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Preclinical studies of the falnidamol as a highly potent and specific active ABCB1 transporter inhibitor

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Abstract

Background ABCB1 overexpression is a key factor in causing multidrug resistance (MDR). As a result, it is crucial to discover effective medications against ABCB1 to overcome MDR. Falnidamol, a tyrosine kinase inhibitor (TKI) targeting the epidermal growth factor receptor (EGFR), is currently in phase 1 clinical trials for the treatment of solid tumors. In this work, we assessed whether falnidamol could act as an inhibitor of ABCB1 to reverse ABCB1-mediated MDR.

Methods The reversal effect of falnidamol on MDR was assessed by MTT, colony formation, 3D microsphere, and xenograft model assays. The protein expression or cellular localization was tested by western blot and immunofluorescence analysis. The intracellular doxorubicin accumulation and efflux were assessed by flow cytometry. The ATPase activity of ABCB1 was detected by a microplate reader. The interaction between falnidamol and ABCB1 was evaluated by docking analysis and cellular thermal shift assay.

Results Our data showed that falnidamol specifically reversed ABCB1-mediated MDR but not ABCG2-mediated MDR in vitro and in vivo. Mechanistic studies suggested falnidamol had no effect on ABCB1 expression or cellular localization, nor on the AKT or ERK pathways. Further studies found that falnidamol reduced ABCB1's efflux function, resulting in enhanced intracellular agent accumulation and thus overcoming MDR. ATPase assay showed that falnidamol suppressed the ABCB1 ATPase activity. Furthermore, docking analysis and cellular thermal shift assay indicated that falnidamol bound directly to the drug-binding site of ABCB1 transporter.

Conclusion The present study proves that falnidamol acts as a highly potent and specific active ABCB1 transporter inhibitor, and can reverse ABCB1-mediated MDR, implying that combining falnidamol with ABCB1 substrate chemotherapeutic agents has the potential to overcome ABCB1-mediated MDR.

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Keywords Falnidamol, ABCB1, Multidrug resistance, Reversal effect, Cancer

Introduction

Multidrug resistance (MDR) is well known to be one of the leading causes of failed cancer treatments [1, 2]. Various MDR mechanisms have been identified, including increased intracellular drug efflux, change in medication metabolism, apoptotic suppression, improved DNA repair capacity, and oncogene mutations [3, 4]. Of these, drug resistance mediated by ATP-binding cassette (ABC) transporter is one of the major contributors to cancer MDR [5, 6].

ABC transporters, a superfamily consisting of seven subfamilies from ABCA to ABCG, are found on cell membranes and have a variety of pharmacological and physiological functions [7]. Of them, ABCB1 and ABCG2 have been recognized as the main causes of MDR [8]. Of particular interest is ABCB1, also known as P-gp or MDR1, which is widely distributed in different tissues, including the intestines, blood-brain barrier and placenta, and when it is overexpressed in cancer cells, it reduces intracellular chemotherapeutic drug concentration [9, 10]. ABCB1 substrates include doxorubicin, paclitaxel, vincristine and others [11]. As a result, it is essential to research for effective inhibitors to overcome ABCB1-mediated MDR.

Recently, several tyrosine kinase inhibitors (TKIs), such as imatinib, sorafenib, gefitinib, sapitinib and erlotinib, have been found to inhibit the ABC transporter's activity, and are therefore expected to reverse MDR [12, 13].They have the potential to be employed as reversal medicines in combination with chemotherapeutic agents for the treatment of MDR malignancies. Falnidamol (Fig. 1A), also known as BIBX1382, is a selective TKI of epidermal growth factor receptor (EGFR) in phase 1 clinical trials for the treatment of solid malignancies [14]. In this study, we assessed whether falnidamol could act as an inhibitor of ABCB1, reversing ABCB1-mediated MDR in drug-resistant cancer cells.

Materials and methods

Chemicals

Falnidamol, doxorubicin, paclitaxel, mitoxantrone, cisplatin, verapamil, Ko143, and other reagents were purchased from MedChemExpress (Shanghai, China). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Thermo Fisher Inc (Shanghai, China). MTT kit, anti-ABCB1, anti-AKT, anti-p-AKT, anti-ERK, anti-p-ERK, anti-GAPDH and the secondary antibodies were bought from Beyotime Biotechnology (Shanghai, China).

Cell lines and cell culture

The following cell lines were used in this study: The human cervical cancer cell line HELA and its ABCB1-overexpressed resistant cell line HELA-Col selected by colchicine. The human colon cancer cell line SW620 and its ABCB1-overexpressed resistant cell line SW620-Adr selected by doxorubicin. The human embryonic kidney cell line HEK293 and their stably transfected cell lines HEK293-Vector (transfected with empty plasmid), HEK293-ABCB1 (transfected with ABCB1 plasmid) and HEK293-ABCG2 (transfected with ABCG2 plasmid). The cell lines, including HELA, SW620 and HEK293, were obtained from Shanghai Cell Bank. The cell lines, including HELA-Col, SW620-Adr, HEK293-Vector and HEK293-ABCB1 were constructed by our laboratory. All cell lines were cultured in DMEM supplemented with 10% FBS.

Cytotoxicity and reversal experiments

The cytotoxicity and reversal experiments were conducted using MTT assay as previously reported [15]. Briefly, cells were seeded into 96-well plates (5×10^3 cells per well) and maintained overnight. For the cytotoxicity assay, different concentrations of falnidamol were added into the wells. For the reversal assay, after pre-incubating with falnidamol or verapamil for 2 h, different concentrations of conventional chemotherapeutic medicines, including doxorubicin, paclitaxel, or cisplatin, were added into the wells. The cells were cultured for 72 h, then MTT was added, and the absorbance was detected using an enzyme reader at 570 nm. Verapamil was utilized as a positive control inhibitor. Cisplatin, a non-substrate agent of ABCB1, was utilized as a negative control anti-cancer agent.

Colony formation and 3D spheroid assays

Colony formation and 3D spheroid assays were performed as described in the previous study [16]. In brief, for colony formation assay, HELA-Col cells were seeded in 6 well plates (1000 cells per well) and treated with falnidamol (5 μM, 6 h), paclitaxel (1 μM, 4 h) or the combination (falnidamol for 2 h followed by paclitaxel for 4 h). After removal of the agents, the cells were cultured for further 10 days. Finally, colonies were stained using 0.1% crystal violet for 30 min after being fixed using methanol for 30 min, and then colonies were counted under a microscope. For 3D spheroid assay, HELA-Col cells were seeded in 6-well plates (1000 cells per well) and treated with falnidamol (5 μM, 6 h), paclitaxel (1 μM, 4 h) or the combination (falnidamol for 2 h followed by paclitaxel for 4 h). After removal of the agents, the cells were seeded

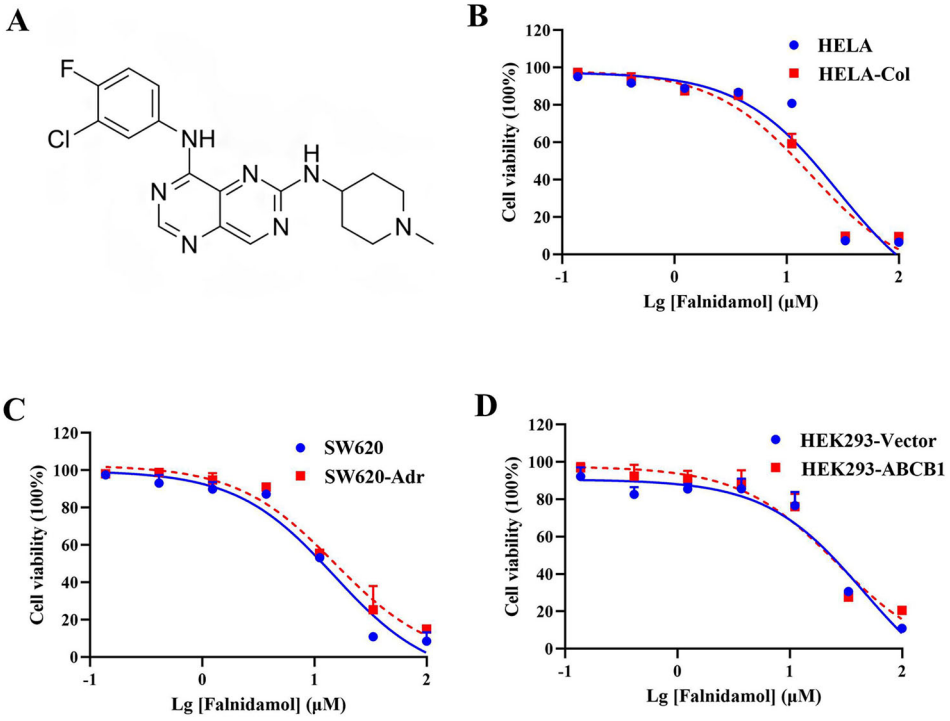

Fig. 1 The cytotoxicity of falnidamol in different cancer cell lines. (**A**) The structure of falnidamol. (**B-D**) The cell viability curve of HELA and HELA-Col cells, SW620 and SW620-Adr cells, and HEK293-Vector and HEK293-ABCB1 cells. Error bars indicate SD

into 96-well plates with minimal surface adhesion, and cultured for further 7 days. Finally, the diameter of the 3D spheroid was measured.

Western blot and immunofluorescence assays

Western blot was performed as our previous report [17]. Briefly, HELA-Col cells were treated with different concentrations of falnidamol for different times, and lysed. Subsequently, proteins were transferred to PVDF membrane after SDS-PAGE. After sequential incubation of the membrane with primary antibody (1:1000) and secondary antibody (1:1000), the protein bands were measured using an ECL kit. Immunofluorescence was carried out as previously described [18]. In short, HELA-Col cells were seeded in a confocal dish, and treated with 5 μM falnidamol for 0–72 h. Cells were fixed in 4% formaldehyde for 20 min and incubated overnight with anti-ABCB1 antibody (1:250). Subsequently, cells were incubated with a secondary antibody (1:500) and DAPI and photographed with a confocal microscopy.

Doxorubicin accumulation and efflux assays

Doxorubicin accumulation assay was assessed as in previous study [19]. In brief, HELA and HELA-Col cells were seeded in 6-well plates (1×10^6 cells per well) for 24 h, and then treated with 5 μM falnidamol or verapamil for 2 h. Subsequently, 10 μM doxorubicin was added and incubated for another 2 h. Finally, cells were collected for measurement by flow cytometry. Doxorubicin efflux assay was evaluated as reported previously [20]. In short, HELA and HELA-Col cells were seeded in 6-well plates (1×10^6 cells per well) for 24 h, and then treated with 10 μM doxorubicin for 30 min. Afterwards, cells were incubated with 5 μM falnidamol or verapamil, and harvested at various time points for flow cytometry examination.

ATPase assay

The ATPase activity of ABCB1 was tested as described in previous study [8]. Briefly, cell membranes overexpressed ABCB1 were incubated with various concentrations of falnidamol for 5 min. Subsequently, the ATPase reaction was initiated by the addition of 5 mM Mg^{2+} ATP. At last, luminescence signals of Pi were detected after 30 min incubation. The variations in relative light units were calculated by comparing the samples treated with Na_3VO_4 with those treated with falnidamol.

Docking analysis

Docking analysis was performed using Maestro v11.1 (Schrödinger LLC, MA, USA) as previously described [16]. In short, human ABCB1 protein model (7A69) was downloaded from RCSB Protein Data Bank, and the molecular structure of falnidamol was downloaded from PubChem. After receptor/ligand preparation, glide XP docking was performed, followed by induced fit docking using the default protocol.

Cellular thermal shift assay

The cellular thermal shift assay was carried out as mentioned previously [21]. HELA-Col cells (2×10^7 cells) were washed, resuspended in PBS, and then the cell suspension was frozen-thawed five times with liquid nitrogen. Protein samples were harvested by centrifugation at 10,000 × g for 10 min at 4 ℃, and then treated with 50 μM of falnidamol or DMSO for 30 min at room temperature, respectively. Next, the same quantities of protein were taken and treated at various temperatures for 3 min. Finally, protein samples were analyzed by western blot.

In vivo assay

In short, HELA-Col cells (1×10^7 cells per mouse) were inoculated subcutaneously in nude mice. Subsequently, the mice were divided into four groups and given one of the following treatments every 3 days: (1) normal saline control group (30 mL/kg, *p.o*); (2) falnidamol group (30 mg/kg, *p.o.*); (3) paclitaxel group (15 mg/kg, i.p.); (4) combination of falnidamol (30 mg/kg, *p.o.*) and paclitaxel group (15 mg/kg, i.p.). The tumor's growth was monitored every 3 days, and the tumor's volume was determined by the following formula: volume (mm^3) = length × $width^2\times0.5$. After the mice were executed by deep anesthesia, and the tumors were harvested and weighed, while the liver and kidney were collected for histopathological examination.

Histopathological examination

Histopathological examination was performed as mentioned previously [22]. In short, tissue samples were fixed with 10% neutral buffered formalin. After paraffin embedding, the samples were sectioned at approximately 4 μm thick and stained with hematoxylin-eosin (HE).

Statistical analysis

Data were analyzed by ANOVA using SPSS 18.0 software. Data were expressed as mean ± SD, with $P<0.05$ representing statistical significance. The experiments were repeated three times.

Results

Falnidamol increases chemotherapeutic sensitivity in ABCB1-overexpressing cells

At first, MTT assay was performed to select the concentration of falnidamol that had no significant cytotoxic effect. The results suggested that more than 80% of the cells, including HELA and HELA-Col cells, SW620 and SW620-Adr cells, and HEK293Vector and HEK293-ABCB1 cells, survived at concentrations of falnidamol below 5 μM (Fig. 1B-D). Thus, 1, 2.5 and 5 μM of falnidamol were used in the following assays.

Subsequently, the reversal effect of falnidamol on MDR was assessed. As shown in Fig. 2, drug-resistant cells that overexpressed ABCB1 (HELA-Col and SW620-Adr) exhibited significantly higher IC_{50} for ABCB1-substrate drugs, including doxorubicin and paclitaxel, than

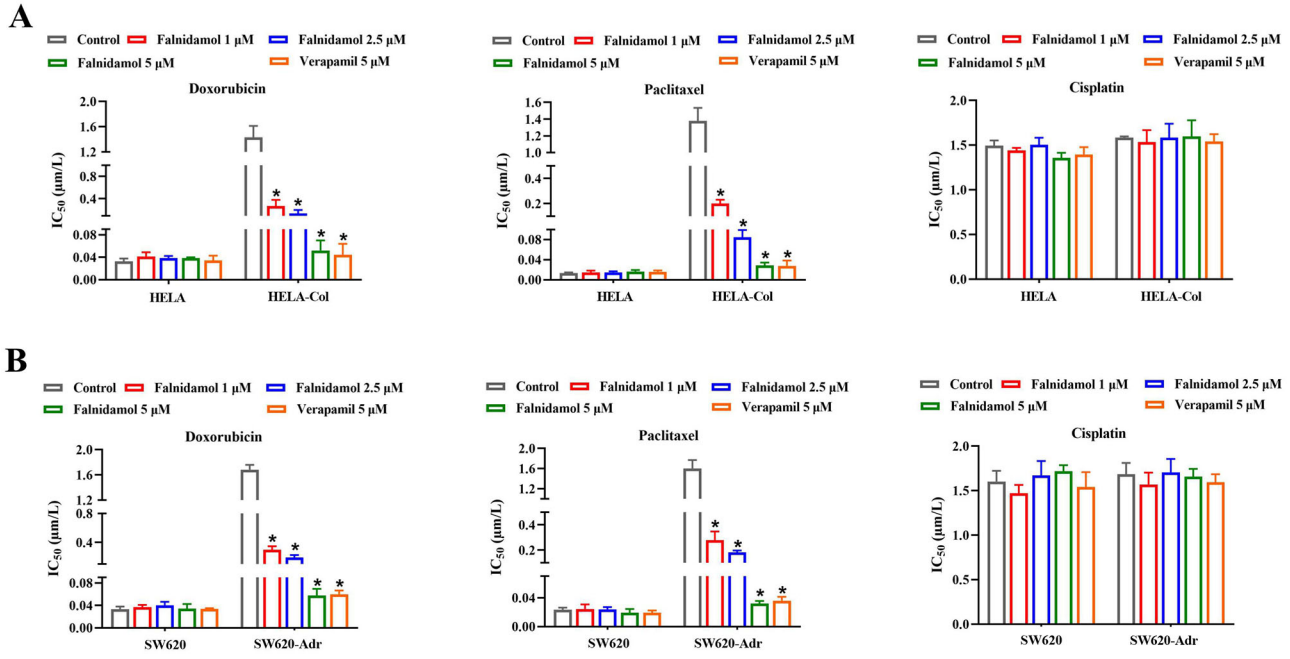

Fig. 2 Falnidamol increases chemotherapeutic sensitivity in MDR cells. (**A-B**) IC_{50} of paclitaxel, doxorubicin and cisplatin in HELA and HELA-Col cells, SW620 and SW620-Adr cells with or without inhibitor for 72 h. Verapamil at 5 μM was utilised as a positive reversal agent. Error bars indicate SD. *$P<0.05$

sensitive cells (HELA and SW620). Interestingly, in MDR cells that overexpressed ABCB1, falnidamol significantly reduced the IC_{50} of chemotherapeutic drugs (doxorubicin and paclitaxel, not cisplatin) in a dose-dependent manner ($P<0.05$), with the reversal effect of 5 μM falnidamol being comparable to that of an equivalent concentration of verapamil. However, no similar reversal effect was observed in their corresponding sensitive cells. Meanwhile, HEK293-ABCB1 cells also showed significantly higher sensitivity to substrate chemotherapeutic drugs after falnidamol treatment (Fig. 3A-C). In addition, our results showed that falnidamol did not reverse ABCG2-mediated MDR (Fig. 3D). The above results suggested that falnidamol could specifically reverse ABCB1-mediated MDR.

Falnidamol improves chemotherapeutic drug's ability to inhibit colony and 3D microsphere formation in HELA-Col cells

To confirm the reversal effect of falnidamol in ABCB1-mediated MDR, colony formation assay was performed. The data showed that falnidamol significantly increased paclitaxel's inhibitory effect on HELA-Col cell colony formation (Fig. 4A, $P<0.05$). Furthermore, 3D microsphere analysis suggested that falnidamol in combination with paclitaxel significantly suppressed the growth of 3D microspheres compared to the paclitaxel alone (Fig. 4B, $P<0.05$). These results proved that falnidamol could reverse ABCB1-mediated MDR.

Falnidamol reverses ABCB1-mediated MDR in vivo

To further validate the ability of falnidamol to reverse MDR in vivo, HLEA-Col cells were injected subcutaneously into nude mice to create a xenograft model. As shown in Fig. 5A-C, the inhibitory effect of falnidamol or paclitaxel alone on tumour growth was not significant, whereas the combination of falnidamol and paclitaxel inhibited tumour growth more effectively, with tumour volume and weight significantly lower than that of the control group ($P<0.01$). Importantly, there was no significant weight loss during treatment (data not shown). HE analysis revealed that no apparent lesions were observed in the liver and kidney of the mice in the combination group compared with the control group (Fig. 5D). These data suggested that falnidamol could reverse ABCB1-mediated MDR in vivo with no additional toxicity.

Falnidamol does not alter ABCB1 expression or cellular localization

Considering that mechanisms for reversing MDR include down-regulation of ABCB1 expression or alteration of its localization in the cell [23, 24], western blot and immunofluorescence assays were conducted to exclude these possible causes. As depicted in Fig. 6A-B, treatment with

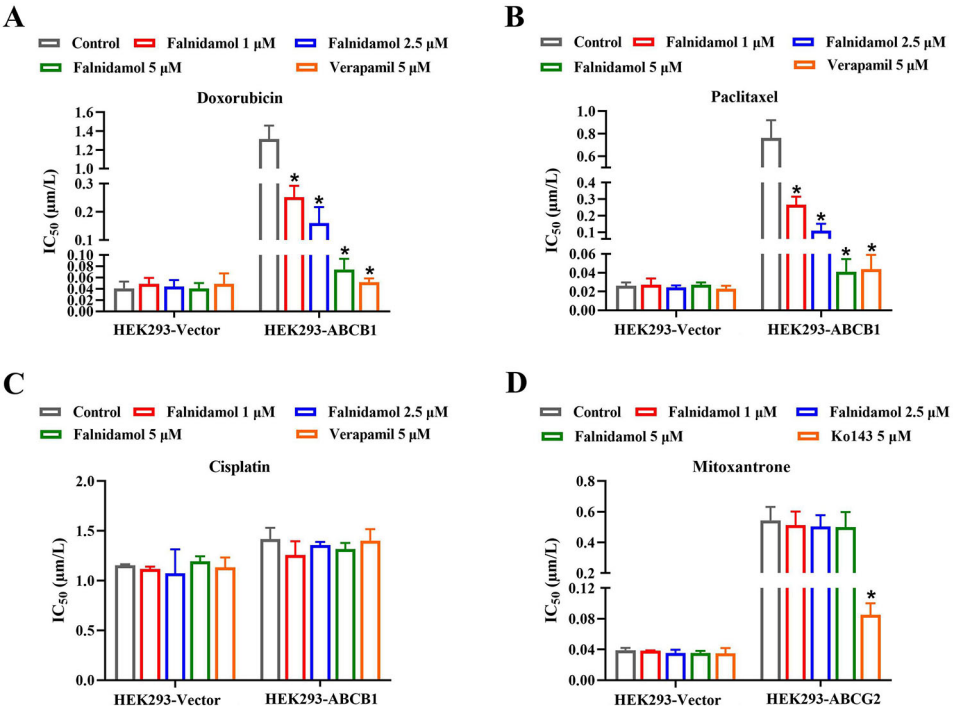

Fig. 3 Falnidamol increases chemotherapeutic sensitivity in HEK293-ABCB1 cells but not HEK293-ABCG2 cells. (**A-C**) IC_{50} of paclitaxel, doxorubicin and cisplatin in HEK293-Vector and HEK293-ABCB1 cells with or without inhibitor for 72 h. (**D**) IC_{50} of mitoxantrone in HEK293-Vector and HEK293-ABCG2 cells with or without inhibitor for 72 h. Verapamil or Ko143 at 5 μM were utilised as a positive reversal agent. Error bars indicate SD

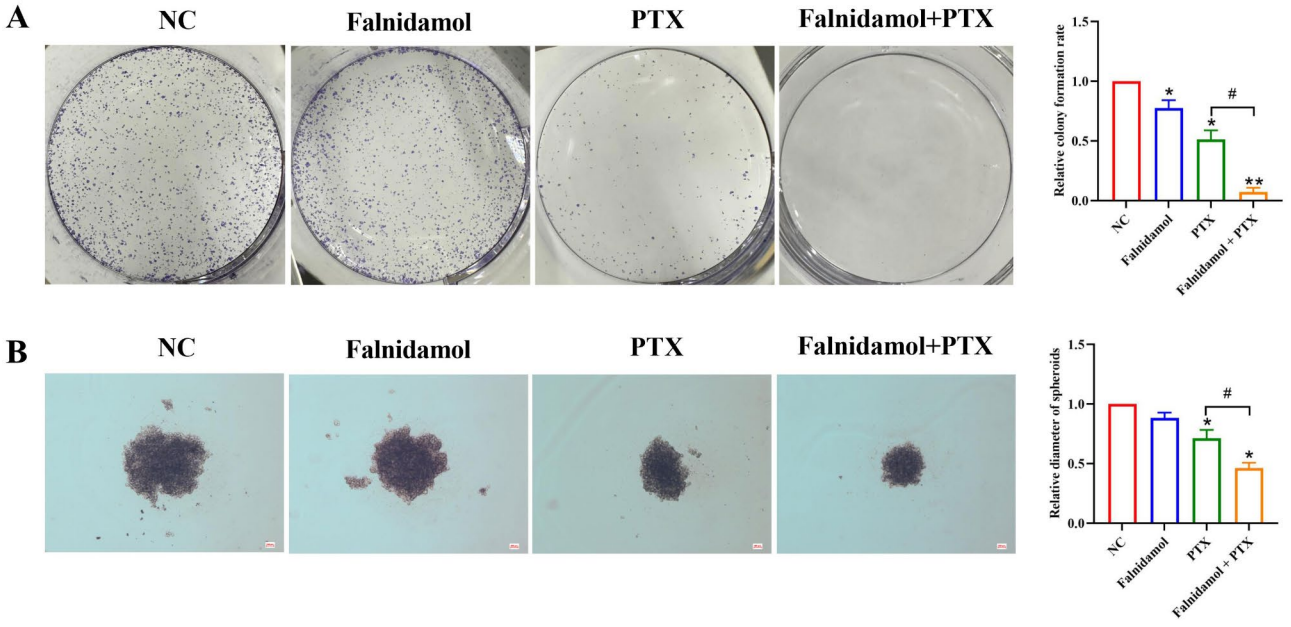

Fig. 4 Falnidamol improves chemotherapeutic drug's ability to inhibit colony and 3D microsphere formation in HELA-Col cells. (**A**) Colony formation of HELA-Col cells following treatment with falnidamol, paclitaxel and their combined. (**B**) 3D microsphere formation of HELA-Col cells following treatment with falnidamol, paclitaxel and their combined. PTX: paclitaxel. Error bars indicate SD. *$P<0.05$, **$P<0.01$ vs. NC group. $^{\#}P<0.05$ vs. PTX group

5 μM of falnidamol for 72 h did not affect the protein expression or cellular localization of ABCB1. These data revealed that falnidamol had no effect on ABCB1 expression or cellular localization.

Falnidamol has no effect on AKT or ERK pathways at MDR reversing concentration

Previous studies have shown that the anti-cancer effect of chemotherapeutic drugs is enhanced by blocking the AKT and ERK pathways [25], the effect of falnidamol on AKT and ERK phosphorylation was examined in HELA-Col and HELA cells. As shown in Fig. 7, the phosphorylation of AKT and ERK was significantly inhibited by 15 μM of falnidamol, but it remained unchanged by treating with 5 μM (MDR reversing concentration) of falnidamol for up to 72 h, indicating that AKT and ERK pathways are not involved in the reversal of ABCB1-mediated MDR by falnidamol.

Falnidamol enhances the intracellular accumulation of doxorubicin and suppresses the efflux of doxorubicin in ABCB1-overexpressing cancer cells

In view of the above data showing that falnidamol did not alter ABCB1 expression and cellular localization or affect the AKT/ERK pathway, we speculated that falnidamol could suppress ABCB1 activity. Therefore, the effect of falnidamol on intracellular doxorubicin accumulation and efflux was assessed by flow cytometry. The data suggested that falnidamol increased the accumulation of doxorubicin in drug-resistant HELA-Col cells, with an effect comparable to that of the positive control verapamil group, whereas the accumulation of doxorubicin in drug-sensitive HELA cells was unchanged (Fig. 8A-B). Further efflux experiment showed that falnidamol significantly impeded doxorubicin efflux in resistant HELA-Col cells (Fig. 8C-D, $P<0.05$), but not in sensitive HELA cells. These data indicate that falnidamol can inhibit ABCB1 activity, which suppresses doxorubicin efflux and thus increases doxorubicin accumulation in drug-resistant cancer cells.

Falnidamol suppresses the ABCB1 ATPase activity

The above results showed that the reversal of the effects of falnidamol was associated with inhibiting the ABCB1 efflux function, possibly affecting ABCB1 ATPase activity. Thus, to assess whether falnidamol affects ABCB1 ATPase activity, ABCB1-mediated ATP hydrolysis was tested at different concentrations of falnidamol. The result showed that falnidamol concentration-dependently inhibited ABCB1 ATPase activity, reducing it to approximately 0.3-fold of the basal activity at 15 μM falnidamol (Fig. 9). This data indicates that falnidamol might inhibit ABCB1 ATPase activity through interaction with the drug-binding domains.

Falnidamol binds directly to ABCB1

To investigate the interaction between falnidamol and ABCB1, docking analysis was performed. The data revealed that falnidamol could bind tightly to ABCB1 (Fig. 10A-B), and the affinity value for the docking was

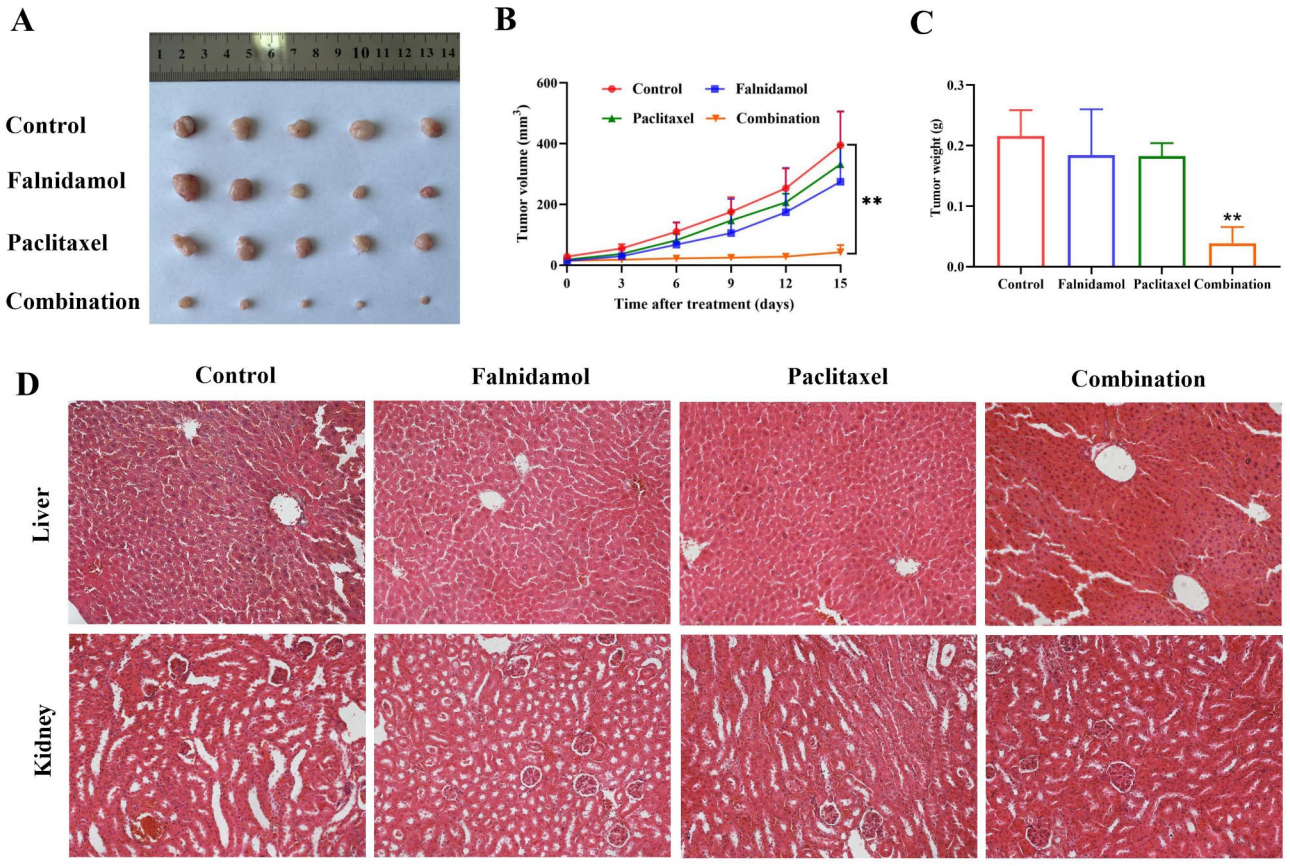

Fig. 5 Falnidamol reverses ABCB1-mediated MDR in vivo. HELA-Col cells were injected subcutaneously into the nude mice, and then the nude mice treated with normal saline (30 mL/kg/3 days), falnidamol (30 mg/kg/3 days), paclitaxel (15 mg/kg/3 days), and falnidamol (30 mg/kg/3 days) plus paclitaxel (15 mg/kg/3 days) for 15 days. (**A**) Photographs of tumors. (**B**) Tumor volume. (**C**) Tumor weight. (**D**) HE analysis of liver and kidney. Error bars indicate SD. $^{**}P < 0.01$

−8.853 kcal/mol. Ligand-receptor interaction details are presented in Fig. 10C-D. The hydrophobic interaction was the main mechanism by which falnidamol bound to ABCB1. Falnidamol located in a hydrophobic pocket made up of Ile306, Tyr307, Tyr310, Phe728, Phe336, Leu339, Ile340, Ala342, Phe343, Val982, Phe983, and Met986. Additionally, falnidamol was further stabilized by pi-pi stacking interactions generated with Tyr307 and Phe728, and hydrogen bonds generated with Gln725.

To further validate the interaction of falnidamol with ABCB1, a cellular thermal shift assay was carried out. The results showed that falnidamol significantly increased the thermal stability of ABCB1 compared to the control group, suggesting that falnidamol directly bound to ABCB1 in HELA-Col cells (Fig. 10E).

Discussion

ABCB1 overexpression in tumor cells is well recognized to cause MDR and consequently chemotherapeutic failure [16, 26]. As a result, it is crucial to find drugs that can effectively counteract ABCB1-mediated MDR. Unfortunately, due to suboptimal effectiveness and various unfavourable side effects associated with the candidate agents, FDA has not yet approved any ABCB1 inhibitors [15, 27, 28]. Recent research has demonstrated that combining chemotherapeutic medicines and TKIs, including imatinib, sorafenib, gefitinib, sapitinib, erlotinib, can successfully overcome ABCB1-mediated MDR [12, 13]. Falnidamol, also known as BIBX1382, is a TKI of EGFR for the treatment of cancer in phase 1 clinical studies [14]. This study assessed if falnidamol could act as an inhibitor of ABCB1, reversing ABCB1-mediated MDR in drug-resistant cancer cells.

Here, our data proved that falnidamol significantly reverses MDR mediated by ABCB1. To begin, an MTT test was done to determine the toxicity of falnidamol and the non-toxic concentration for the reversal research. According to the findings, 1, 2.5 and 5 μM of falnidamol were employed in the reversal research. our data revealed that falnidamol significantly reverses ABCB1-mediated MDR, equivalent to the reversal impact of verapamil, a recognized ABCB1 inhibitor [29]. Importantly, the effect of falnidamol in reversing MDR was confirmed by in vitro assays, including MTT, colony and 3D microsphere

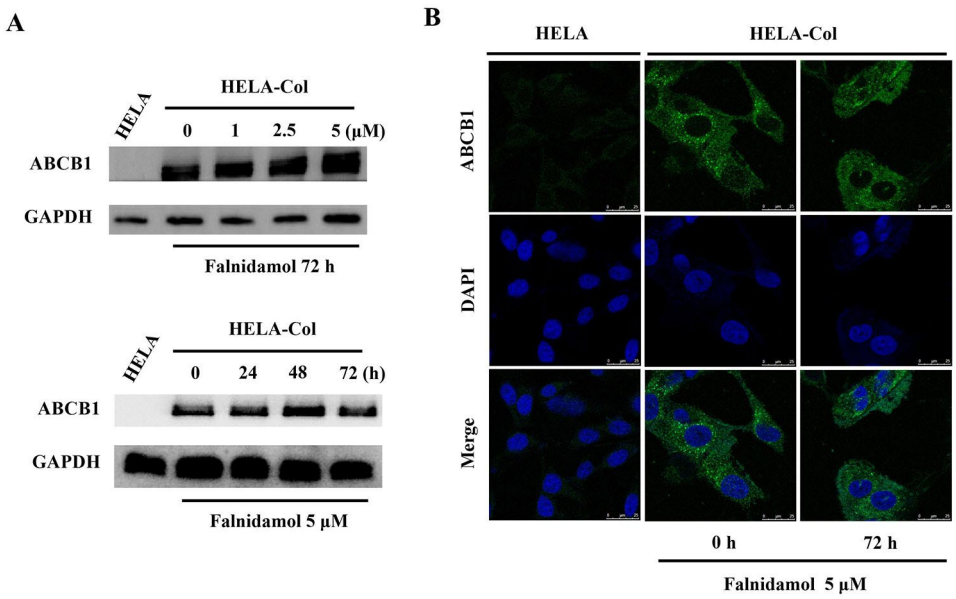

Fig. 6 Falnidamol does not alter ABCB1 expression or cellular localization. (**A**) The effect of falnidamol on ABCB1 expression in HELA and HELA-Col cells. (**B**) The effect of falnidamol on ABCB1 localization in HELA and HELA-Col cells. Error bars indicate SD. Blue: nuclei. Green: ABCB1. Full-length blots are presented in Supplementary Fig. 1

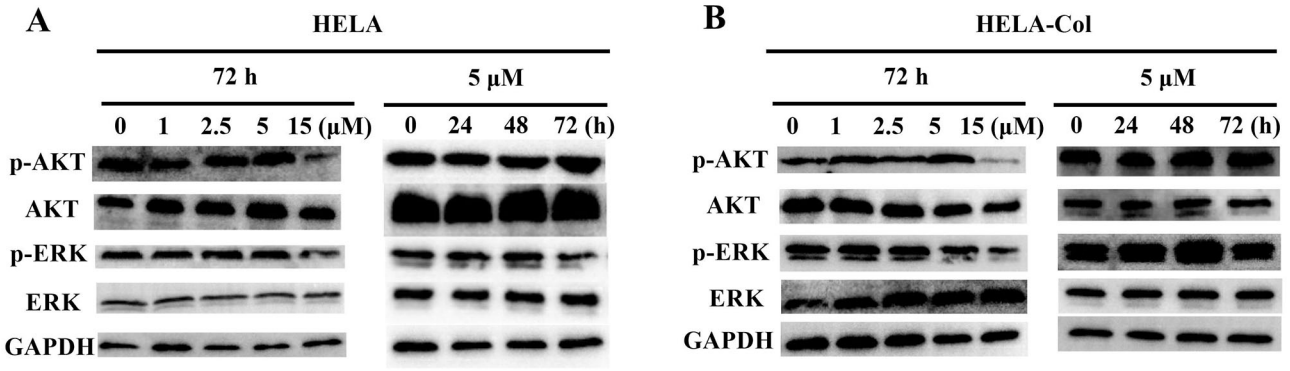

Fig. 7 Falnidamol have no effect on AKT or ERK pathways at MDR reversing concentration. (**A-B**) The total and phosphorylation level of AKT and ERK were tested by western blot analysis in HELA and HELA-Col cells. Full-length blots are presented in Supplementary Fig. 2

formation, and in vivo experiments. These data suggest that falnidamol effectively reverses ABCB1-mediated MDR. In addition, although histopathologic testing indicated no acute toxicity from falnidamol, there are still limitations in its safety evaluation, such as the absence of chronic toxicity trials. Future studies will focus on assessing the safety of falnidamol in reversing ABCB1-mediated MDR.

Previous studies reported that that the reversal effect could be attributable to ABCB1 down-regulation or altered subcellular localization, so western blot and immunofluorescence tests were undertaken to evaluate these possibilities [23, 24]. Our results showed that the protein expression or subcellular localization of ABCB1 was not affected by 72 h treatment with 5 μM falnidamol. Therefore, falnidamol's reversal impact is not mediated by changing ABCB1's subcellular location or its protein expression. Further research is required to determine if falnidamol can impact ABCB1 protein levels and subcellular location at higher concentrations or longer time. Moreover, previous research has also demonstrated that inhibiting the AKT and ERK pathways may improve the anti-cancer effect of chemotherapeutic medicines [25, 30], so the effect of falnidamol on AKT and ERK phosphorylation was investigated. The data revealed that, when treated with effective MDR reversal concentration (5μM), falnidamol had no significant effect on the phosphorylation of AKT and ERK, suggesting that falnidamol's ability to reverse ABCB1-induced MDR is not mediated by the AKT or ERK pathways.

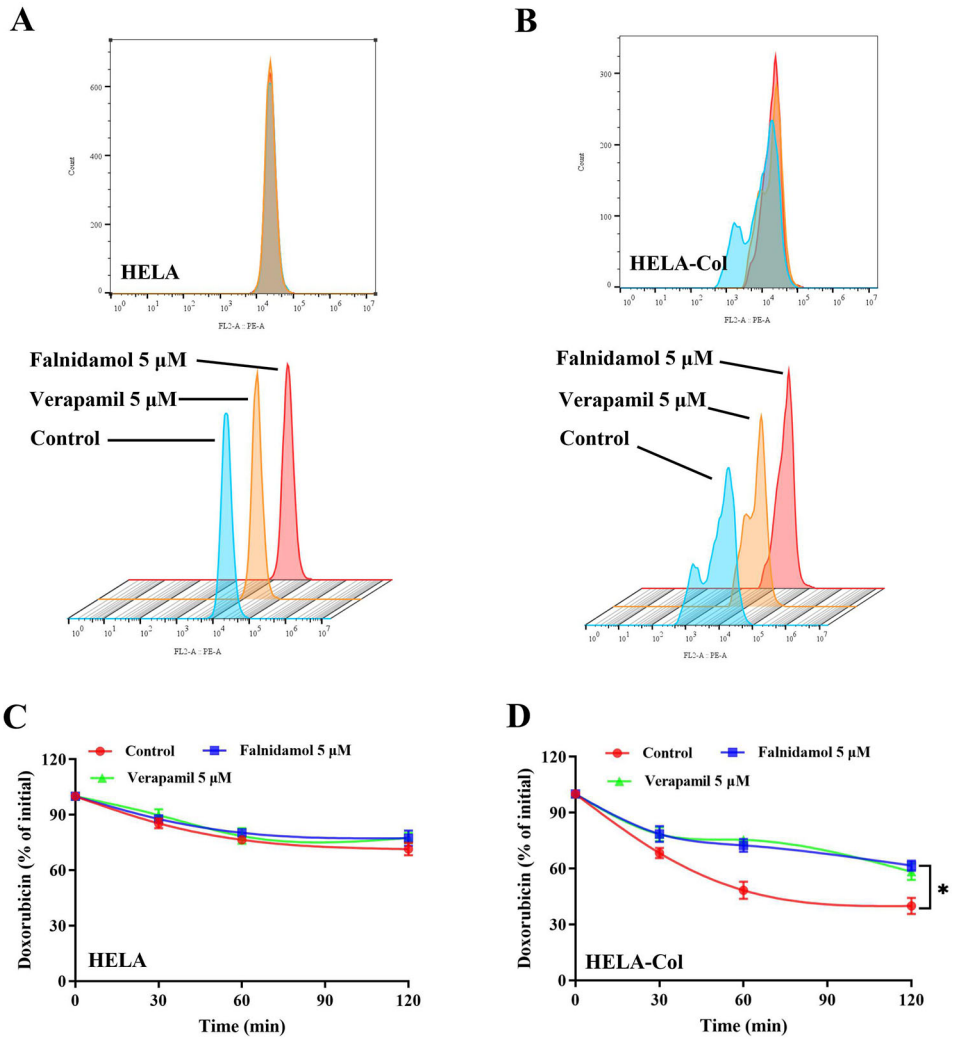

Fig. 8 Falnidamol enhances intracellular accumulation of doxorubicin and suppresses the efflux of doxorubicin in ABCB1-overexpressing cancer cells. (**A-B**) The effect of falnidamol on the accumulation of doxorubicin in HELA and HELA-Col cells. (**C-D**) The effect of falnidamol on the efflux of doxorubicin in HELA and HELA-Col cells. *$P < 0.05$

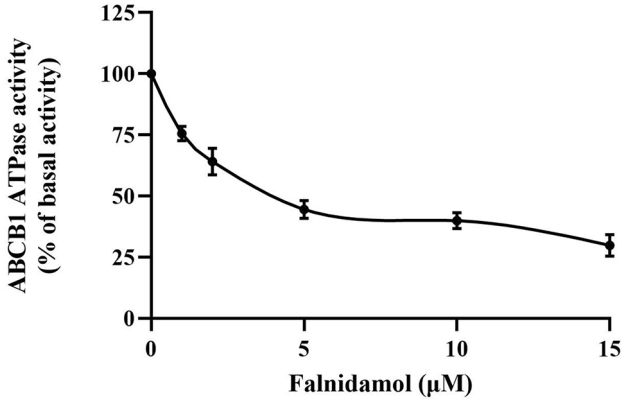

Fig. 9 Falnidamol suppresses the ABCB1 ATPase activity. Error bars indicate SD

To further understand how falnidamol reverses ABCB1-mediated MDR, accumulation and efflux assays were undertaken. Our findings implied that falnidamol markedly enhances intracellular accumulation of chemotherapeutic agents in drug-resistant cells, possibly due to the inhibition of ABCB1 efflux function. The data were consistent with other studies, in which they reported that some TKIs could reverse ABCB1-mediated MDR [31, 32]. Furthermore, the ABCB1 is well known for using ATP hydrolysis energy to remove xenobiotics [33, 34]. Thus, we investigated if falnidamol might impact ABCB1's ATPase activity. Out data showed that falnidamol decreased ABCB1's ATPase activity in a concentration-dependent manner, with a maximum inhibition of 0.3-fold, implying that falnidamol might interact with the drug-binding region of ABCB1.

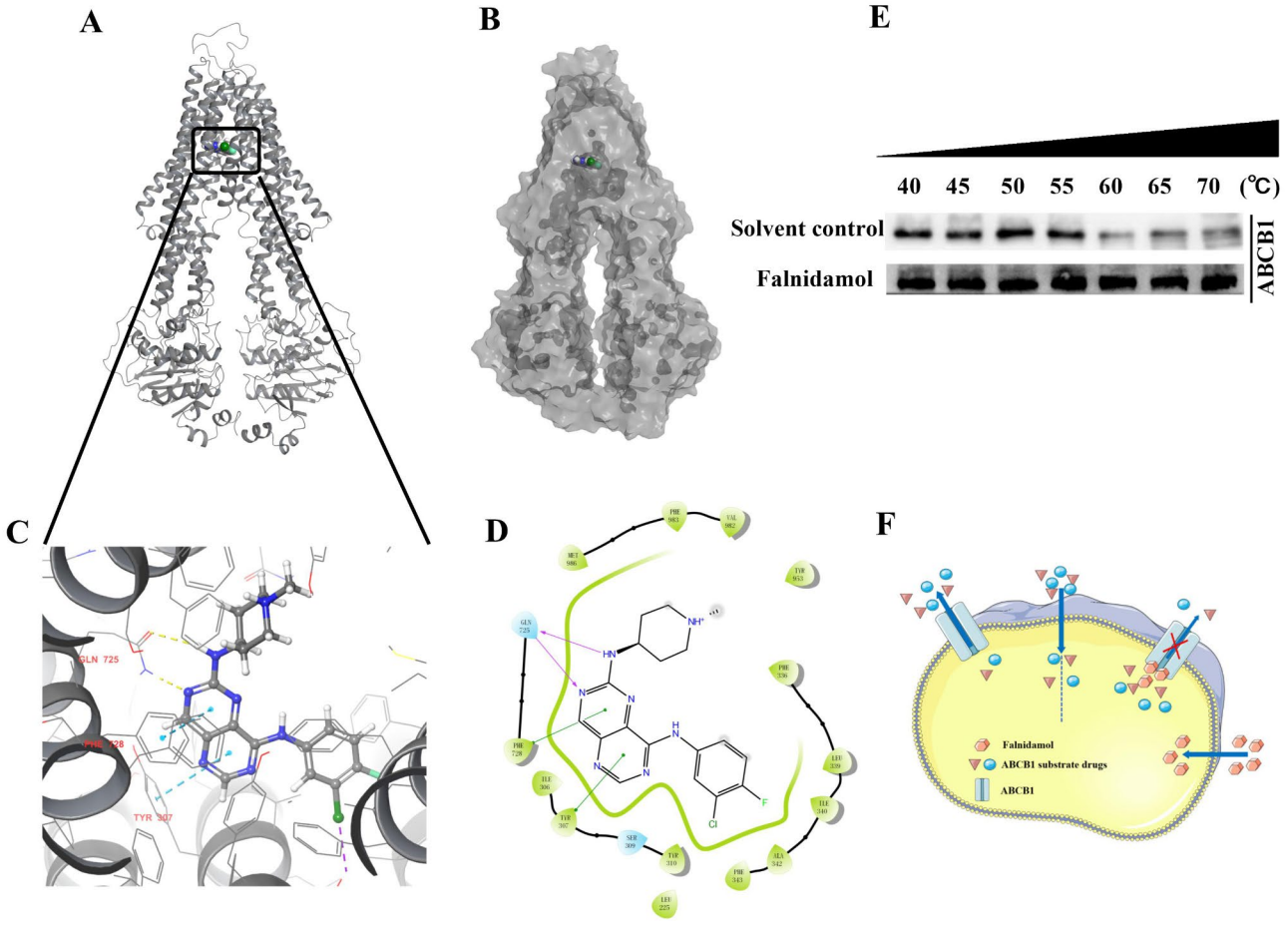

Fig. 10 Falnidamol binds directly to ABCB1. (**A**) Overview of falnidamol binding pattern with ABCB1. (**B**) Docking model showing ABCB1 and ligand surfaces. (**C**) Interaction details between falnidamol and ABCB1 binding pocket. ABCB1 is shown as a grey band and falnidamol is shown as colored sticks. pi-pi stackings, hydrogen bonds and halogen bonds were displayed as cyan, yellow and purple dash lines respectively. (**D**) 2D falnidamol-ABCB1 interaction. Amino acids were displayed as color bubbles. pi-pi stacking interactions and hydrogen bonds are indicated with green and purple lines respectively. (**E**) The interaction of falnidamol with ABCB1 was assessed by cellular thermal shift assay. (**F**) Proposed mechanism for falnidamol reversing ABCB1-mediated MDR. Full-length blots are presented in Supplementary Fig. 3

It is widely known that docking analysis has been used extensively to study the interaction between molecules [35, 36]. Therefore, docking analysis was performed to investigate the interaction between falnidamol and ABCB1. The results revealed that the affinity value for the docking of falnidamol was −8.853 kcal/mol, suggesting that falnidamol could interact with the ABCB1 drug-binding site. The strong affinity of falnidamol for the ABCB1 drug-binding site could be attributed to hydrophobic interactions with ABCB1 residues, pi-pi stacking interactions, and hydrogen bonds. Besides, our finding of cellular thermal shift assay showed that falnidamol significantly increased the thermal stability of ABCB1 compared to the control group. It is widely acknowledged that thermal stability is improved when drugs are bound directly to relevant proteins [16, 21]. Thus, our data provided further evidence that falnidamol bound directly to the ABCB1 drug-binding pocket. Based on previous studies, we speculate that the high binding energy of falnidamol allows it to remain bound to the ABCB1 protein without being pumped out of the cells [28, 37]. Consequently, falnidamol can inhibit ATPase activity of ABCB1 and act as a pump inhibitor. Another hypothesis is that falnidamol's interaction with ABCB1 protein causes a conformational shift that suppresses ABCB1 function [28]. Nevertheless, until the actual falnidamol-ABCB1 binding structure is identified, the precise mode of interaction between falnidamol and ABCB1 will remain unknown.

Conclusions

In conclusion, our study suggests that falnidamol, as a highly potent and specific active ABCB1 transporter inhibitor, can inhibit the chemotherapeutic drugs efflux function of ABCB1 protein by interacting with the substrate binding site of ABCB1, thereby increasing the

intracellular concentration of drugs and thus effectively reversing ABCB1-mediated MDR (Fig. 10F). This study finding warrants further evaluation in clinical studies.

Supplementary Information

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Supplementary Material 1

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Not applicable.

Author contributions
Conceptualization, Weiguo Feng and Zhifang Pan; methodology, Baojie Liu; software, Junbao Xu; validation, Yongzheng Lu and Ruihui Lin; data curation, Zilin Shang; writing—review and editing, Weiguo Feng; supervision, Xinyu Hou; project administration, Xulong Shao; funding acquisition, Weiguo Feng and Tao Yu.

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Data availability
The datasets used during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval
The animal study protocol was approved by the Animal Ethics Committee of Shandong Second Medical University (2021SDL378, Weifang, China). The guidelines include animal care, housing, and procedures that minimize the suffering and distress of animals. Strict adherence to the ARRIVE guidelines was observed throughout all of the procedures carried out during the research to ensure that the animals received the best possible care. Moreover, all procedures were conducted ethically and humanely at all times.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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