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RESEARCH ARTICLE

Isorhamnetin Inhibits Feline Infectious Peritonitis Virus Replication by Binding PI3K and Suppressing the AKT/NF-κB Pathways

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ABSTRACT

This study investigated the anti-Feline infectious peritonitis virus (FIPV) effects of Isorhamnetin (ISO) and its mechanisms *via* modulation of the serine-threonine kinase/nuclear factor kappa-B (AKT/NF-κB) signalling pathways. These effects of ISO were evaluated by measuring viral levels, the protein expression levels of key components in the AKT/NF-κB pathways, and the production of inflammatory cytokines *in-vitro*. For the first time, we demonstrated that ISO directly targets and binds to phosphatidylinositol 3-kinase (PI3K), inhibiting its lipid kinase activity, reducing the generation of the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3), and consequently effectively blocking the phosphorylation and activation of downstream key proteins in the AKT and NF-κB pathways. This action ultimately led to the suppression of viral replication and the downregulation of key pro-inflammatory cytokines, such as interleukin-8 (IL-8). In conclusion, our findings indicate that ISO exerts anti-FIPV effects by targeting PI3K and inhibiting the AKT/NF-κB pathways, providing a mechanistic basis for further evaluation of its antiviral activity and warranting validation in feline *in-vivo* models.

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INTRODUCTION

Feline infectious peritonitis (FIP) is a frequently fatal feline disease, caused by a pathogenic variant of the feline coronavirus (FCoV) known as the FIP virus (FIPV) (Cook *et al.*, 2022). As one of the serious infectious viruses in cats, FIPV infection can cause severe anorexia, jaundice, pyrexia and weight loss. The disease pathogenesis is characterized by abnormal viral replication within mononuclear phagocyte system cells, leading to their abnormal proliferation or differentiation and ultimately triggering an excessive and destructive inflammatory response (Kennedy, 2020; Gao *et al.*, 2023). Since its detailed description in the 1960s, FIP continues to pose a significant global challenge for feline health, with high mortality rates and substantial emotional and economic burdens for pet owners (Thayer *et al.*, 2022). Current therapeutic options for FIP remain severely limited. Conventional treatments involving glucocorticoid hormone (e.g., cortisol) and immunosuppressive treatment are frequently associated with adverse effects and poor outcomes (Tanaka *et al.*, 2012). While antiviral compounds such as GS-441524 and GC-376 have shown promise as treatments, they remain officially unapproved for veterinary use worldwide (Murphy *et al.*, 2018; Lu *et al.*, 2022). Therefore, it is urgently required to discover novel, safe and effective anti-FIP drugs.

Viral infection represents a dynamic interplay between the pathogen and host defenses (Moschonas *et al.*, 2024). To replicate successfully, viruses often hijack host cellular pathways (Fan *et al.*, 2024). The phosphatidylinositol 3-kinase/serine-threonine kinase (PI3K/AKT) signalling pathways, a central regulator of processes like cell proliferation, apoptosis, and metabolism, is a frequent target for viral manipulation (Jia *et al.*, 2007). AKT is a key kinase in this pathway, and its phosphorylation is a core event following PI3K activation that further regulates downstream signalling pathways such as nuclear factor

kappaB (NF-κB) (Li *et al.*, 2024; Liu *et al.*, 2024). Several viruses, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Duck Tembusu virus, and hepatitis C virus, have developed specialized mechanisms that allow them to hijack the host's signalling pathways to facilitate viral infection (Preciado *et al.*, 2014; Lamprinou *et al.*, 2023; Hahm, 2024). However, it remains unknown whether FIPV infection stimulates the PI3K/AKT/NF-κB pathways and other pathways, and this uncertainty underscores a pressing need for the development of therapeutics targeting this pathway.

To address this research gap, screening active compounds from natural products that can regulate this pathway may provide potential strategies for the development of anti-FIP agents. Given the limited availability of current therapeutic agents, many phytochemicals derived from medicinal plants have emerged as promising candidates for novel drug discovery and screening. Their natural abundance and the reduced likelihood of resistance development make them highly favored candidates. Consequently, Isorhamnetin (ISO), a biologically active O-methylated flavonoid isolated from the berries of *Hippophae rhamnoides* L. and the foliage of *Ginkgo biloba* L., has garnered increasing interest (Mei *et al.*, 2025). As a well-characterized natural PI3K inhibitor, ISO exerts diverse pharmacological effects, including anti-tumor, antioxidant, and anti-inflammatory activities, which are closely associated with the modulation of the PI3K/AKT signalling pathways (Gong *et al.*, 2020; Kong *et al.*, 2025). Significantly, ISO has been shown to have antiviral properties against multiple viruses, including SARS-CoV-2, influenza A strain, and human immunodeficiency virus 1, *in-vitro* (Li *et al.*, 2021; Zhan *et al.*, 2021; Fredsgaard *et al.*, 2023). Given its well-documented anti-inflammatory and PI3K-inhibitory properties, we hypothesized that ISO could suppress FIPV replication through targeted modulation of the PI3K/AKT/NF-κB pathways.

Multiple experimental assays were performed to examine the antiviral properties of ISO and its modulatory effects on Crandell Reese Feline Kidney (CRFK) cell proliferation. For the first time, the experiments demonstrate that FIPV infection promotes PIP3 production, subsequently driving viral replication by inducing the phosphorylation of AKT and NF-κB. Furthermore, this work confirmed that the anti-FIPV activity of ISO is closely associated with the reduction of PIP3 production and the inhibition of AKT and NF-κB phosphorylation. These findings elucidate the antiviral mechanisms of ISO, providing a theoretical basis for its further evaluation against FIPV *in-vitro*.

MATERIALS AND METHODS

Cells and viruses: CRFK cells (Procell, Wuhan, China) cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) were used for propagation of FIPV strain DF2 (GenBank JQ408981.1).

Antibodies, chemicals and reagents: ISO (Fig. 1A) and Baicalin (Fig. S1) (Bide Pharmatech, Shanghai, China; purities 97 and 95%, respectively). MK-2206, SC79, LY294002, BAY 11-7082, HRP-conjugated goat anti-mouse and anti-rabbit IgG (H+L) secondary antibodies, CCK-8 kit, RIPA lysis buffer, Lipo8000, protease inhibitor, and phosphatase inhibitor (Beyotime, Shanghai, China). Rabbit monoclonal anti-p-AKT (Ser473; Cat. No. 4060), anti-AKT (Cat. No. 4691), anti-p- NF-kappa-B inhibitor alpha (p-IκBα) (Ser32; Cat. No. 2859), anti-IκBα (Cat. No. 4812), anti-p-NFκB (Ser536; Cat. No. 3033), and anti-NFκB (Cat. No. 8242) (Cell Signalling Technology, Danvers, USA). UCL-TRO-1938 (MedChemExpress, Monmouth Junction, USA). Mouse monoclonal anti-GAPDH (Cat. No. AB8245) (Abmart, Shanghai, China) and anti-FIPV NP (Cat. No. MCA2194) (Bio-Rad, Shanghai, China). PI3K (P85 ALPHA) Protein (Cat. No. SRP0691) (Sigma-Aldrich, Shanghai, China)

Cytotoxicity assay: CRFK cells were treated with serial dilutions of ISO for 48 h. Cell viability was determined by CCK-8 assay *via* absorbance at 450nM.

Quantitative real-time PCR (qRT-PCR): Total RNA extracted from CRFK cells was reverse-transcribed into cDNA, followed by qPCR using the gene-specific primers listed in Table 1. The relative expression levels of target genes were determined using the $2^{-(\Delta\Delta Ct)}$ method.

Table 1: Sequences of the primers used in this study

Name	Sequences 5' to 3'
FIPV-NP-F	AAGGCCAGCGTAAGGAACTC
FIPV-NP-R	GCATTTGGCAGCGTTACCAT
GAPDH-F	GGTCATCATCTCTGCCCCTT
GAPDH-R	TCATGAGTCCCTCCACGATG
TNF-a N-F	GCCTCTTCTCCTTCCTCCTC
TNF-a N-R	TACTGGCTTGTCACTCGGAG
IL-1β N-F	CTGATGGCCCCGAAAAGATG
IL-1β N-R	GGTTGTGAGCAGGCAAAAGA
IL-8 N-F	CTCTGTGTGAAGCTGCAGTT
IL-8 N-R	ACCTTCTGCACCCACTTTTG
IL-6 N-F	TGCCAAGTCTGTGTACACCA
IL-6 N-R	CCTGAGGCTGAACTGAAGGA

Western blotting (WB): Protein levels were determined *via* standard Western blot procedures, using commercial antibodies against target proteins, followed by ECL detection and ImageJ-based densitometric analysis.

Antiviral activity assay: Serial dilutions of viral samples were inoculated onto CRFK cells. Cytopathic effects (CPE) were recorded after incubation. $TCID_{50}$ was then calculated according to the Reed-Muench.

Time-of-addition assay (TOA): The antiviral activity of ISO was tested in FIPV-infected CRFK cells using pre-, co-, and post-treatment protocols. Protein and mRNA levels were measured by WB and qRT-PCR. Viral titers were determined by endpoint dilution.

PIP3 levels assays: The PIP3 levels were determined by ELISA and quantified *via* a standard curve with dilution correction.

Plasmid preparation and cell transfection: A PI3KCG expression plasmid carrying the feline PI3KCG gene (NCBI Gene ID: 101096215) was constructed in pcDNA3.1(+) vector. The plasmid was transfected into CRFK cells using Lipo8000 and cells were harvested 24 h later.

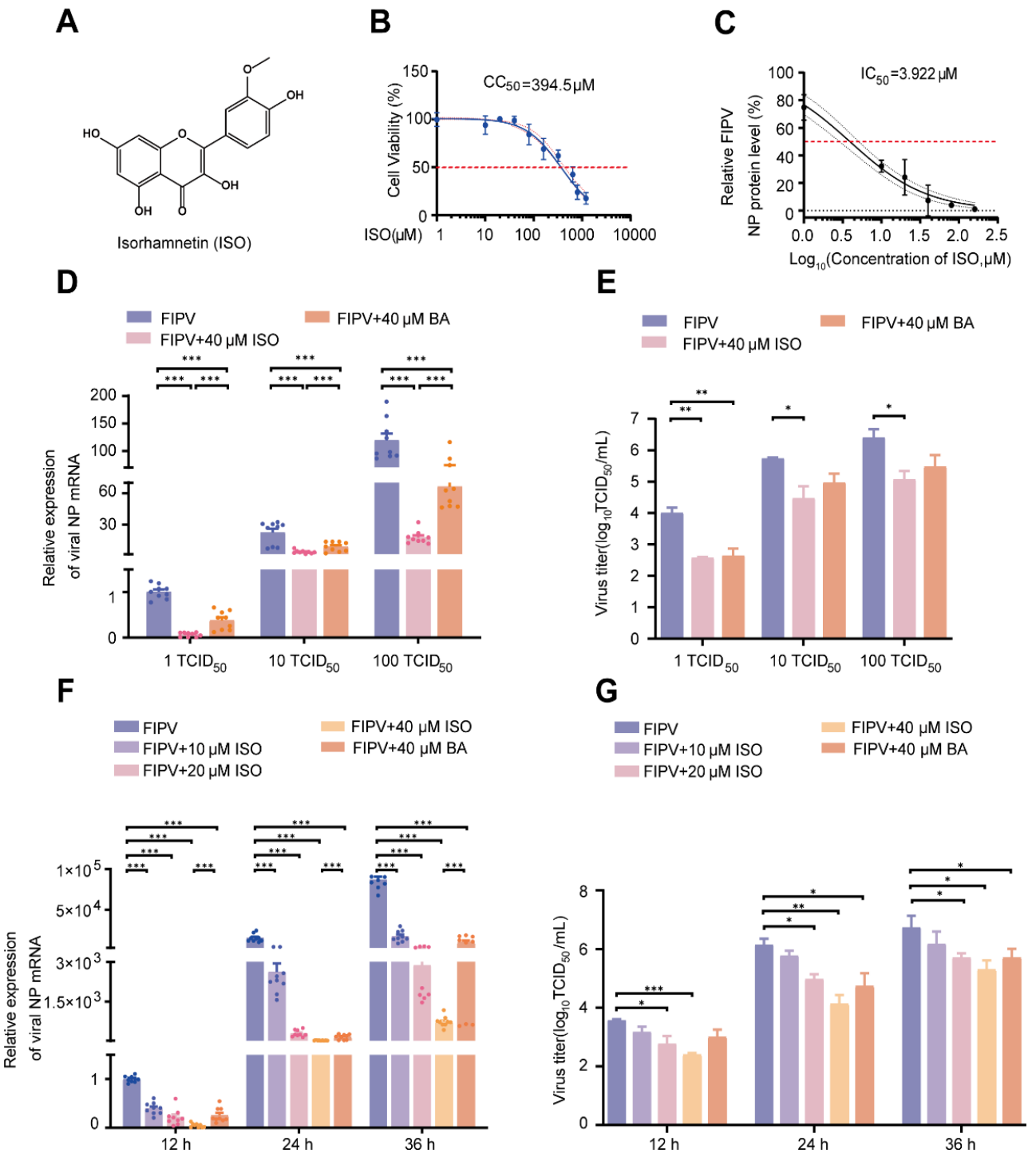


Fig. 1: ISO inhibits FIPV replication in CRFK cells. (A) Molecular structure of ISO, (B) The CC_{50} of different concentrations of ISO in CRFK cells. (C) The IC_{50} of different concentrations of ISO in CRFK cells. (D and E) Effect of ISO on viral RNA levels and viral titres under different (1, 10 and 100) $TCID_{50}$ values.(F and G) Effect of ISO on viral RNA levels and viral titres under different concentrations of ISO (10, 20 and 40μM) for 12, 24, and 36 hpi.

Cellular thermal shift assay (CETSA): Soluble cell lysates were pretreated with ISO (40μM) or DMSO for 1 h at RT. Protein stability was then assessed by WB after heat pulses at 50-70°C for 5 min.

Isothermal titration calorimetry (ITC): ITC assays were performed at 25°C in HEPES buffer (pH 7.5), with 1μM PI3K in the cell and 50μM ISO in the syringe. Titration proceeded *via* 20 consecutive 2μL injections (120-s intervals, 350rpm) into 300μL of sample, alongside a buffer-only control. Data analysis to calculate the dissociation constant (K), stoichiometry (N), enthalpy (ΔH), and entropy (ΔS) was performed with NanoAnalyzer software.

PPI Network Analysis: The overlapping targets of ISO and FIPV genes were obtained *via* Venny and then utilized to build a STRING-based protein-protein interactions (PPI) network.

Molecular dynamics (MD) simulations: Molecular docking of ISO to PI3K (NCBI XP_019680903) was performed using AutoDock Vina, followed by MD simulations using GROMACS (CHARMM36/TIP3P,

NPT, 300 K). Complex stability was evaluated by Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), Radius of Gyration (Rg) and hydrogen bonds (H-bonds) analyses.

Statistical analysis: Data were analyzed using GraphPad Prism version 9.5 and are presented as mean ± SEM from three independent experiments (n=3 per group). Unpaired/paired t-tests or one-way analysis of variance (ANOVA) with Tukey's multiple comparison test were applied to analyze the significance of data. Statistical significance was considered when the P-value was less than 0.05.

RESULTS

ISO inhibits FIPV replication: In order to assess the inhibition of FIPV replication by ISO, a CCK-8 assay was first used to screen for appropriate concentrations of ISO in CRFK cells. Results showed that a non-cytotoxic concentration (>90%) was observed at 40μM, and the half-cytotoxic concentration (CC_{50}) was 394.5μM (Fig. 1B). Subsequently, the antiviral activity of ISO against FIPV infection in CRFK cells was evaluated using immunofluorescence assays (IFA). The results indicated that ISO substantially inhibited FIPV replication in CRFK cells (Fig. S2A), with a half-maximal inhibitory concentration (IC50) of 3.922μM (Fig. 1C). Based on this dose-response profile, submaximal concentrations (10, 20 and 40μM) were selected for subsequent mechanistic studies to avoid complete cytotoxicity. Baicalin (BA), which has been demonstrated to inhibit FIPV replication in CRFK cells (Cao *et al.*, 2024), was employed as a positive control in our study.

To further validate the ability of ISO to inhibit FIPV replication, its impact on viral gene levels and progeny virus titer was evaluated. Initially, ISO was tested against different FIPV infectious doses (1, 10 and 100 $TCID_{50}$). Treatment with 40μM ISO resulted in significant decreases in viral gene levels and progeny virus titer at 24 hpi, suggesting that ISO exerts stable antiviral effects (Fig. 1D, E). Subsequently, the antiviral effect of ISO was evaluated at 12, 24 and 36 hpi. Results showed that 10, 20, and 40μM ISO significantly reduced FIPV gene levels at 12, 24 and 36 hpi. The inhibition rate of 40μM ISO was significantly greater than that of 40μM BA ($P<0.05$) (Fig. 1F). Meanwhile, 20 and 40μM ISO significantly inhibited the virus titer of FIPV at 12, 24 and 36 hpi, demonstrating dose-dependent antiviral activity (Fig. 1F, G). Taken together, these findings demonstrate that ISO consistently inhibits FIPV replication in a dose-dependent manner *in-vitro*.

ISO inhibits FIPV replication through different treatment modes: To evaluate the antiviral mechanism of ISO, a time-of-addition assay was conducted. Briefly, CRFK cells were treated with 40μM ISO under pre-, co-, or post-treatment conditions relative to FIPV infection (Fig. 2A). Pre-treatment with ISO for 3 h ,6 h or 12 h, as well as post-treatment with ISO for 3 h, 6 h, 9 h, 12 h and 24 h, significantly decreased FIPV mRNA levels (Fig. 2B), progeny virus titres (Fig. 2C) and protein expression levels (Fig. 2D, E). Notably, compared with pre- and post-treatment, the ISO co-treatment revealed a weakened inhibitory activity. This finding indicated no direct interaction between ISO and FIPV, which was supported by the direct evaluation results of their interaction (Fig. S4). Additionally, ISO substantially inhibited FIPV proliferation under pre-treatment conditions in a dose-dependent manner (Fig. S5), indicating that it effectively modulates CRFK cell susceptibility to FIPV infection. Collectively, these findings suggest that ISO exerts an inhibitory effect on FIPV replication, probably through the modulation of cellular pathways.

ISO inhibits FIPV replication by reducing p-AKT levels: To determine the possible targets of ISO in inhibiting FIPV replication, network pharmacology analysis was performed. A PPI network was constructed to identify key factors mediating ISO's inhibition of FIPV replication and to explore the underlying mechanism. It was found that AKT had the highest enrichment score (Fig. 3A), indicating that it was critical for the anti-FIPV effect of ISO. Given that activation of AKT requires phosphorylation (Romano *et al.*, 2021), we investigated the effect of ISO on p-AKT in FIPV-infected CRFK cells by WB. Indeed, WB confirmed that p-AKT levels were significantly increased in CRFK cells at 24 and 36 hpi (Fig. 3B), and ISO significantly decreased p-AKT levels in a dose-dependent manner (Fig. 3C).

Subsequently, to investigate the mechanism by which ISO exerts anti-FIPV activity by inhibiting the AKT signalling pathway, the AKT inhibitor MK2206 and the inducer SC-79 were used *in-vitro*. The results showed that p-AKT levels decreased in a dose-dependent manner following MK2206 exposure but increased similarly following SC-79 exposure in FIPV-infected CRFK cells. Meanwhile, consistent differences in viral protein levels were observed among the treatment groups, suggesting that the reduction of p-AKT was critically involved in inhibiting FIPV replication and alleviating cellular damage (Fig. 3D, E). Furthermore, ISO treatment counteracted the SC-79-mediated upregulation of p-AKT levels in both mock- and FIPV-infected CRFK cells (Fig. 3F), a finding consistent with the effects observed with MK2206 (Fig. 3G). These results suggest that ISO can inhibit FIPV replication by reducing the levels of p-AKT.

ISO decreases p-AKT levels by reducing PIP3 levels; PIP3 functions as a key signalling molecule in the PI3K/AKT pathways by recruiting and activating downstream AKT (Carrillo *et al.*, 2024). Given that previous experimental results confirmed that ISO can directly bind to PI3K, we further measured the levels of p-AKT and PIP3 to assess the effect of ISO on PI3K activity. After ISO intervention, the levels of PIP3 were significantly decreased (Fig. 4A). LY294002, a known PI3K inhibitor, showed effects similar to those of ISO on the levels of PIP3, p-AKT and FIPV protein (Fig. 4B, D). Conversely, UCL-TRO-1938 stimulated PI3K and increased the level of PIP3, a process that facilitated the rapid phosphorylation of AKT and promoted FIPV replication in CRFK cells (Fig. 4C, E). Furthermore, ISO and LY294002 treatment individually counteracted the UCL-TRO-1938-induced upregulation of p-AKT levels and viral protein expression (Fig. 4F, G). Therefore, ISO may inhibit FIPV replication by decreasing PIP3 levels.

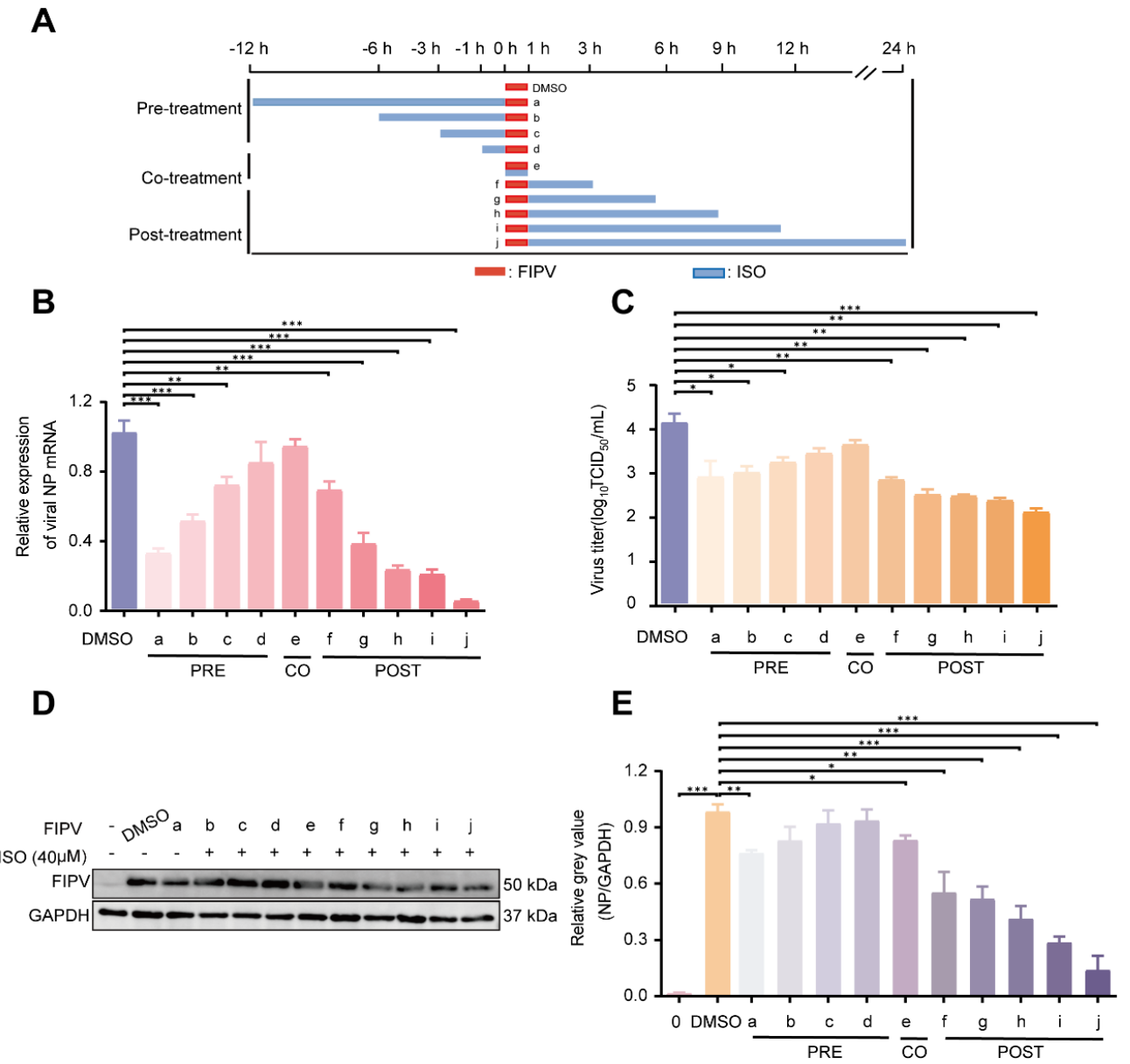


Fig. 2: Efficacy of ISO in CRFK cells with respect to the administration time. (A) Schematic diagram of different administration modes of ISO. (B) Effect of ISO on viral RNA levels under different administration modes. (C) Effect of ISO on viral titres levels under different administration modes. (D) Effect of ISO on protein expression levels under different administration modes. (E) Relative quantification results of Fig D.

ISO inhibits FIPV replication by binding to the PI3K protein: To investigate the functional associations, an analysis of KEGG pathway enrichment was conducted and the PI3K/AKT signalling pathway was significantly overrepresented among the shared targets (Fig. 5A). Given that ISO has been reported to inhibit PI3K activity *via* direct binding (Kim *et al.*, 2011), we further investigated its effect. Specifically, we performed CETSA and ITC assays to determine whether PI3K is ISO's key target in PI3K/AKT-mediated inhibition of FIPV replication. The result showed that binding of ISO to PI3K stabilized the protein against heat-induced degradation in CRFK cells (Fig. 5B). Consistent with the results from CETSA, the ITC analysis also demonstrated that ISO directly interacted with PI3K, with a dissociation constant (Kd) of 2.79 μM (Fig. 5C).

Subsequently, the molecular docking predictions revealed that ISO has a hydrophobic effect on PI3KCG protein residues, including ARG-851, TYR-789 and GLN-297. ISO exhibited a binding energy of -6.2 kcal·mol^{-1} with PI3KCG (Fig. 5D). Molecular dynamics simulations were conducted to further confirm the ability of ISO to bind PI3KCG. As shown in Fig. 5E, the PI3KCG-ISO complex was stable after 20 ns, which indicated that the binding of ISO to PI3KCG resulted in no substantial conformational change in the protein and maintained relative stability. Specifically, the continuous RMSD curve indicated stable binding of the compound to the PI3KCG protein pocket without dissociation. Meanwhile, the PI3KCG-ISO complex exhibited a stable Rg, indicating that the protein conformation was stable (Fig. 5F). Moreover, the RMSF analysis in Fig. 5G indicated low mobility in most residues, with significantly higher fluctuations observed only at the head, tail, and around residue 448. Finally, the stable number of H-bonds in the PI3KCG-ISO complex (mean 1.48 ± 0.90) further supports that ISO exerts its pharmacological effects through direct binding to PI3KCG (Fig. 5H).

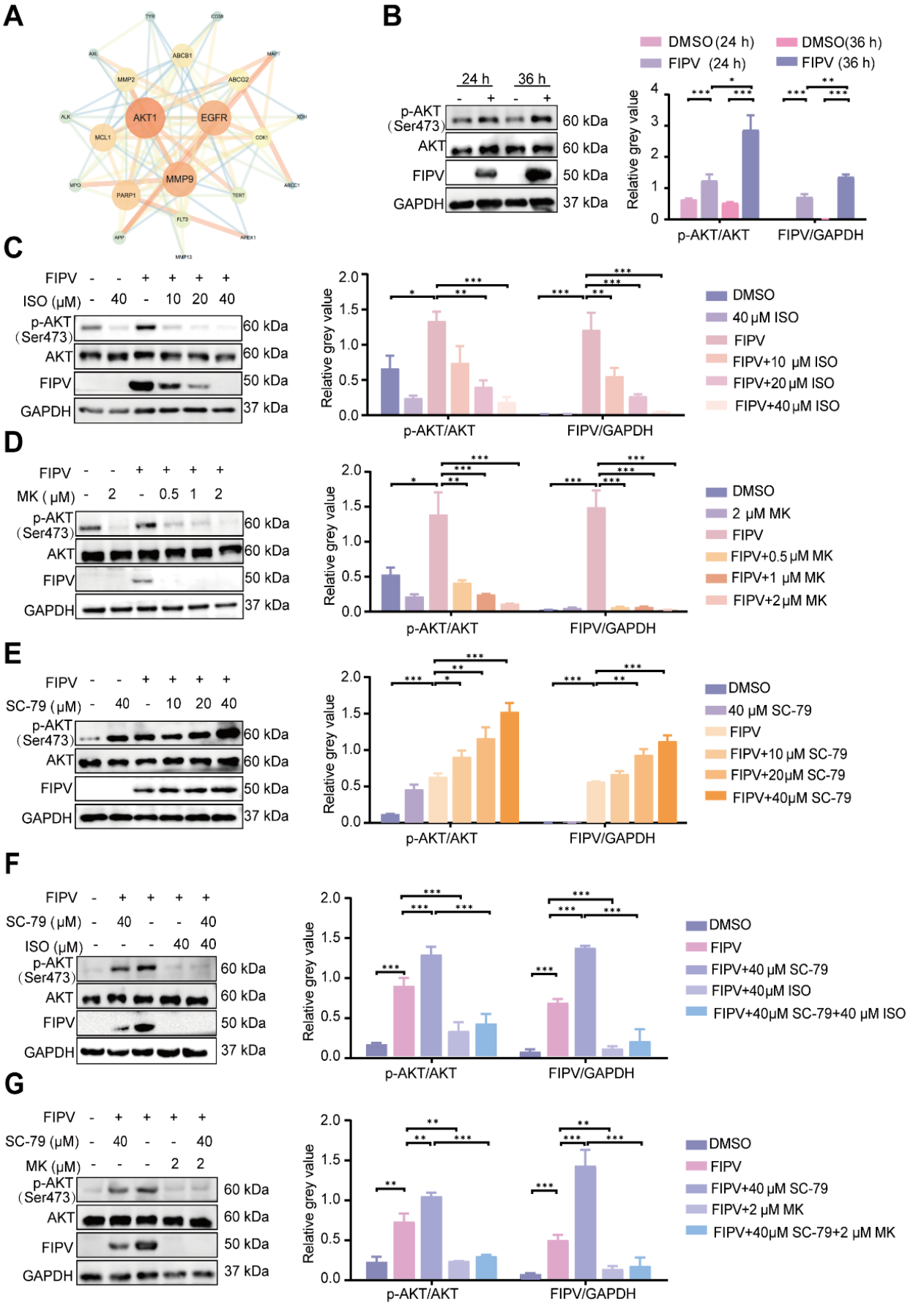


Fig. 3: ISO inhibits FIPV replication by reducing p-AKT levels. (A) PPI network derived from STRING (confidence cutoff = 0.9). (B) Quantitative assessment of the designated protein expression at 24 and 36 hpi. (C and E) Effects of different concentrations with ISO, MK2206 and SC-79 on indicated proteins levels and quantitative evaluation. (F and G) Effects of cotreatment with ISO, MK2206 and SC-79 on indicated proteins levels and quantitative evaluation.

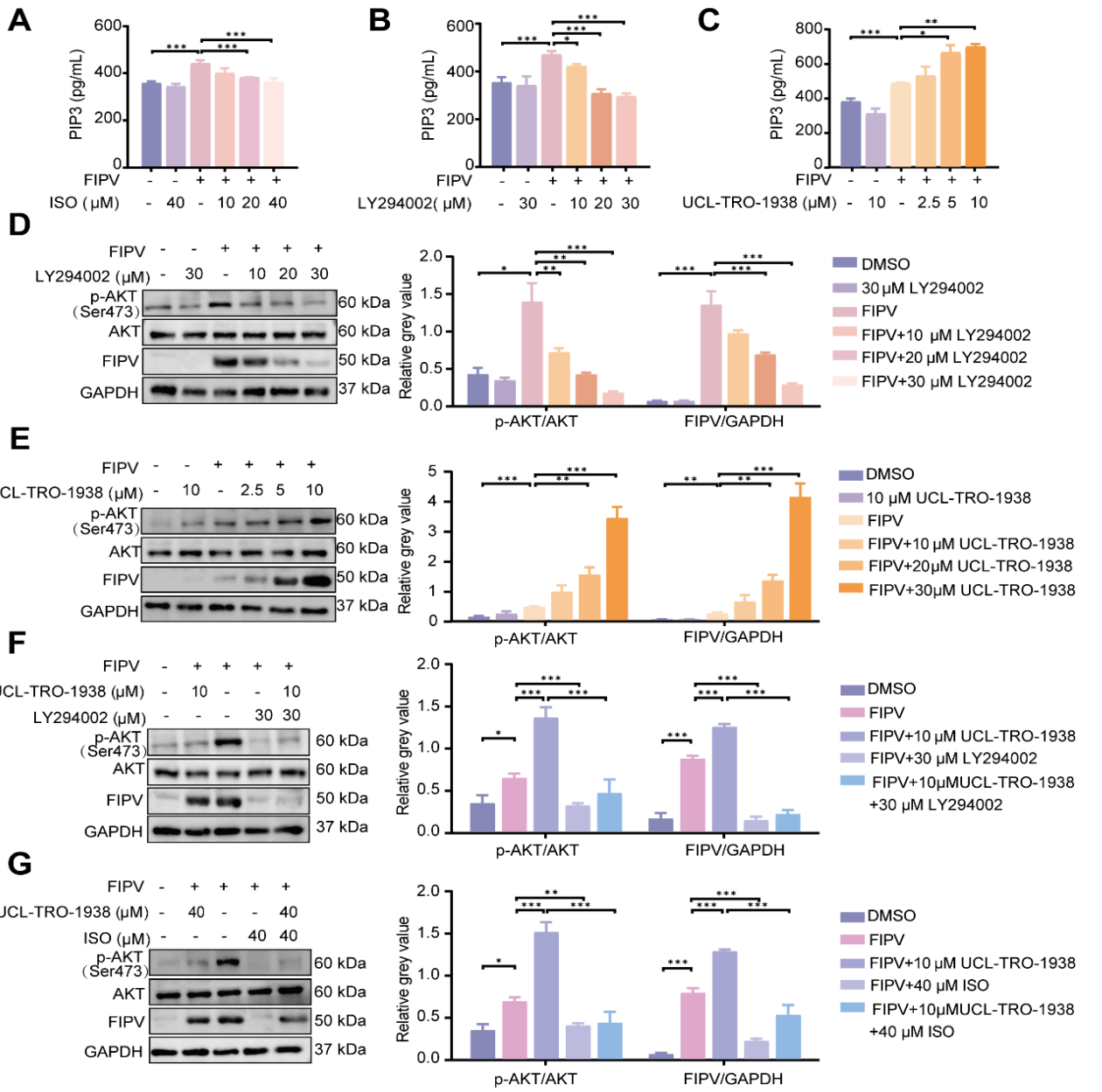


Fig. 4: ISO decreases p-AKT levels by reducing PIP3 levels. (A–C) Effects of ISO, LY294002 and UCL-TRO-1938 on the levels of PIP3 in FIPV-infected CRFK cells. (D and E) Effects of different concentrations with LY294002 and UCL-TRO-1938 on indicated proteins levels and quantitative evaluation. (F and G) Effects of co-treatment with ISO, LY2940026 and UCL-TRO-1938 on indicated proteins levels and quantitative evaluation.

The MM/PBSA calculations revealed that ISO binds to PI3KCG with free energy of -21.24±5.12 kcal·mol^{-1}, indicating that the binding is spontaneous and potent. Van der Waals (VDWAALS) forces and electrostatic (EEL) interactions serve as the key driving forces, with key binding residues including TRP203, ARG279, etc (Fig. 5I, J). Collectively, the results from CETSA, ITC, molecular dynamics simulations, and free energy calculations demonstrated that ISO directly binds to PI3K, thereby inhibiting FIPV replication.

ISO inhibits FIPV replication by inhibiting the AKT/NF-κB signalling pathways: The NF-κB signalling pathway is known to be downstream of the PI3K/AKT signalling pathways (Liu *et al.*, 2024), with IκBα and p65 serving as key regulatory activators (Pu *et al.*, 2022). Upon activation, these kinases regulate apoptosis and aggravate virus-mediated cellular damage through multiple mechanisms. Therefore, we further evaluated the influence of ISO on NF-κB activity during FIPV infection. Indeed, our findings indicated that p-IκBα and p-p65 levels were elevated at 24 and 36 hpi following FIPV infection (Fig. 6A), suggesting the activation of the NF-κB signalling pathway. ISO significantly suppressed p-IκBα and p-p65 levels in a dose-dependent manner (Fig. 6B). Subsequently, the NF-κB pathway inhibitor BAY11-7082 was employed to further clarify the association between NF-κB and viral replication. At concentrations ranging from 2.5 to 10 μM, BAY11-7082 decreased p-IκBα, p-p65, and viral protein levels in a dose-dependent manner, indicating that the NF-κB signalling pathway plays a critical role in FIPV replication (Fig. 6C). Moreover, LY-294002, a specific PI3K inhibitor, simultaneously downregulated AKT/NF-κB signalling by decreasing the levels of p-IκBα and p-p65 proteins in FIPV-infected CRFK cells (Fig. 6D).

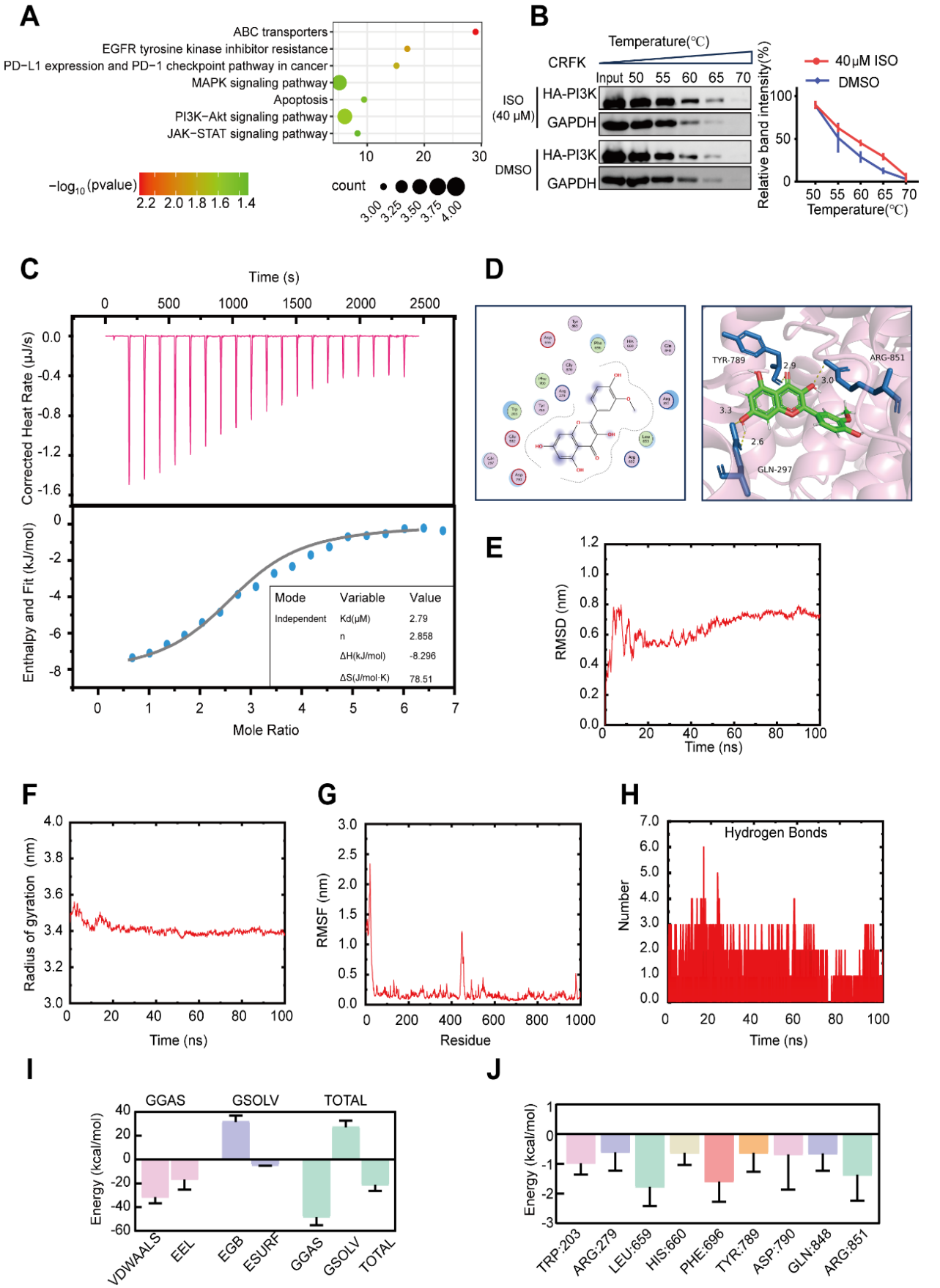


Fig. 5: ISO inhibits FIPV replication by binding to the PI3K protein. (A) Bubble plot of common targets in ISO and HA-PI3K. (B) CESTA of the direct interaction between ISO and HA-PI3K in CRFK cells. (C) ITC analysis of the direct interaction between ISO and PI3K. (D) Molecular docking patterns of ISO with the PI3KCG target. (E) Conformational stability analysis based on backbone RMSD. (F) Overall structural compactness analysis based on Rg. (G) Analysis of local residue flexibility based on RMSF. (H) Analysis of internal packing density based on intramolecular H-bonds. (I and J) Decomposition of binding free energies for the ISO-FIPV PI3KCG complex.

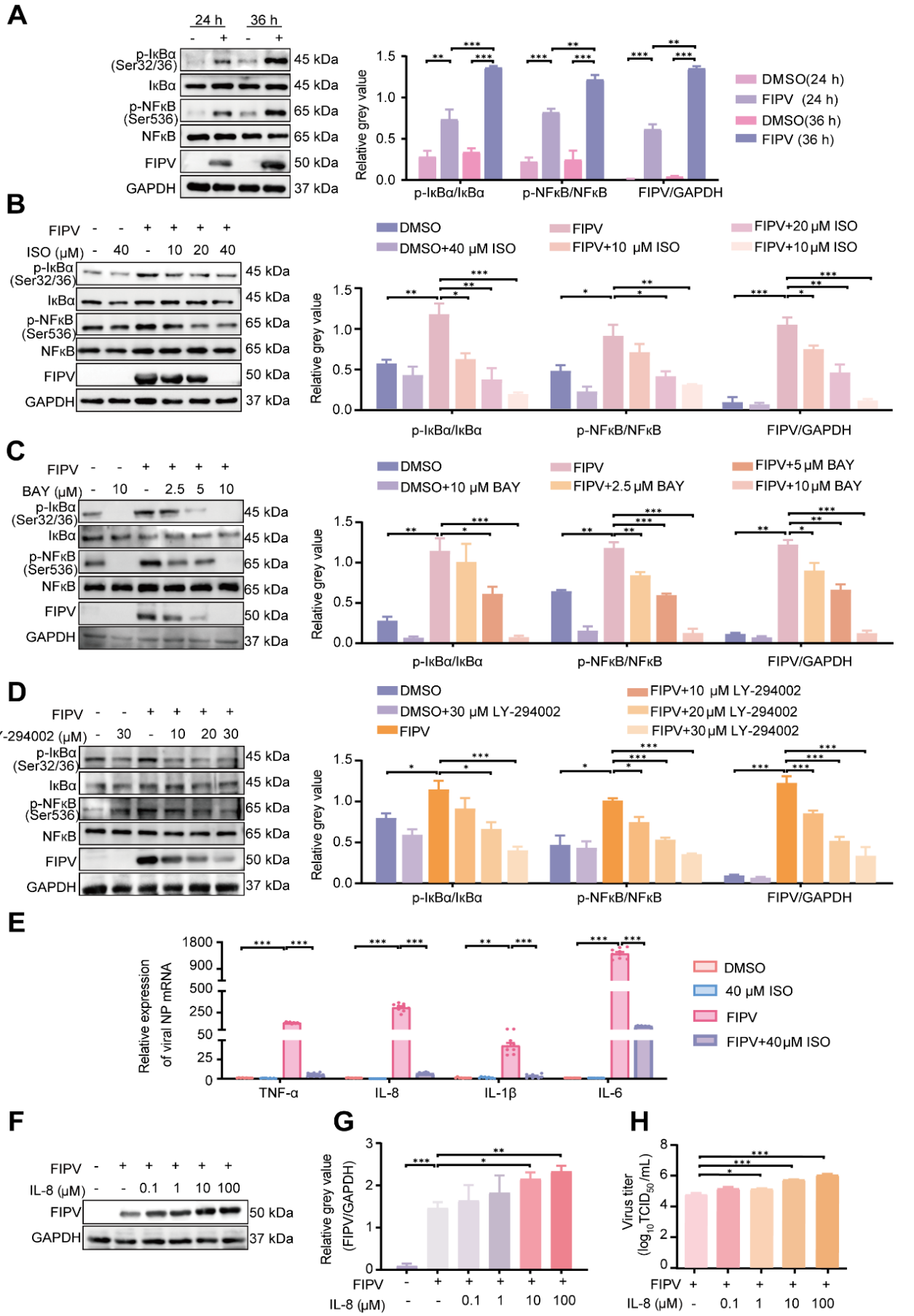


Fig. 6: ISO inhibits FIPV replication through blocking the AKT/NF-κB signalling pathways. (A) Quantitative assessment of the designated protein expression at 24 and 36 hpi. (B-D) Effects of different concentrations with ISO, BAY and LY294002 on the expression and quantitative evaluation of the indicated proteins. (E) Effects of ISO on the TNF-α, IL-8, IL-1β and IL-6 mRNA levels. (F and G) Effects of different concentrations with IL-8 on the expression levels of the indicated proteins. (H and I) Effects of IL-8 on the viral titre and FIPV replication in CRFK cells.

Next, the role of key inflammatory cytokine expression in FIPV-infected CRFK cells was explored. A significantly increased expression of cytokines TNF-α, IL-8, IL-1β and IL-6 mRNAs was observed in FIPV-infected CRFK cells. Furthermore, ISO strongly suppressed IL-8 and IL-6 expression in both mock- and FIPV-infected CRFK cells (Fig. 6E). Given these results, we chose to further investigate the specific role of IL-8 and IL-6 in FIPV replication. The cells were pretreated with increasing concentrations of the exogenous cytokines IL-8 and IL-6 (0.1 to 100ng/mL) over 4 h, and then infected with FIPV (Fig. 6F, S6). As a result, the addition of exogenous cytokine IL-8 promoted FIPV replication in CRFK cells, whereas the addition of IL-6 had no significant effect (Fig. 6G, H). These results suggest that ISO suppresses AKT phosphorylation, which in turn blocks NF-κB activation and IL-8 production, ultimately reducing FIPV replication in CRFK cells.

DISCUSSION

Although FIP was identified more than half a century ago, no effective vaccines or approved therapeutic drugs have been developed to counteract FIPV infection, highlighting the critical need for novel treatment strategies. Our research demonstrates that FIPV infection promotes PIP3 expression and AKT phosphorylation. This subsequently enhances NF-κB phosphorylation and creates a cellular environment conducive to viral replication. Concurrently, the upregulation of the NF-κB pathway further promoted cytokine synthesis and FIPV gene expression, thereby facilitating viral replication. Importantly, we identified the natural compound ISO as a potent inhibitor of this signalling pathway. Our findings provide substantial evidence indicating that ISO interacts with PI3K, leading to a reduction in PIP3 levels and the suppression of the AKT/NF-κB signalling pathways, which ultimately inhibits FIPV replication.

Natural products, especially those derived from Chinese herbal medicines, are valuable sources of antiviral agents, as evidenced by the approval of several such agents by China's National Medical Products Administration (NMPA) for treating viral infections (Qian *et al.*, 2024). Among these, ISO exhibits a wide spectrum of therapeutic bioactivities, including antioxidant, anti-inflammatory, immunomodulatory, and notably, antiviral effects (Gong *et al.*, 2020). The anti-coronavirus potential of ISO is evidenced by its inhibition of SARS-CoV-2 spike pseudotyped virus entry (Zhan *et al.*, 2021; Li *et al.*, 2021). Given the similarities between FIPV and SARS-CoV-2 in host adaptation and clinical manifestations (Sweet *et al.*, 2022), ISO shows promising anti-FIPV activity. Nevertheless, further studies are needed to confirm its efficacy and elucidate its mechanisms.

To experimentally validate this potential, we first assessed the antiviral efficacy of ISO against genotype II FIPV in CRFK cells. The results showed that ISO exhibited significant antiviral activity against FIPV. Importantly, the compound showed a high selectivity index (SI ≈ 100.6), which was calculated from a CC_{50} of 394.5μM and an IC_{50} of 3.922μM in CRFK cells, indicating a favourable therapeutic window for its observed antiviral effects. Additionally, we confirmed the efficacy of ISO against FIPV *in-vitro* by conducting qRT-PCR and endpoint dilution assays. These findings revealed that ISO inhibited FIPV in CRFK cells in a dose- and time-dependent manner.

The TOA assay serves as an analytical tool for monitoring the various stages of drug therapy. Our findings suggest that ISO does not exhibit a direct interaction with FIPV, which was supported by the results of the virus-ISO interaction experiments (Fig.S4). However, the pre-treatment results indicate that ISO inhibits FIPV replication by influencing host cells. Specifically, our data indicate that the suppressive effect of ISO on FIPV replication occurs through a host-centric mechanism that involves its interaction with and disruption of key pathways in host cells. This host-directed mechanism fundamentally distinguishes ISO from directly acting antiviral drugs. It thus provides a critical theoretical foundation for further investigating the specific cellular targets and signalling pathways of ISO, offering clear direction for future research.

The interaction between the compound and disease was investigated by constructing a PPI network. Ultimately, AKT1 was recognized as the most effective target of ISO for inhibiting FIPV infection. AKT plays a critical role in controlling cell survival and apoptosis. Upon phosphorylation, activated AKT influences various cellular targets, consequently suppressing apoptosis and enhancing metabolism, growth, and synthetic processes (Plas and Thompson, 2005). In our present study, the p-AKT was significantly induced by FIPV infection in CRFK cells (Fig. 3B). The AKT inhibitor MK2206 similarly reduced p-AKT levels and viral replication (Fig. 3D), thereby confirming the previously reported relationship between FIPV infection and AKT activation (Cao *et al.*, 2024). Furthermore, ISO reduced basal p-AKT levels in uninfected CRFK cells and counteracted the SC-79-induced increase in p-AKT levels in both mock-infected and FIPV-infected cells (Fig. 3F). These findings indicate that ISO inhibits FIPV replication by decreasing p-AKT levels, suggesting that this pathway is a potential target for antiviral intervention.

Recent reviews have thoroughly described the role of the PI3K/AKT pathways in the host response, revealing its ability to trigger both anti- and proviral functions (Dunn and Connor, 2012). Given the multiple effects of ISO on disease treatment *via* the PI3K/AKT signalling pathways (Tan *et al.*, 2025; Gao *et al.*, 2022), we aimed to investigate its potential therapeutic value in FIPV infection. Consistent with a potential proviral role, our data indicated that FIPV infection significantly increased the cellular levels of PIP3. However, this increase was markedly attenuated by ISO treatment (Fig. 4A). The functional role of the PI3K/AKT pathway was validated using the PI3K inhibitor LY294002 and the inducer UCL-TRO-1938. Treatment with LY294002 suppressed the expression and phosphorylation of PI3K/AKT signalling-related proteins, thereby inhibiting FIPV infection. In contrast, UCL-TRO-1938 exerted the opposite effect by promoting pathway activation and enhancing viral

infection (Fig. 4D, E). These opposing outcomes confirm the critical involvement of this pathway in FIPV infection. Importantly, our results further showed that ISO similarly inhibits FIPV replication by suppressing PI3K/AKT pathway activation, as indicated by reduced phosphorylation of key components. Furthermore, the elevation of p-AKT levels induced by UCL-TRO-1938 was effectively reversed by cotreatment with ISO (Fig. 4G).

The relationship between ISO and PI3K was further investigated using the CESTA and ITC assays, which provided evidence for the interaction between ISO and PI3K (Fig. 5B, C). Subsequent in silico studies, including molecular docking and MD simulations, were conducted to assess the binding affinity and stability of the ISO-PI3K interaction. The MD simulations confirmed the binding mechanism and the stability of the complex between ISO and the target protein PI3KCG, thereby validating the reliability of the screening results. Collectively, these results indicate that ISO binds to PI3K, suppressing PIP3 production and enhancing the inhibition of downstream AKT kinase, ultimately inhibiting FIPV replication.

AKT is essential for the upstream activation of the NF-κB signalling pathways (Santoro *et al.*, 2003). Studies indicate that FIPV employs the NF-κB signalling pathway as a key mechanism to counteract host innate immune defences (Chen *et al.*, 2020). Consistent with this, our data indicate that FIPV infection strongly activates NF-κB signalling in CRFK cells, as evidenced by the increased phosphorylation of IκBα and p65 at 24 and 36 hpi (Fig. 6A). Both the NF-κB inhibitor BAY 11-7082 and ISO dose-dependently suppressed NF-κB activation and FIPV replication in these cells (Fig. 6B and C). Similarly, the PI3K inhibitor LY-294002 and the AKT inhibitor MK-2206 substantially reduced the levels of p-IκBα and p-p65 in FIPV-infected CRFK cells (Figs. 6D and S3). Mechanistically, the anti-FIPV activity of ISO appears to be associated with its suppression of the NF-κB pathway, mediated by the PI3K phosphorylation cascade in CRFK cells. Consequently, viral replication was suppressed through the partial inactivation of the PI3K/AKT/NF-κB signalling pathways.

Among the numerous strategies that viruses employ to evade host immune defences is the production of viral cytokines and their receptors (Barry and McFadden, 1997). The available data suggest that TNF-α, IL-8, IL-1β and IL-6 are readily secreted into the peritoneal effusion supernatant of FCoV-positive animals (Safi *et al.*, 2017). Our experimental results indicate that the mRNA expression levels of these cytokines are significantly increased in CRFK cells following FIPV infection, which is consistent with the findings reported in previous studies. Among the cytokines assessed, IL-6 and IL-8 mRNA levels were markedly suppressed by ISO in CRFK cells, regardless of whether they were infected with FIPV (Fig. 6E). Moreover, a comparative transcriptome analysis of the FIPV-infected group revealed that NF-κB acts as an upstream regulatory molecule of IL-8, promoting neutrophil activation and function (Lee *et al.*, 2025). In our study, the addition of exogenous IL-8 facilitated FIPV replication, whereas the addition of IL-6 had no significant effect (Fig. 6F and G). These results suggest that ISO inhibits FIPV replication at least partly by reducing the level of IL-8. Finally, a schematic summary illustrating the proposed mechanisms by which ISO inhibits FIPV replication (Fig. 7).

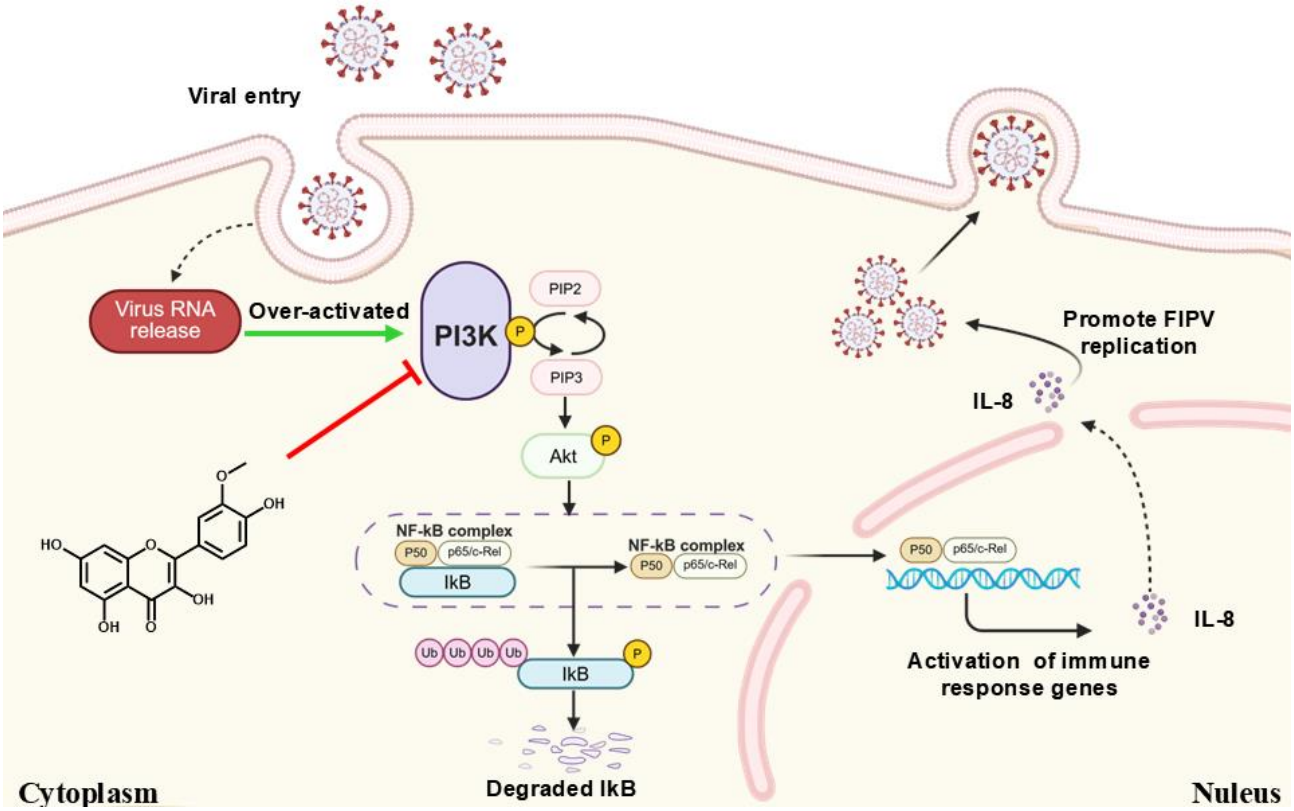


Fig. 7: Schematic overview of the mechanisms underlying the inhibitory effects of ISO on FIPV replication.

To preliminarily assess the *in-vivo* druggability potential of ISO, we employed the open-source computational model PKSmart to predict its pharmacokinetic parameters, and the results showed favorable drug-like properties. However, computational predictions cannot replace actual animal experimental data, and further studies are needed to systematically evaluate the pharmacokinetic, pharmacodynamic, and toxicological profiles of ISO in feline *in-vivo* models to fully validate its clinical translational potential.

Conclusions: In conclusion, these findings demonstrate that ISO inhibits FIPV replication by directly binding to PI3K and blocking the AKT/NF-κB signalling pathways. These findings not only provide direct evidence for the antiviral mechanism of ISO but also robustly support its feasibility as a candidate anti-FIPV drug. However, these findings warrant further validation in feline *in-vivo* models and natural infection cases to assess their translational potential. Future investigations should focus on the development of PI3K/AKT-targeted antiviral drugs for FIPV and assess their potential for clinical application. Such contributions will facilitate the formulation of innovative preventive and regulatory measures within the cat industry, decrease FIPV transmission and infection rates in Felis catus, and consequently alleviate economic losses while enhancing the overall health of the pet industry.

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