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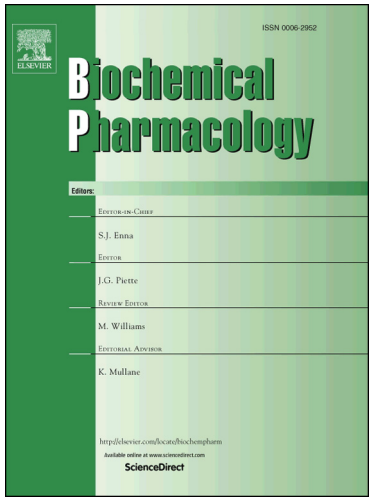

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Genipin, a natural AKT inhibitor, targets the PH domain to affect downstream signaling and alleviates inflammation

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Abstract

The iridoid compound genipin (GNP) is a geniposide hydrolysate of β-glucosidase. GNP has many pharmacological effects, including antioxidant, anti-apoptotic, and anti-inflammation effects. However, its exact target and mechanism of action remain poorly understood. In this study, the binding of GNP to AKT protein was demonstrated via a GNP-modified magnetic microspheres (GNP-MMs) capture and immunofluorescence co-localization test. GNP-MMs fishing coupled with competitive testing and AKT plasma transport experiments indicate that GNP may act on the PH domain of AKT, and affect AKT plasma transport. The specific binding directly inhibits phosphorylation of AKT, affecting the downstream activation, and reducing inflammatory responses. The results indicate that GNP targets the PH domain region of AKT, inhibits the phosphorylation of AKT, and attenuates the transduction of AKT based inflammation signal pathway.

Keywords: genipin; AKT; PH domain; anti-inflammation; PI3K/AKT pathway

1. Introduction

Inflammation is an essential part of immune defense[1]. Under normal physiological conditions, inflammation maintains homeostasis of the interactions between immune cells and the cytokine environment[2]. However, when autoimmune tolerance is disrupted, the cytokine environment becomes unbalanced, and the local environment transforms into a pro-inflammatory state leading to tissue damage [3]. Many experimental clinical studies have shown that chronic inflammation is an important factor in triggering various human diseases[4], especially chronic metabolic disorders, cardiovascular diseases, cancer, and diabetes [5]. Therefore, it is imperative to discover safe and effective anti-inflammatory drugs and interventions.

Some effective drugs for the treatment of inflammation have been developed. Non-steroidal anti-inflammatory drugs (NSAIDs) are the most frequently prescribed drugs [6]. Lipopolysaccharide (LPS) is a major component of the cell membrane of Gram-negative bacteria and initiates downstream signaling pathways by activating the Toll-like receptor 4 (TLR4), such as phosphatidylinositol 3-kinase (PI3K)/AKT, nuclear factor -κB (NF-κB), thereby stimulating inflammation[7]. NSAIDs can inhibit LPS-induced leukocyte migration and show its anti-inflammatory activity[8, 9]. The PI3K/AKT signaling pathway is closely related to leukocyte migration[10]. As a key target in the pathway, protein kinase B, also known as AKT, enhances the chemotaxis of activated neutrophils and stabilizes F-actin polymerization. Therefore, AKT is regarded as an important node for inhibiting neutrophilic inflammation[11].

Studies have shown that a large number of small molecules in the dietary composition exhibit significant anti-inflammatory activity[12]. Inflammation also can be effectively modulated by the dietary intervention[13]. For example, Gardenia

jasminoides as traditional Chinese medicine is commonly used to treat angina and jaundice, and is also used for the prevention and treatment of respiratory infectious diseases [14]. Geniposide (GPS) is the main active ingredient of gardenia [15]. Genipin (GNP) is an aglycone of GPS, which is produced by GPS metabolism in body tissues [16]. GNP has many pharmacological effects, including antioxidant, anti-apoptotic, and anti-inflammatory effects [17]. Studies have shown that GNP inhibits inflammatory mediators in BV2 microglia by activating the Nrf2 signaling pathway[18], and inhibits croton oil-induced ear edema in mice[17], indicating that GNP has a significant anti-inflammatory effect. Delayed neutrophil apoptosis is critical for inflammation [19], and the PI3K/AKT signaling pathway is essential for neutrophil survival [20]. GNP reduces neutrophil recruitment, thereby protecting flagellin-induced lung inflammation[21], and numerous experiments have demonstrated that GNP exerts its anti-inflammatory effect through the PI3K/AKT pathway[22]. However, the specific targets and mechanisms of GNP anti-inflammatory, especially pulmonary infection, have not been thoroughly investigated.

Based on the traditional experience, a GNP molecular probe (GNP-P) was synthesized, then a GNP-modified magnetic capture method and an immunofluorescence co-localization test were used to demonstrate the binding of GNP to target protein in lung tissue and human bronchial epithelial cells (BEAS-2B) cells. The competitive test and AKT membrane translocation experiments indicated that GNP might act on the PH domain of AKT and affect its function. At the same time, we verified the effect of GNP on the downstream pathway that was activated by LPS, through which the relevant mechanism of action was clarified.

2. Materials and Methods

2.1 Materials

GNP (purity ≥98%, S31404) was obtained from Shanghai Yuanye Biotechnology Co. Ltd. (Shanghai, China). AKT competitors, including SC79 (S7863) AKT allosteric inhibitor VIII (S1012), PHT-427 (S1556) and AT7867 (S1558) were purchased from Selleckchem (TX, USA). LPS (L2630) and dexamethasone (Dex, D1756) were purchased from Sigma Corporation (MO, USA). Primary antibodies for AKT (4776), IKKα/β (2694), p-IKKα/β (2697), NF-κB (p65, 8242), p-NF-kB (p65, 3033) and β-actin (3700) were purchased from Cell Signaling Technology (MA, USA). p-AKT (Thr308 (ab38449) and Ser473 (ab81283)) antibody and Alexa Fluor® 594-conjugated goat anti-rabbit antibody (SA00006-4) were purchased from Abcam (MA, UK). Coomassie Brilliant Blue kit (ST030) was purchased from Beyotime Biotechnology (Shanghai, China). AKT Activity Assay kit (GMS50054.1) was purchased from GenMed Scientific Inc. (MA, USA). N3-tag (3-azido-7-hydroxy-2H-chromen-2-one, 9361) was purchased from WuXi AppTec (Beijing, China). Sulfo-SADP (Sodium1-((3-((4-azidophenyl) disulfanyl) propanoyl) oxy)-2, 5-dioxopyrrolidine-3-sulfonate, CB9178443) was obtained from Bioworld (MN, USA). DiI (1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate, D-282) was purchased from Invitrogen (Karlsruhe, Germany). pcDNA3 AKTPH(R25)-GFP (#74165) and AKTPH(R25C)-GFP (#25548) plasmids were purchased from Addgene (MA, USA).

2.2 Cell Culture

HEK 293T cells and BEAS-2B cells were purchased from American Type Culture Collection (Rockville, MD) and maintained in 10% fetal bovine serum （FBS, F8687, Sigma Corporation, MO, USA） and 1% penicillin/streptomycin (15140-122) contained DMEM (12491-015) and RPMI 1640 medium (61870-044 Gibco BRL Life

Technologies, NY, USA), respectively. All the cells were cultured in a humidified atmosphere containing 5% CO2 at 37°C.

2.3 Synthesis of the Alkynyl-Modified GNP-P

GNP (50 mg, 0.22 mM) was dissolved in 5 mL of DMF, then propargyl alcohol (15.6 μL, 0.26 mM), and 500 μL of concentrated hydrochloric acid was added, and the reaction was carried out by incubation for 3 h in an oil bath at 60°C. The completion of the reaction was indicated using TLC plate chromatography. The product obtained by purification was the GNP probe (GNP-P) (47.0 mg, 85.4%). The NMR and HRMS of GNP-P were: ^{1}H NMR (400 MHz, Chloroform-d) δ 7.49 (s, 1H), 5.84 (s, 1H), 4.79 (d, J = 8.0 Hz, 1H), 4.50 – 4.38 (m, 2H), 4.28 (s, 3H), 3.72 (s, 3H), 3.21 (q, J = 8.4 Hz, 1H), 2.89 (dd, J = 16.9, 8.6 Hz, 1H), 2.67 (t, J = 8.2 Hz, 1H), 2.50-2.45 (m, 1H), 2.10 (dd, J = 16.8, 8.6 Hz, 1H); ^{13}C NMR (100 MHz, Chloroform-d) δ 167.8, 151.8, 142.9, 129.1, 111.5, 99.6, 75.9, 75.4, 61.4, 56.3, 51.4, 46.4, 39.1, 35.8. HRMS $[M + Na]^{+}$ calculated 287.0890, found 287.0892.

2.4 Dual-Luciferase Reporter Assay for NF-kB

HEK293 cells were added to 96-well plates and incubated for 24 h as described above. Then the cells were transfected with the dual-luciferase reporter plasmid pGL4.32 (Promega, WI, USA; 100 ng/well), and the internal control vector pRL-TK (Renilla luciferase) plasmid (9.6 ng/well). After 18 h, the cells were exposed to TNF-α (5 ng/mL) for 6 h, and then administered with different doses of GNP, GNP-P, or Dex (10 μM) for 6 h. The relative Luciferase activity for NF-kB was measured according to the manufacturer's protocol (Promega, WI, USA).

2.5 Target Proteins Prediction

The GNP sdf file was uploaded to the PharmMapper server (http://lilab.ecust.edu.cn/pharmmapper/), and all parameters were set to their default values. The GO and KEGG pathway information of the relevant protein targets were obtained from the MAS 3.0 server (http://bioinfo.capitalbio.com/mas3/). The resulting anti-inflammatory related pathway was analyzed using the KEGG PATHWAY database (https://www.genome.jp/kegg/pathway.html) to predict the target protein. Finally, STRING 10 (http://string-db.org/) was used to generate predictive association networks.

2.6 Target Fishing

GNP-P modified functionalized magnetic microspheres (GNP-MMs) were prepared by the click reaction for fishing target proteins. Specifically, 2 mL Fe_3O_4 amino magnetic microspheres suspension (Tianjin baseline Chromtech Research Center, China) was mixed in 10 mL borate buffer (pH 8.5), Sulfo-SADP (1 mM) was added, and reacted at room temperature for 12 h. After magnet separation, GNP-P (1.5 mM) was added to the reaction mixture and incubated with the catalyst solution (1 mM $CuSO_4$ and 1 mM sodium ascorbate) in PBS for 12 h, and then the GNP-MMs were obtained. Next, 1 mL supernatant of the lung tissue homogenate (100 μg/mL) was added to the GNP-MMs and incubated at 4 °C for 12 h. The magnetically captured protein was washed and released via a disulfide bond reduction by 10 mM DL-dithiothreitol (DTT) for 30 min, and then was evaluated by SDS-PAGE and Western blot.

2.7 Co-Localization

BEAS-2B cells that had been treated with 10 μM GNP-P for 6 h were fixed with 4% paraformaldehyde at 4 °C, and continued to incubate with 10 μM N_3-tag and a catalyst solution (10 μM $CuSO_4$ and 10 μM sodium ascorbate in PBS) for 1 h. Then the cells

were stained with rabbit anti-AKT antibody (1:200) and Alexa Fluor® 594-conjugated secondary antibody (1: 500), and the fluorescence image was obtained with a confocal microscope (Leica TCS SP8, Japan). The excitation and emission wavelengths of Alexa Fluor® 594 were set at 594 nm and 617 nm, and for GNP-P were set at 365 nm and 440 nm, respectively. Finally, an ImageJ program rendering method with a Pearson Correlation Coefficient (PCC) co-localization plug-in was used to statistically quantify the co-localization ratio of the two fluorophores.

2.8 Competitive Test

GNP-MMs was incubated with lung homogenate for 12 h at 4°C, followed by magnetic separation. The magnetically captured proteins were eluted by incubating the beads with 10 mM of each AKT competitor (PHT-427, AKT inhibitor VIII and AT7876) for 12 h at 4°C. The eluates were probed for AKT dissociation by Western blot.

2.9 AKT Plasma Translocation Assay

For evaluating the effects of GNP on AKT plasma translocation, HEK293 transiently transfected cell lines that expressed $AKT^{PH(R25)}$-GFP or $AKT^{PH(R25C)}$-GFP fusion protein were utilized, respectively. The cells cultured on glass-bottomed dishes (MatTek, MA, USA) were firstly incubated with GNP (0.1, 1 and 10 μM) for 6 h or SC79 (10 μM) for 30 min, then the pcDNA3 plasmids ($AKT^{PH(R25)}$-GFP or $AKT^{PH(R25C)}$-GFP) were transfected separately. After specific membrane staining by Dil for 10 min, the images were finally obtained by the confocal microscope. The excitation and emission wavelengths of GFP were 488 nm and 507 nm, and Dil were 549 nm and 565 nm, respectively. Finally, PCC was also used to represent the co-localization ratio of the two fluorophores.

2.10 Western blot and cytokines assay

BEAS-2B cells were cultured in 6-wells plates, an AKT agonist SC79 (10 μM) was used to induce phosphorylation of AKT for 30 min. Then the cells were treated with GNP (0.1, 1 and 10 μM) for 6 hours. The cell lysates were used to detect the inhibition effect of phosphorylation of AKT by Western blot. To evaluate the effect of GNP on inflammatory pathways, BEAS-2B cells were treated with LPS (10 μg/mL) for 15 min to induce AKT activation. The inhibition effect with or without GNP (0.1, 1 and 10 μM) for 6 hours was tested by Western blot via phosphorylation detection of AKT, IKK, NF-kB, respectively. Next, the cytokines expression levels of IL-8 and TNF-α in the supernatants were analyzed and quantified using ELISA kits (Wes Tang Bio-Tech, Shanghai, China).

2.11 AKT activity assay

BEAS-2B was cultured in 75 cm^2 flasks for 24 h. Then the cells were collected and treated with GNP (0.1, 1 and 10 μM) or SC79 (10 μM) in 37°C for 30 min. The cell lysate was used for AKT enzyme activity assay according to the manufacturer's manuals (GMS50054.1, GENMED, MA, USA).

2.12 Statistical Analysis

All the data were performed as the mean ± standard deviation (SD). The statistical comparison between two groups was performed by t-tests, and for multiple groups by analysis of variance (ANOVA) followed by Bonferroni's test with a single pooled variance test, and $p < 0.05$ was considered to be statistically significant.

3. Results

3.1 Identification of AKT as a target protein for GNP

To capture the target protein of GNP, an alkynyl-modified GNP-P was synthesized (Fig. 1A). To investigate whether GNP-P altered the activity of GNP, the efficacy against

NF-κB expression was tested by dual-luciferase reporter assay. As shown in Fig. 1B, GNP-P did not change the inhibition of NF-κB activity when compared to GNP at 10 μM condition. Therefore, we can conclude that GNP-P has the same potency as GNP regarding anti-inflammatory activity, and it can be used for target fishing or tracing.

After GNP-MMs fishing, the magnetically captured proteins were released by DTT and detected by SDS-PAGE (Fig. 1C). The total protein in lung tissue lysates was shown in Fig. 1C (lane 1). Compared to the negative MMs group (lane 2), which without GNP-P modified, some proteins were enriched by GNP-MMs (lane 3).

To analyze potential targets of GNP, top 50 candidate proteins were predicted by bioinformatic tools. 15 proteins involved in inflammatory signaling pathways were demonstrated via protein-protein interaction network by STRING 10 (Fig. 1D). As a result, six major inflammatory pathways, including TNF, Toll-like, AMPK, T-cell receptor, MAPK, and PI3K/AKT signaling pathways were suggested[23, 24]. Compared to other protein targets, it was deduced that AKT was the key node in six pathways. To further verify the above prediction, a target fishing experiment was carried out, and western blot was used to verify whether AKT protein was enriched. As a result, an approximately 56 kDa AKT protein was significantly enriched (Fig. 1C, right panel) when compared to lane 2 (negative control). Hence, we speculated that AKT may be a key target protein of GNP.

3.2 Co-Localization of GNP-P and AKT

To further confirm a relationship between GNP and AKT, we created a fluorescent GNP probe using the click reaction between GNP-P and N3-tag of the fluorescent probe 7-hydroxy coumarin (Fig. 2A). As shown in Fig. 2B, the pseudo green fluorescence of GNP-P distribution can be visualized in the cytoplasm (PCC = 0.592 ±

0.107, n=6), while GNP showed little fluorescence (PCC = 0.129 ± 0.100, n=6) (Fig. 2B). The pseudo red fluorescence shows the location of AKT, and particular merged with the GNP-P (yellow). From the results, we speculated that GNP might act on AKT in cells and affect the function of it.

3.3 GNP Affects the Function of AKT

To assess the effect of GNP on AKT activity levels, an AKT kit was used to measure AKT activity. SC79 is an Akt activator that activates Akt phosphorylation (Fig. 3A), while GNP could effectively reverse the increase of AKT activity induced by SC79.

To test the effect of GNP on AKT phosphorylation, western blot was used to assess the phosphorylation levels of p-AKT (308) and p-AKT (473). The results showed that there was no significant change in the expression of AKT, while GNP reduced the magnitude of SC79-induced activation of AKT in a concentration dependent manner.

To clarify the effect of GNP on AKT function, an AKT-PH membrane translocation experiment was designed. The expression of green fluorescent protein fusion of AKTPH (R25)-GFP was used to observe the plasma transport phenomenon of AKT. $AKT^{PH\ (R25C)}$-GFP, a PH domain mutant cell line, acted as a positive control to show the inhibitory effect of membrane translocation [25]. Compared with the specific membrane staining with DiI, SC79 inhibited its transfer to the membrane after binding to the AKT-PH domain (Fig. 3C). And the plasma transport of AKT-PH domain can also be blocked by GNP. The PCC of cell membrane showed significant statistical differences in a dose-dependent manner (0.1–10 μM) (Fig. 3C).

3.4 GNP Acts on the PH Domain of AKT

We designed a competitive assay to characterize the site of GNP binding in AKT. In this assay, AKT captured onto GNP-MMs was incubated with the various AKT

inhibitors, then the eluates were subjected to Western blot to detect eluted AKT. We used PHT427, AT7867 and AKT inhibitor VIII, which act on the PH domain, ATP binding pocket, and kinase domain respectively. [26, 27]. Our results show that GNP and PHT-427 were effective in competing AKT bound to GNP-MMs (Fig. 4A), indicating that GNP might bind to the PH-domain pocket in AKT.

For investigating the interaction between GNP and AKT-PH domain, molecular docking was performed by AutoDock4.2 and MOE software to provide the binding position to PH domain (PDB: 1 UNQ). And the best docking model with the lowest energy level was shown in Fig. 4B. As a selective AKT activator, SC79 (-6.55 kcal/mol) may interact with Lys14, Glu17, Arg23, Arg25, and Arg86 in the binding pocket (Fig. 4B, left panel), and the residues of Arg23, Arg25, and Arg86 had been regarded as the key amino acid for the formation of the AKT-PH domain binding sites [28]. GNP (-6.11 kcal/mol) may interact with Arg23, Arg25, Leu52, and Arg86 in the binding site (Fig. 4B, right panel). Based on the molecular docking results, we hypothesized that GNP might also act on the AKT-PH domain. However, the detailed interaction effects need to keep going.

3.5 GNP Affects Downstream Signaling and Alleviates Inflammation

To further estimate the inhibitory effect of GNP on AKT inflammasome activation, Western blot was used to study the effect of GNP on AKT, IKK, and NF-kB phosphorylation. LPS induced phosphorylation of AKT, IKK and NF-κB were significantly inhibited by GNP (Fig. 5A).

Also, the expression of inflammatory factors was examined. BEAS-2B cells were treated with LPS (10 μg/mL) for 15 minutes, then treated with GNP (0.1, 1 and 10 μM) for 6 hours, and then the supernatants were assayed for IL-8 and TNF-α cytokine

expression levels by ELISA kit. Treatment with GNP significantly reduced the levels of TNF-α and IL-8 secretion into the cell supernatant (Fig. 5B). The above results indicated that GNP could effectively regulate downstream factors of AKT, and alleviate the inflammatory process induced by LPS.

4. Discussion

Bacterial pneumonia is one of the most important infectious diseases in terms of incidence, mortality, and impact on quality of life [29, 30]. In these cases, Gram-negative bacterial infection are more frequent in clinic [31]. When the innate defenses of the respiratory tract are altered, bacteria invade into the alveoli easily, and induce inflammatory factor cascades and lung damage [32]. While the TNF, MAPK, and NF-κB pathways are closely linked to acute lung injury caused by inflammation[33]. As a key node of these pathways, the serine/threonine kinase AKT plays an important role in regulating multiple inflammations. AKT can affect various cellular physiological processes by targeting effector proteins[34].

AKT is known to include the amino-terminal PH domain, the kinase domain region, and the C-terminal regulatory domain[35]. The PH domain of AKT affects the membrane translocation of AKT from the cytoplasm to plasma by membrane lipid-binding to phosphatidylinositol (PtdIns)(4,5)P2 and PtdIns(3,4,5)P3 [36]. AKT is subsequently phosphorylated, leading to further kinase activation [37]. The resulting inactive form has a closed conformation while the active form has an open one [38].

PtdIns(4,5)P2 is phosphorylated by PI3K to PtdIns(3,4,5)P3, and it induces a conformational change in AKT. Ser473 and Thr308 phosphorylation sites are exposed and promote AKT activation due to AKT conformational changes[39]. In the AKT-PH domain, Arg25 is required for the binding of AKT and 4IP (third carbon atom at inositol),

and AKT will not be transferred to the plasma membrane after inhibition of Arg25 [40]. Arg25 and Arg86 form the bottom of the PH domain binding pocket, while Arg23 is located on the wall of the pocket, and these residues are the key to enabling AKT activity[28]. Based on the docking results, GNP may bind to the AKT-PH domain at key residues of Arg23, Arg25, and Arg86. The binding of GNP to the AKT-PH domain blocks its recruitment to the plasma membrane, thus affecting the binding of the AKT-PH domain to PtdIns(3,4,5)P3, and locking AKT in an inactive state (Fig. 6). In the closed conformation, its phospholipid binding sites at Ser473 and Thr308 are blocked. The inhibited AKT remains in the cytoplasm and it is not activated by phosphorylation, further affecting the PI3K/AKT signaling pathway and NF-kB pathway.

In conclusion, this study shows that GNP inhibits AKT activity by acting on the AKT-PH domain, affects AKT plasma transport, and that inhibition of AKT activity affects its downstream pathways and provides a possibility for the treatment of inflammation.

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Author Contributions

Y.J. performed cell culture experiments and analyzed the data. F.S., Z.W. and M.Z. discussed the results and contributde to the manuscript preparation. Y.J., F.S. and L.Y.

wrote the manuscript. J.G., Y.H. and G.B. designed and supervised the project. All authors have read and approved the final manuscript.

Conflict of Interest

The authors have declared no conflicts of interest.

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Fig. 1. Capture and identification of target proteins by GNP-MMs. (A) Synthetic route of GNP-MMs and target protein capture process. (B) The effect of anti-inflammatory activity of GNP or GNP-P was evaluated by dual-luciferase reporter assay of NF-κB. (Each bar represents the mean ± SD (n=3); ** p<0.01, *** p<0.001 vs. model, ### p<0.001 vs. control; ns, no significance, between GNP and GNP-P in 10 μM). (C) SDS-PAGE (left panel) and Western blot analysis (right panel) were used to detect the enrichment effect of GNP-MMs. Lane 1 includes lung tissue lysates as loading control; Lane 2, fishing lysates by non-GNP probe modified microspheres as a negative control; Lane 3, captured and released proteins by GNP-MMs. All protein samples are of equal cell lysates concentration before fishing. And the same samples were used to detect Western blot. (D) Protein-protein interaction network of GNP by STRING 10.

Fig. 2. Co-localization of GNP with AKT. (A) Synthesis of the GNP fluorescent probe by click reaction of GNP-P and N_3-tag. (B) Co-localization of AKT antibody (pseudo red) and GNP-P fluorescent probe (pseudo green) in BEAS-2B cells.

Fig. 3. The effect of GNP on AKT activity inhibition, phosphorylation and plasma transport. (A) GNP inhibited SC79-induced AKT activity in a dose-dependent manner (0.1–10 μM); (B) GNP inhibited SC79-induced AKT phosphorylation. (Each bar represents the mean ± SD (n=3); * p<0.05, ** p<0.01, *** p<0.001 vs. SC79 group, # p<0.05, ## p<0.01 vs. control). (C) GNP-affected membrane translocation of AKT-PH in a dose-response manner (0.1–10 μM). Representative images show the membrane translocation of the AKT-PH domain with SC79 (10 μM) or GNP (0.1–10 μM) treatment. $AKT^{PH(R25)}$-GFP and $AKT^{PH(R25C)}$-GFP were used as a positive and negative control,

respectively. The histogram presented the fluorescence ratios of AKTPH(R25)-GFP and DiI on HEK 293T membranes by PCC. (Each bar represents the mean ± SD (n = 6); * p<0.05, ** p<0.01, *** p<0.01 vs. R25 group).

Fig. 4. GNP acts on the PH domain of AKT. (A) The competitive test was carried out by different inhibitors against the captured target protein via GNP-MMs. The effect was detected by western blot, and the histogram presented the efficiency of each inhibitor (each bar represents the mean ± SD (n=3); * p<0.05, ** p<0.01 vs. the control group). (B) Molecular docking of SC79 and GNP with the AKT PH domain (PDB: 1UNQ). AutoDock4.2 software was used to display the 3D maps of AKT PH domain-ligand interaction, and MOE software was used to display the 2D depiction of the protein-ligand interaction.

Fig. 5. GNP inhibits downstream inflammatory signaling. (A) The effect of GNP (0.1, 1 and 10 μM) on attenuating phosphorylation of AKT, IKK, and NF-κB. (Error bars indicate mean ± SD (n=3); * p<0.05, ** p<0.01, *** p<0.001 vs. the LPS group, ## p<0.01 vs. the control group). (B) After treatment with GNP (0.1, 1 and 10 μM), the level of inflammatory factors TNF-α (left panel) and IL-8 (right panel) in the culture medium was significantly lower compared with LPS induced model group. (Error bars indicate mean ± SD (n=3); ** p<0.01, *** p<0.001 vs. model group, ## p<0.01, ### p<0.001 vs. the control group).

Fig. 6. Schematic model of GNP-induced AKT deactivation.

The conformational change of AKT exposes the phosphorylation sites of Ser473 and Thr308, and promotes AKT activation by mTOR and PDK1. GNP binds to the AKT-PH domain and blocks its recruitment to the plasma membrane, thus affecting the binding of the AKT-PH domain to PtdIns(3,4,5)P3, and locking AKT in an inactive state. The coloration is as follows: kinase domain (pink), PH domain (purple), Thr308 (green), and Ser473 (blue). The dotted line represents the linker between the kinase and PH domains of AKT.