mitochondria to induce ROS production and thus suppress tumors.

In vivo experiments indicated that treatment with H/D@FA-CaP-NPs markedly upregulated M1 macrophages in ID8 tumors (Figs. 4g and h). All procedures in this study were approved by the Institutional Animal Care and Use Committee at Renji Hospital, School of Medicine, Shanghai Jiao Tong University (Shanghai, China).

In summary, we found that both BACH1 and CD47 were upregulated in cisplatin-resistant ovarian cancer, then, we used the BACH1 inhibitor hemin to reverse drug resistance by down-regulating the BACH1/CD47 axis. The tumor-targeted nanoparticles H/D@FA-CaP-NPs could promote the apoptosis of cisplatin-resistant ovarian cancer cells and enhance the phagocytosis of macrophages and target mitochondria to induce ROS production to reversed the drug resistance of ovarian cancer cells through down-regulating the BACH1/CD47 axis, which provided a novel idea for clinical treatment of platinum-based chemotherapy-resistant patients. The NPs have certain adsorption capacity and require avoiding the adsorption of other substances during the preparation process; moreover, the organic solvent residues need to be avoided in the prepared NPs. This study shows that BACH1 and CD47 are hyper-expressed in platinum-resistant ovarian cancer, the BACH1 inhibitor hemin can synergistically kill ovarian cancer with DDP, and the H/D@FA-CaP-NPs nanomedicines prepared in the study of-

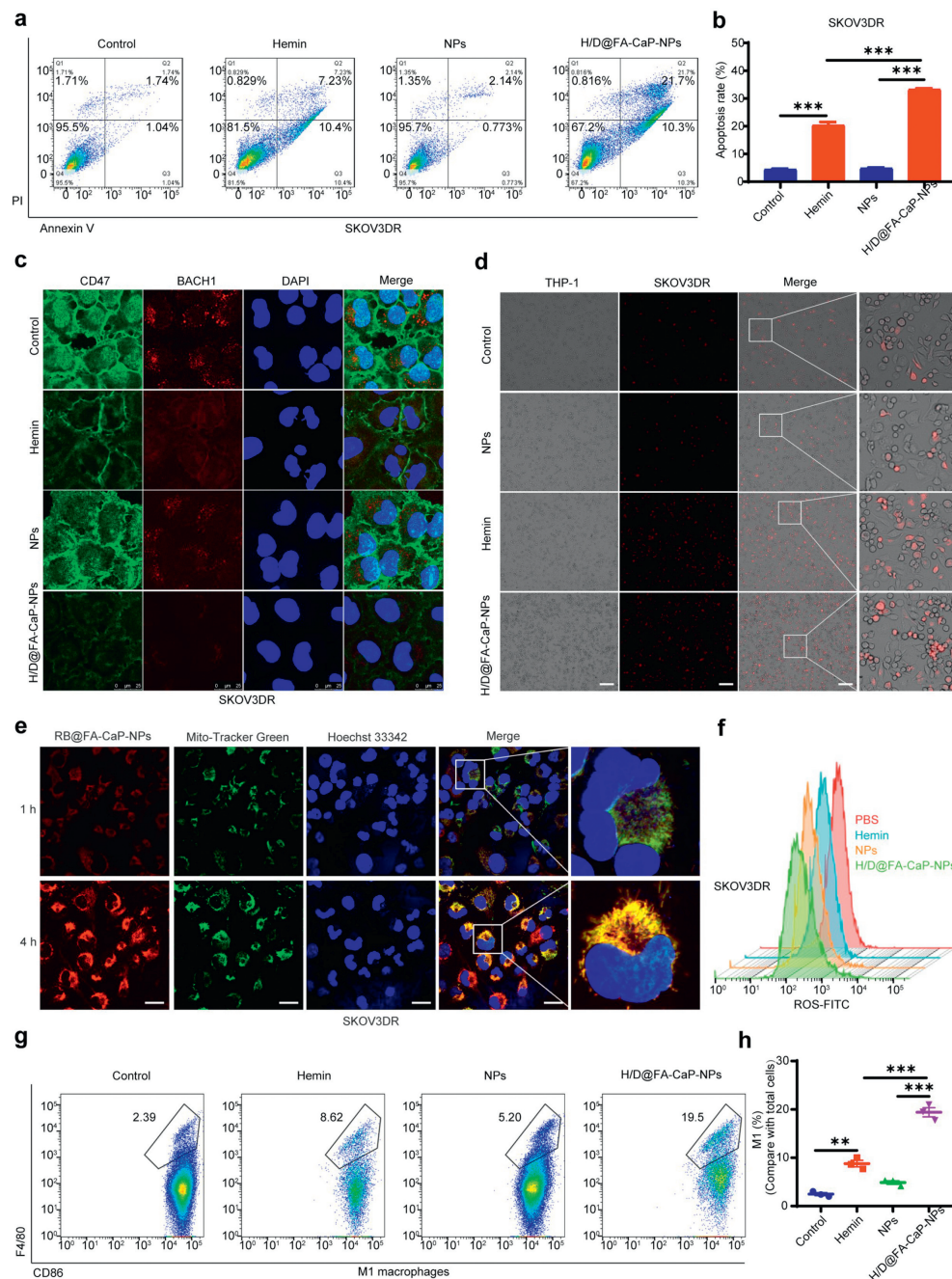


Fig. 4. H/D@FA-CaP-NPs promoted apoptosis and induced phagocytosis in DDP-resistant ovarian cancer cells by downregulating BACH1/CD47. (a) Apoptosis assay showed the apoptotic rate of human SKOV3DR cells treated with hemin, H/D@FA-CaP-NPs, and NPs for 2 days. (b) Statistical results of apoptosis rate of SKOV3DR cells. Mean \pm SEM, $n=3$. (c) The result of confocal microscopy assay showed that BACH1 and CD47 protein levels in SKOV3DR cells after treatment with hemin, H/D@FA-CaP-NPs, and NPs for 2 days. DAPI stains nuclei. Scale bar: 25 μ m. (d) The result of phagocytosis assay showed the phagocytosis of SKOV3DR cells by macrophages after treatment with hemin, H/D@FA-CaP-NPs, and NPs. The red area represents SKOV3DR cells, and the uptake of red blood cells by macrophages represents phagocytosis (40 \times). (e) The result of nanoparticle targeting to mitochondria within SKOV3DR in different treatment time (1 h and 4 h). Red were RB@FA-CaP-NPs, green was mitochondria, and blue was nuclei. Where red and green superimposed in orange indicate nanoparticles targeting mitochondria; red and green separated indicate nanoparticles not targeting mitochondria, scale bar: 25 μ m. (f) The result of flow cytometry assays showed that ROS production within SKOV3DR in different treatment groups. (g) The result of the flow cytometry assays showed that M1 macrophages in ID8 tumor of mouse. M1 macrophages were labeled as live⁺/CD45⁺F4/80⁺CD86⁺. (h) Statistical results for M1 macrophages, Mean \pm SEM, $n=3$. ** $P < 0.01$, *** $P < 0.001$.

fer new insights for clinical treatment of drug-resistant ovarian cancer.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ccl.2023.108888.

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