

The Novel ROCK Inhibitor Fasudil Derivative Fasudil-D-6h Prevents Tumour Progression by Regulating the Adherens Junction Signalling Pathway in Triple-Negative Breast Cancer

Jinghui Liang¹, Mu Tang², Lieliang Wang²

¹Department of Comprehensive Radiotherapy, Jiangxi Cancer Hospital & Institute, Nanchang City, Jiangxi Province, People's Republic of China;

²Department of Breast Surgery, Jiangxi Cancer Hospital & Institute, Nanchang City, Jiangxi Province, People's Republic of China

Correspondence: Lieliang Wang, Department of Breast Surgery, Jiangxi Cancer Hospital & Institute, 518 East Beijing Road, 518 East Beijing Road, Nanchang City, Jiangxi Province, People's Republic of China, Email zoya1302@163.com

Background: Triple-negative breast cancer (TNBC) is an aggressive malignancy with limited therapeutic options. Rho-associated coiled-coil containing protein kinase (ROCK) signaling is a promising target, known to drive tumor progression through cytoskeletal remodelling and epithelial-mesenchymal transition (EMT). However, its role in disrupting epithelial integrity via adherens and tight junctions in TNBC remains underexplored. Fasudil-D-6h is a novel derivative of the clinically approved ROCK inhibitor fasudil. This study investigates the anti-tumor efficacy of fasudil-D-6h and its novel mechanism of action in TNBC.

Methods: The effects of fasudil-D-6h and the reference inhibitor HA-1100 on cell proliferation (CCK-8) and apoptosis (flow cytometry) were assessed in MDA-MB-231 and MCF-7 cells. Transcriptome sequencing of fasudil-D-6h-treated MDA-MB-231 cells identified differentially expressed genes (DEGs) and enriched pathways. A protein-protein interaction (PPI) network was constructed. The in vivo efficacy was evaluated in a nude mouse model of subcutaneous MDA-MB-231 tumors. qRT-PCR and Western blotting validated the expression of ROCK1/2 and key adherens junction pathway components (Cdc42, Rac3, Src, ZO-1, Occludin, Claudin-1).

Results: Fasudil-D-6h significantly inhibited proliferation and induced apoptosis in TNBC cells. Transcriptomic analysis revealed 8,092 DEGs, with significant enrichment in the adherens junction pathway. Accordingly, fasudil-D-6h treatment in vitro and in vivo significantly downregulated ROCK1/2 and robustly upregulated the expression of CDC42, RAC3, SRC, ZO-1, OCCLUDIN, and CLAUDIN-1. In the mouse model, fasudil-D-6h treatment led to a significant reduction in tumor mass and volume.

Discussion: Our findings demonstrate that the novel ROCK inhibitor fasudil-D-6h exerts potent anti-tumor effects in TNBC. Its mechanism of action is distinctively linked to the restoration of genes critical to adherens and tight junction integrity. Given its derivation from the clinically viable fasudil, fasudil-D-6h presents a strong translational potential as a novel therapeutic agent for TNBC.

Keywords: ROCK inhibitor, fasudil-D-6h, triple-negative breast cancer, adherens junction, breast cancer signalling pathway

Introduction

Breast cancer remains the most frequently diagnosed cancer and a leading cause of cancer-related mortality among women worldwide.¹ Its clinical management is profoundly influenced by tumor molecular subtypes, which are classified based on the expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2).² These biomarkers guide the use of targeted therapies, such as endocrine agents for ER/PR-positive disease and HER2-targeted antibodies for HER2-positive disease, significantly improving patient outcomes.³

In stark contrast to these subtypes, triple-negative breast cancer (TNBC), defined by the absence of ER, PR, and HER2 amplification, represents approximately 15–20% of all breast cancers and is clinically the most aggressive.⁴ The lack of actionable therapeutic targets renders TNBC patients unresponsive to endocrine and HER2-targeted therapies, leaving conventional chemotherapy as the primary standard of care.⁵ While initially often responsive, TNBC tumors

frequently develop rapid chemoresistance, leading to high rates of early recurrence, visceral metastasis, and significantly poorer overall survival compared to other subtypes.⁶ This stark therapeutic gap and dire prognosis underscore the critical need to identify novel molecular drivers and develop effective targeted therapies for TNBC.

The Rho-associated coiled-coil containing protein kinases (ROCK1 and ROCK2), key effectors of the Rho GTPase pathway, are master regulators of fundamental cellular processes, including actomyosin contractility, cell migration, and invasion.⁷ It is this ROCK-mediated contractile force that forms the critical mechanistic link to the disruption of epithelial integrity. Adherens junctions (AJs), which are anchored to the cortical actin cytoskeleton via α - and β -catenin, are primary targets of this force. Excessive ROCK activation applies mechanical stress to E-cadherin complexes, promoting their endocytosis and lysosomal degradation, thereby dismantling the key structure for stable cell-cell adhesion.⁸ Furthermore, this contractile force disrupts epithelial homeostasis by directly compromising tight junctions (TJs). TJs, located apically to AJs, are crucial for creating a paracellular barrier. ROCK-mediated contraction can lead to the displacement and internalization of key TJ proteins, including Occludin, Claudins, and their cytoskeletal linker ZO-1.^{9,10} The frequent downregulation of these proteins in human tumors is a testament to this phenomenon. Consequently, the loss of junctional integrity increases epithelial permeability, disrupts cell polarity, and creates a permissive environment for tumor cell dissemination.¹¹ Therefore, while the role of ROCK in promoting EMT is well-established, its function in directly dismantling AJs and TJs through the fundamental mechanism of actomyosin hyper-contraction represents a compelling and complementary therapeutic target. Inhibiting ROCK is predicted not only to curb cell motility but also to actively restore epithelial stability by preserving these critical junctional complexes.¹²

Pharmacological inhibition of ROCK has been validated as a viable anti-cancer strategy in preclinical models using compounds like Y-27632.¹³ Importantly, fasudil is the only ROCK inhibitor approved for clinical use (for cerebral vasospasm), demonstrating a established safety profile and the inherent “druggability” of this pathway.¹⁴ Repurposing fasudil or developing next-generation derivatives with improved potency, specificity, or pharmacokinetic properties is a logical strategy for expanding its application to oncology. In our previous work, we developed fasudil-D-6h, a novel chemical derivative designed to enhance anti-tumor efficacy.

In our research, we hypothesize that hyperactive ROCK signaling drives TNBC progression by disrupting junctional integrity and that fasudil-D-6h exerts potent anti-tumor effects by uniquely targeting this mechanism. To test this, we employed an unbiased transcriptomic approach to delineate the global gene expression changes induced by fasudil-D-6h in TNBC cells. We further aimed to validate its functional role in restoring the expression and localization of key junctional proteins (Occludin, Claudins, ZO-1) and to assess its efficacy in suppressing tumorigenesis both *in vivo* and *in vitro*. This study is the first to characterize fasudil-D-6h, aiming to establish a novel link between ROCK inhibition, junctional protein restoration, and the suppression of TNBC progression, thereby identifying a promising new therapeutic strategy.

Materials and Methods

Cell Culture and Treatment

The TNBC cell lines MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection (ATCC; Maryland, USA). The cells were cultured in DMEM/F12 medium (Thermo, USA) containing 10% foetal bovine serum (FBS; Thermo, USA) in a humidified incubator at 37 °C and 5% CO₂. In addition, MCF-7 and MDA-MB-231 cells were treated with fasudil-D-6 h (0 μ M, 5 μ M or 10 μ M, synthesised in our laboratory)¹⁴ or HA-1100 (10 μ M, Catalogue No. S8208, Selleck) for 24 hours.

mRNA Sequencing

Post administration of a 0 μ M fasudil-D-6h dose (serving as the negative control) or a 10 μ M fasudil-D-6 h dosage, we gathered MDA-MB-231 cells and promptly submerged them in liquid nitrogen for preservation. Subsequently, we assessed the RNA levels in the specimens using a Qubit[®] RNA Assay Kit within a Qubit[®] 2.0 framework to conduct an initial quantification, followed by adjusting the concentration of the samples to a uniform 1 ng/ μ L. Evaluation of the insert size utilized the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA), confirming consistency with the anticipated parameters. Following this, precise quantification of the appropriate insert size was carried out employing

the TaqMan fluorescence probe in conjunction with the AB Step One Plus Real-Time PCR system, ensuring the library concentration exceeded 2 nM. These satisfactory libraries underwent sequencing using the Illumina/MGI technology, yielding single-end reads of 50 base pairs in length.

Differential Expression Analysis

The tumour-bearing nude mice were subsequently randomly divided into the following three groups ($n = X$ mice per group): peritumoral injection of 0.2 mL normal saline (NS) for the control group; peritumoral injection of 0.2 mL of 10 mg/kg fasudil-D-6h; and peritumoral injection of 0.2 mL of 20 mg/kg fasudil-D-6h. Given the premise that mRNA read counts adhere to a binomial distribution, DEGseq's methodology, rooted in the utilization of MA plots, enjoys widespread application in the assessment of differential gene expression. Each gene received a designated P value, which was modified using Benjamini and Hochberg's method to manage the rate of false discoveries. miRNAs that exhibited a q -value below 0.05 and an absolute $\log_2$ ratio of 1 or higher were classified as differentially expressed genes (DEGs).

GO Analysis

GO enrichment analysis was performed to functionally annotate genes associated with fasudil-D-6h treatment and to classify the cellular component (CC), biological process (BP), and molecular function (MF) domains. Functional annotation clustering and enzyme classification assessments were conducted using the DAVID bioinformatics resource (<http://david.abcc.ncifcrf.gov/>). For the GO enrichment analysis, the significantly enriched GO terms of the DEGs were compared with a control gene set consisting of 8092 DEGs, including 4123 upregulated genes and 3969 downregulated genes. After multiple test correlations, a BP P value <0.01 and an MF P value <0.1 were set as significance thresholds.

Protein–Protein Interaction (PPI) Network

The PPI network was constructed using the STRING database with DEGs, including genes related to the adherens junction signalling pathway, ROCK1 and ROCK2, which were selected as inputs (version 11.0; www.string-db.org). A PPI score (medium confidence) ≥ 0.4 was used as the cut-off value.

Cell Counting Kit-8 (CCK8) Assay

A CCK8 assay was also performed according to the manufacturer's instructions (WST-8, Japan). MCF-7 and MDA-MB-231 cells treated with fasudil-D-6h (0 μ M, 5 μ M or 10 μ M) or HA-1100 (10 μ M) for 24 hours and the corresponding controls were incubated in a 96-well plate at a density of 5000 cells/well. A total of 10 μ L of CCK8 reagent was added to each well at 24, 48, 72, and 96 h, after which the cells were incubated for 2 h. The absorbance at 450 nm was determined using a spectrophotometer.

Cell Apoptosis Assay

The induction of cell death in MCF-7 and MDA-MB-231 cells was evaluated using the Annexin V-FITC kit for apoptosis (BestBio, Shanghai, China), following the manufacturer's instructions. RPTECs were cultured in 6-well plates with a seeding density of 1.5×10^5 cells per well. At 48 hours post-transfection, the cells underwent digestion using trypsin that was free of 0.08% EDTA and were subsequently rinsed in chilled PBS twice. The use of propidium iodide (PI) and Annexin V conjugated with fluorescein isothiocyanate (FITC) facilitated the assessment of apoptotic and necrotic cell proportions. Apoptotic cells were quantified using a Beckman Coulter flow cytometer (KBB, CA, USA), and FlowJo software was employed for data analysis.

Subcutaneous Tumour Model of Breast Cancer

Female BALB/c nude mice (6 weeks old, 21.8 ± 1.6 g, purchased from Vital River Laboratory Animal Technology, Beijing, China) were subcutaneously injected with 4×10^6 MDA-MB-231 cells to cause tumorigenesis, while PBS was used as a negative control. The tumours were visible two weeks after inoculation and had a diameter of 6 mm 10 days after inoculation. The tumour-bearing nude mice were subsequently randomly divided into the following three groups: peritumoral injection of 0.2 mL normal saline (NS) for the control group; peritumoral injection of 0.2 mL of 10 mg/kg fasudil-D-6h; and

peritumoral injection of 0.2 mL of 20 mg/kg fasudil-D-6h. All the groups were injected every week from days 10 to 34. All mice were executed by cervical dislocation on day 40. The animal experiments were performed in accordance with the Chinese laws governing the use of medical laboratory animals (authorisation no. 551998, 2013, by the Ministry of Health).

Quantitative Real-Time PCR (qRT–PCR)

Cells and tissues were subjected to Total RNA extraction via the TRIzol method (Takara), adhering to the instructions provided by the producer. The specific primers employed are detailed in [Supplementary Table 1](#). For the conversion of RNA (1µg) to cDNA, a PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Beijing, China) was utilized. Quantification of gene expression was carried out using the comparative 2- $\Delta\Delta$ CT technique, employing GAPDH as the internal standard.

Western Blot Analysis

Protein extracts were obtained from both the MDA-MB-231 cellular structures and the subdermal neoplastic samples, then preserved at a temperature of minus twenty degrees Celsius. Quantitative analysis of the protein content for each of these extracts was accomplished through the application of the bicinchoninic acid (BCA) assay. Post quantification, uniform quantities of the protein extracts underwent electrophoretic segregation using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequent to this fractionation, the proteins were transitioned onto PVDF membranes by means of electrophoretic apparatuses (Tanon VE180 and Tanon VE186, from Shanghai). Blocking of the PVDF membranes was performed using a 5% solution of skim milk in w/v for a duration of two hours, followed by an overnight incubation at a temperature of 4°C with a series of primary antibodies, which included the following: rabbit polyclonal anti-CDC42 at a dilution ratio of 1:2000 (catalog ab187643), anti-RAC3 at 1:1000 (catalog ab129062), anti-SRC at 1:1000 (catalog ab133283), anti-ZO-1 at 1:2000 (catalog ab307799), anti-Occludin at 1:2000 (catalog ab216327), and anti-Claudin-1 at a 1:1000 dilution (catalog ab307692). Subsequent to being triple-rinsed in 1× phosphate-buffered saline (PBS) procured from Sangon in Shanghai, the membrane sheets underwent a period of incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody specific to goat anti-rabbit IgG (ab254552), which was employed at a dilution of 1:10,000. To detect immunoreactive sites, an advanced chemiluminescence (ECL) detecting solution provided by Thermo Fisher in the United States was utilized. Imagery of the gels was captured using a Bio-Rad Gel Doc XR+ system, stationed in the US, while Bio-Rad's Image Lab Software (version 5.1) along with SPSS 20.0 served as tools for image processing and the execution of statistical evaluations. GAPDH functioned as an intrinsic standard to verify consistent protein application.

Statistical Analysis

SPSS 22.0 (IBM Corporation, USA) and GraphPad Prism 5.0 (GraphPad Inc., USA) were used for the statistical analyses. The data are presented as the mean $\pm$ the standard deviation (SD). Independent group comparisons were performed via Student's *t* test or one-way ANOVA with the Bonferroni post hoc correction. All studies were repeated independently at least 3 times. A value of $P < 0.05$ indicated statistical significance.

Results

Fasudil-D-6h Inhibited Cell Proliferation and Promoted Apoptosis in the TNBC Cell Lines MCF-7 and MDA-MB-231

To clarify the effects of ROCK1 and ROCK2 downregulation on cell proliferation and apoptosis in the TNBC cell lines MCF-7 and MDA-MB-231, the mRNA levels of ROCK1 and ROCK2 were detected by qRT–PCR in MDA-MB-231 cells treated with fasudil-D-6h. The results revealed significantly lower levels of ROCK1 and ROCK2 in MDA-MB-231 cells after treatment with 5 µM or 10 µM fasudil-D-6h or HA-1100 (positive control) than in the 0 µM fasudil-D-6h treatment group (negative control) ([Figure 1A and B](#)). These results suggested that fasudil-D-6h can significantly inhibit the expression of ROCK1 and ROCK2 in TNBC cell lines. Furthermore, the CCK8 assay revealed that, compared with the negative control, treatment with 5 µM and 10 µM fasudil-D-6h or HA-1100 significantly decreased the proliferation

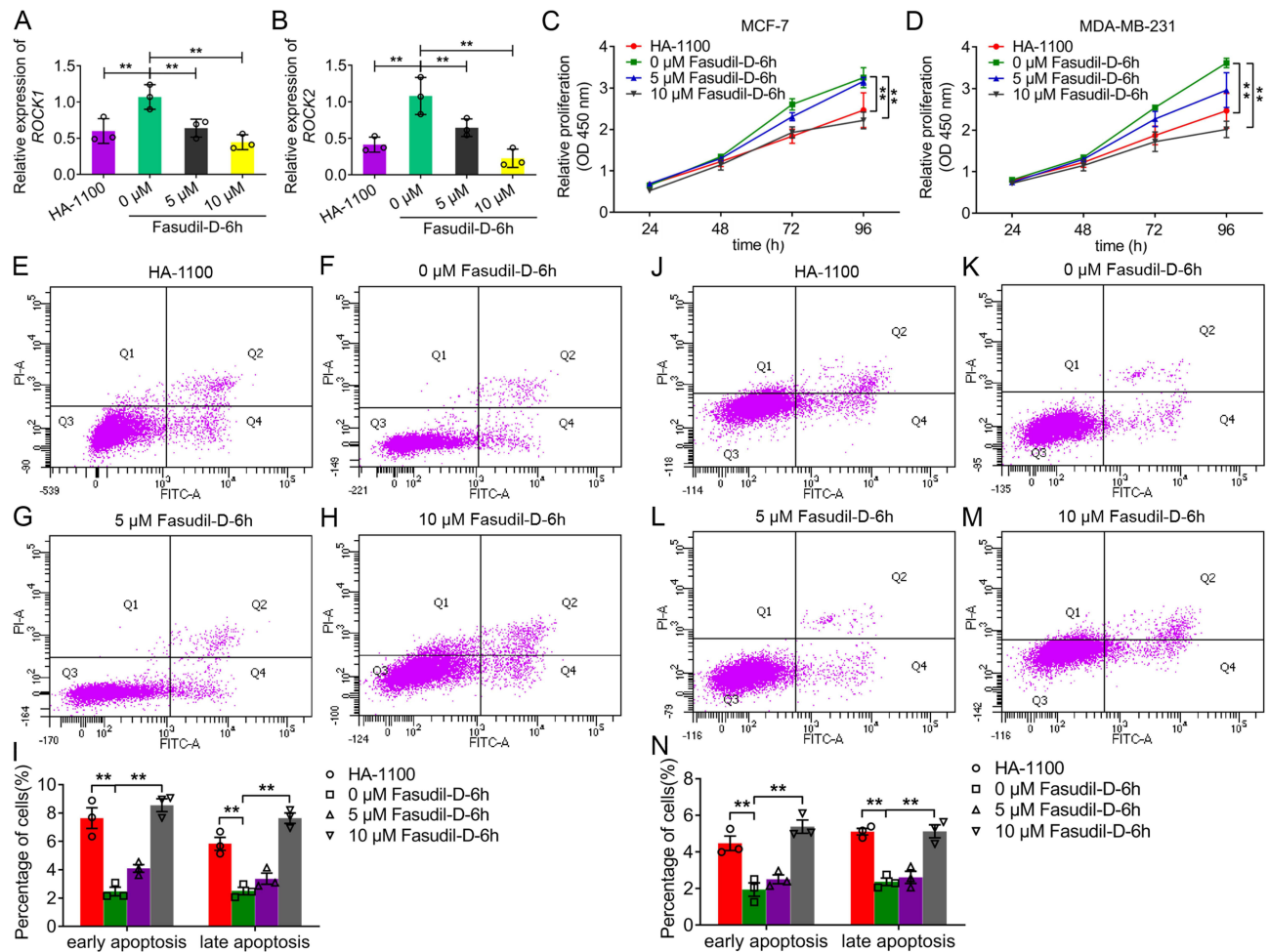


Figure 1 Fasudil-D-6h inhibited cell proliferation and promoted apoptosis in the TNBC cell lines MCF-7 and MDA-MB-231. (**A** and **B**) ROCK1 (**A**) and ROCK2 (**B**) expression was detected via qRT-PCR in MDA-MB-231 cells after treatment with 0 μ M, 5 μ M or 10 μ M fasudil-D-6h or HA-1100. (**C** and **D**) Proliferation of MCF-7 (**C**) and MDA-MB-231 (**D**) cells treated with 0 μ M, 5 μ M or 10 μ M fasudil-D-6h or HA-1100 was detected via the CCK8 assay. (**E**–**H**) Apoptotic MCF-7 cells treated with 0 μ M, 5 μ M and 10 μ M fasudil-D-6h or HA-1100 were stained with PI and analysed by flow cytometry. (**I**) Statistical analysis of the percentage of MCF-7 cells in the early and late stages of apoptosis. (**J**–**M**) Apoptotic MDA-MB-231 cells treated with 0 μ M, 5 μ M or 10 μ M fasudil-D-6h or HA-1100 were stained with PI and analysed by flow cytometry. (**N**) Statistical analysis of the percentage of MDA-MB-231 cells in the early and late stages of apoptosis. $^{**}P < 0.01$. The bars represent the means $\pm$ SEMs.

of MCF-7 and MDA-MB-231 cells after 96 h ($^{**}P < 0.01$), Fasudil-D-6h demonstrated a significantly decreased the proliferation (10 μ M) compared to HA-1100 (10 μ M) in MCF-7 and MDA-MB-231 cells is powerful (Figure 1C and D). Flow cytometry further validated the changes in cell apoptosis. Early and late cell apoptosis were significantly increased in 10 μ M fasudil-D-6h-treatment compared to 5 μ M HA-1100-treated MCF-7 cells (Figure 1E–I) and MDA-MB-231 cells (Figure 1J–M and Figure 1N). Overall, fasudil-D-6 h downregulated ROCK1/ROCK2 mRNA expression, inhibited cell proliferation and promoted apoptosis in the TNBC cell lines MCF-7 and MDA-MB-231.

The Adherens Junction and Breast Cancer Signalling Pathways Were Involved in the Effects of Fasudil-D-6h Treatment on MDA-MB-231 Cells

After data preprocessing, the expression matrix of 14,150 genes was obtained from six samples. At a threshold of $|\log_2\text{FC}| \geq 0.5$, 8092 DEGs were selected for subsequent analysis, including 4123 upregulated and 3969 downregulated DEGs, which were considered significant and are shown in a heatmap (Figure 2A) and volcano plots (Figure 2B). The DEGs between the two groups were identified (Supplementary Table 2).

Furthermore, GO analysis of the genes in this module revealed that ‘cell-substrate adherens junction’ in the CC domain (Figure 3A), ‘positive regulation of the Wnt signalling pathway’, ‘intrinsic apoptotic signalling pathway in

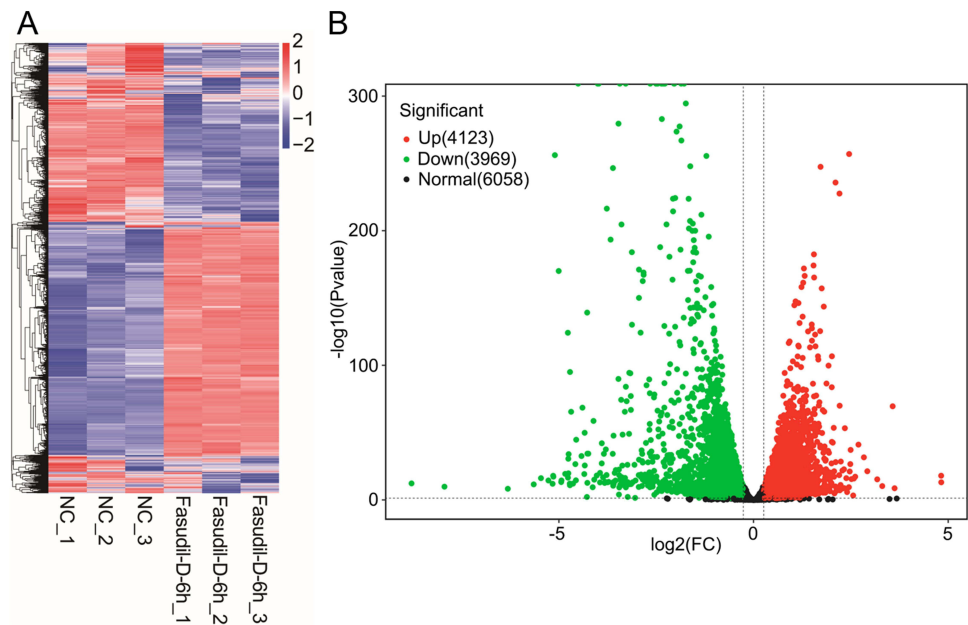


Figure 2 Transcriptomic analysis of MDA-MB-231 cells treated with fasudil-D-6h. **(A)** Heatmap of significant DEGs in the negative control (NC) group and the Fasudil-D-6 h treatment group. X-axis: sample name; Y-axis: gene name. **(B)** Volcano plot of the differentially expressed genes. X-axis: $\log_2(FC)$; Y-axis: $-\log_{10}(FDR)$. Red represents upregulated genes, and green represents downregulated genes.

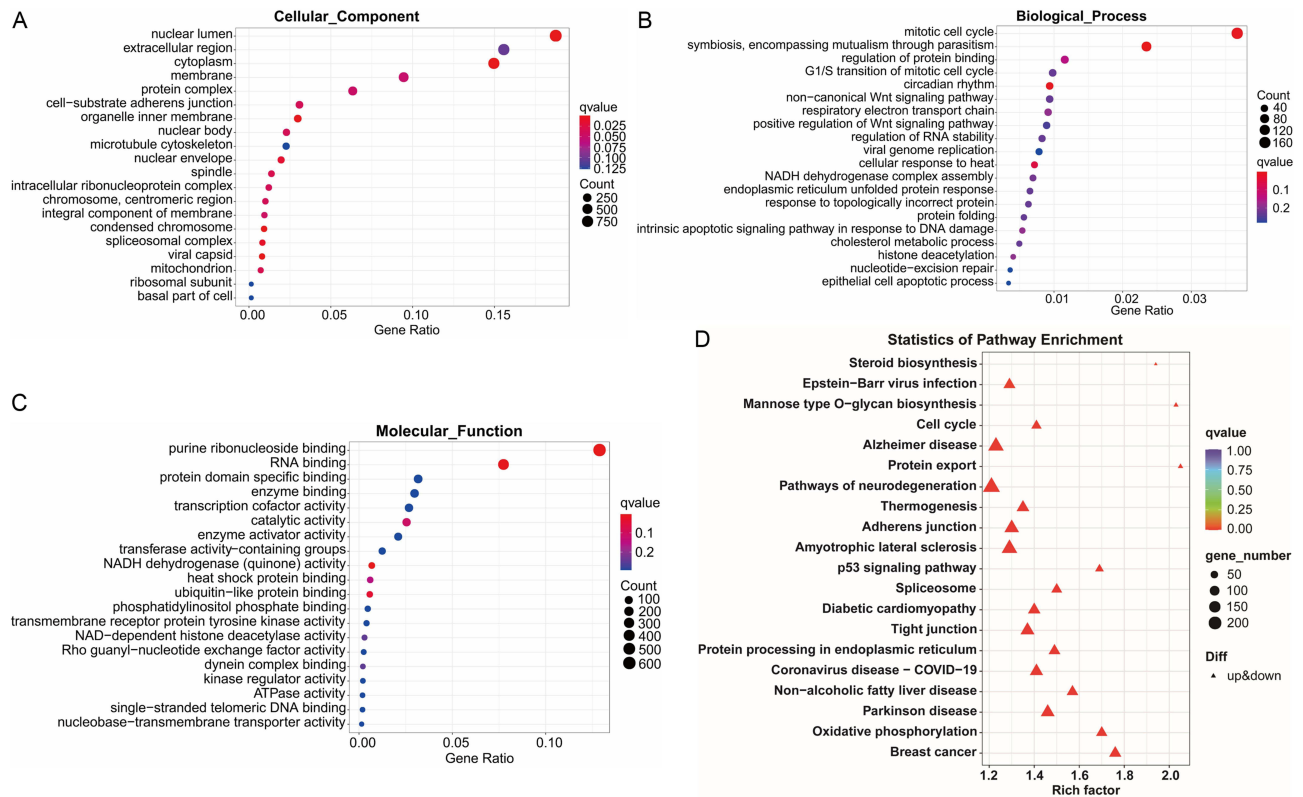


Figure 3 Gene expression profile of MDA-MB-231 cells treated with fasudil-D-6h. **(A)** GO term analysis of the CC **(A)**, BP **(B)** and MF domains **(C)** of the DEGs. The negative logarithm of the P value (x-axis) indicates the significance of the gene set belonging to the predefined categories for the DEGs. The y-axis represents each GO category. The gene number and EC value of the category in the subnetwork are shown in brackets. **(D)** Adherens junction and breast cancer genes were enriched in the significant DEGs, as determined by KEGG pathway analysis.

response to DNA damage” in the BP domain (Figure 3B) and “transferase activity-containing groups”, and “purine ribonucleotide binding” in the MF domain (Figure 3C) were significantly enriched (Supplementary Table 3). Moreover, KEGG pathway analysis of all the DEGs revealed that the adherens junction (Supplementary Figure 1) and breast cancer signalling pathways (Supplementary Figure 2) were enriched (Figure 3D and Supplementary Table 4).

Fasudil-D-6h Upregulated the Expression of Genes Related to the Adherens Junction Signalling Pathway in MDA-MB-231 Cells

To understand the possible interactions among the proteins encoded by ROCK1, ROCK2 and the differentially expressed adherens junction signalling pathway-related genes, a protein–protein interaction (PPI) network was constructed via the STRING online database. The connections among these proteins are shown in (Figure 4A). This network suggested that certain genes that have not been previously reported to be involved in TNBC may be mined from reported microarray data and that novel interactions between these genes may also be identified from the connections in the network.

We further verified that both ROCK1 and ROCK2 inhibited cell proliferation and promoted cell apoptosis by regulating the adherens junction signalling pathway in TNBC. qRT–PCR was performed to detect the mRNA levels of the adherens junction signalling pathway-related genes Cdc42, Rac3, Src, ZO-1, Occludin and Claudin-1 in MDA-MB-231 cells treated with fasudil-D-6h or HA-1100. The results revealed that the expression of Cdc42, Rac3, Src, ZO-1, Occludin and Claudin-1 was increased in MDA-MB-231 cells after treatment with 5 μ M or 10 μ M fasudil-D-6h or HA-1100 compared with cells treated with 0 μ M fasudil-D-6h (Figure 4B–G).

In addition, Western blotting was performed to assess the protein levels of Cdc42, Rac3, Src, ZO-1, Occludin and Claudin-1 in MDA-MB-231 cells that were treated with fasudil-D-6h to further investigate the relationship between ROCK1/ROCK2 and the adherens junction signalling pathway. Higher protein levels of CDC42, RAC3, SRC, ZO-1, Occludin and Claudin-1 were detected in MDA-MB-231 cells after treatment with 5 μ M and 10 μ M fasudil-D-6h or HA-1100 (Figure 4H–N). These results suggested that fasudil-D-6 h can significantly upregulate the expression of genes related to the adherens junction signalling pathway in MDA-MB-231 cells.

Fasudil-D-6h Attenuated the Growth of Subcutaneous Breast Tumours in Nude Mice

The intervention effect of fasudil-D-6h on the growth of subcutaneous breast tumours in nude mice was evaluated by observing tumour growth and the degree of differentiation, as assessed by cell morphology. The time course of subcutaneous tumour development and the subcutaneous injection of fasudil-D-6h in mice is shown in (Figure 5A). Beginning on day 10, the tumour lengths and widths were evaluated every 6 days until seven volume measurements were obtained. Considerable tumour growth retardation was observed in the 10 mg/kg fasudil-D-6 h and 20 mg/kg fasudil-D-6h groups compared with the normal saline (NS) group (Figure 5B). The tumours were subsequently dissected, and their weights and accurate sizes were appraised (Figure 5C). Compared with those in the NS group, the masses and average volumes of the tumours in the 10 mg/kg fasudil-D-6h and 20 mg/kg fasudil-D-6h groups were significantly lower (** $P < 0.01$) (Figure 5D and E). These findings suggested that fasudil-D-6h treatment effectively attenuated tumour growth.

Fasudil-D-6h Upregulated the Expression of Genes Related to the Adherens Junction Signalling Pathway in Subcutaneous Breast Tumours in Nude Mice

qRT–PCR revealed significantly higher levels of ROCK1/ROCK2 mRNAs and lower levels of Cdc42, Rac3, Src, ZO-1, Occludin and Claudin-1 mRNAs in subcutaneous breast tumours from nude mice injected with normal saline than in those from normal mice (Figure 6A–H). Moreover, the expression of ROCK1/ROCK2 decreased significantly, while the expression of Cdc42, Rac3, Src, ZO-1, Occludin and Claudin-1 increased significantly in the 10 mg/kg fasudil-D-6h and 20 mg/kg fasudil-D-6h groups compared with the NS group (Figure 6A–H).

Furthermore, the Western blot results revealed that the protein levels of CDC42, RAC3, SRC, ZO-1, Occludin and Claudin-1 were significantly lower in the NS group than in the normal group but were significantly greater in the 10 mg/kg fasudil-D-6h and 20 mg/kg fasudil-D-6h groups (Figure 6I–O). These results indicated that fasudil-D-6h upregulated the expression of genes related to the adherens junction signalling pathway in vivo.

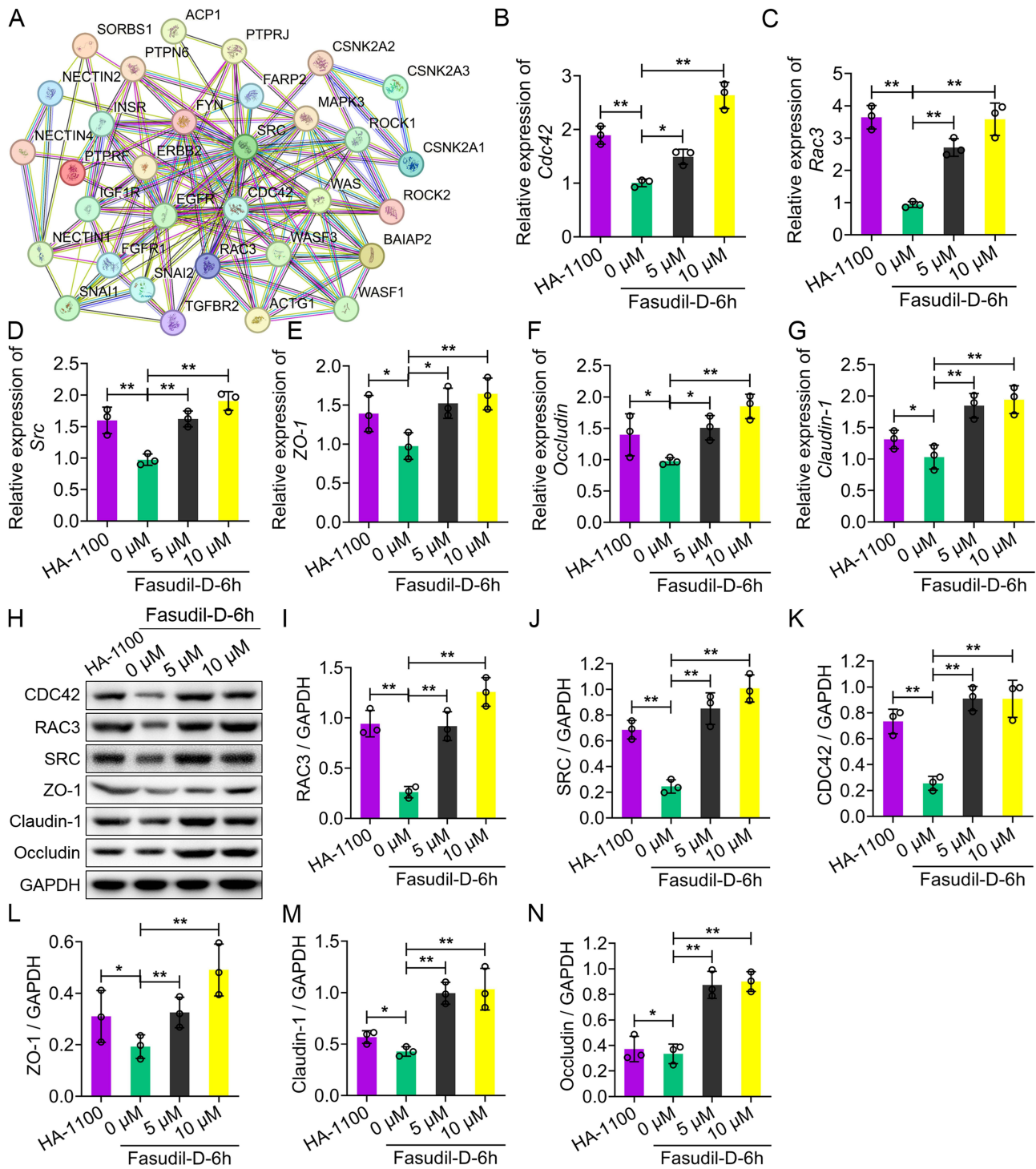


Figure 4 Fasudil-D-6h affected the expression of genes related to the adherens junction signalling pathway in MDA-MB-231 cells. **(A)** A protein-protein interaction (PPI) network of the DEGs involved in the adherens junction signalling pathway and ROCK1/ROCK2 was constructed via the STRING database. **(B–G)** The expression levels of adherens junction signalling pathway-related genes (Cdc42, Rac3, Src, ZO-1, Occludin and Claudin-1) were analysed using qRT-PCR in MDA-MB-231 cells treated with 0 μ M, 5 μ M or 10 μ M fasudil-D-6h or HA-1100. **(H)** The protein levels of CDC42, RAC3, SRC, ZO-1, Occludin and Claudin-1 were detected by Western blotting. **(I–N)** For the quantitative analysis of the protein levels, target protein expression was normalised to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). * P < 0.05, ** P < 0.01. The bars represent the means $\pm$ SEMs.

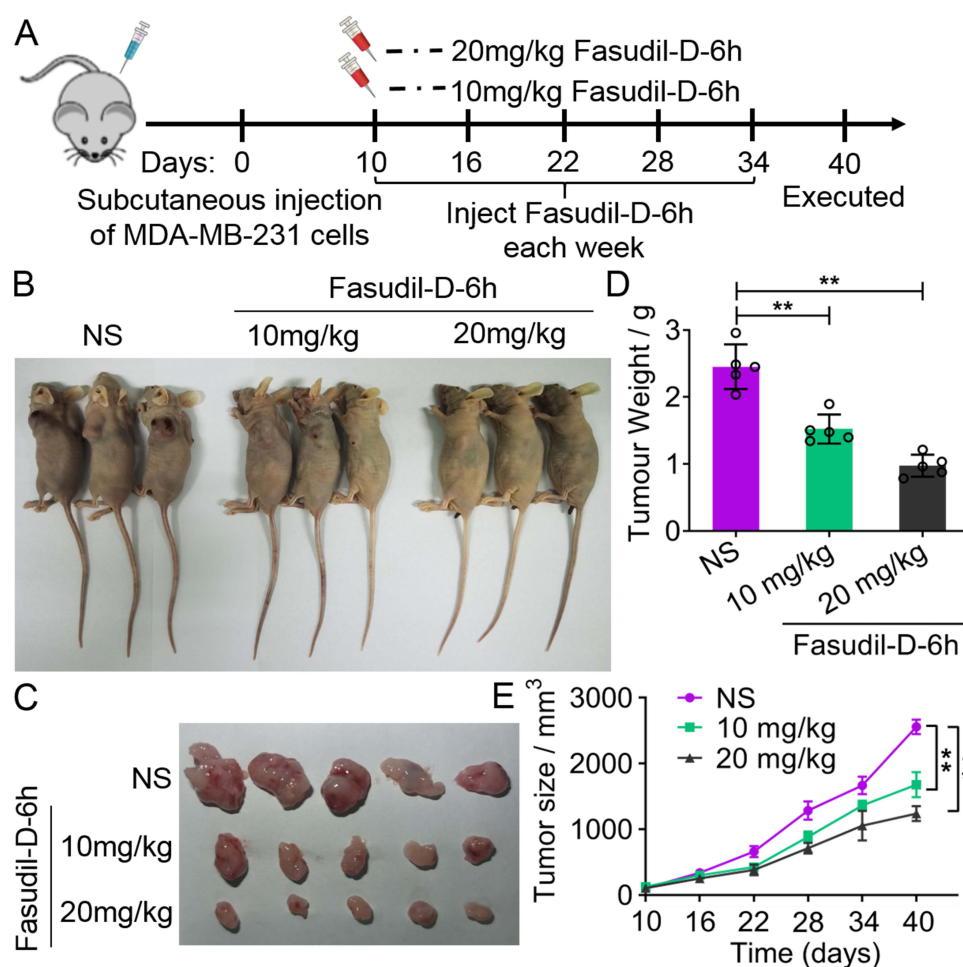


Figure 5 Effects of fasudil-D-6 h on the growth of subcutaneous breast tumours in nude mice. **(A)** Time course of the subcutaneous injection of MDA-MB-231 cells and treatment with fasudil-D-6h in nude mice. **(B)** Representative images of nude mice after subcutaneous injection of MDA-MB-231 cells and treatment with fasudil-D-6h. **(C)** Representative images of excised tumours from the mice. **(D–E)** Quantitative analyses of the tumour weights **(D)** and volumes **(E)**. $**P < 0.01$.

Discussion

In this study, we verified the antitumour activity of the currently marketed generic Rho-kinase (ROCK)-specific inhibitor HA-1100, an active metabolite of fasudil hydrochloride, and fasudil-D-6 h, a novel ROCK inhibitor and fasudil derivative developed by us, in vitro and in animal tumour models. Our study revealed that treatment with HA-1100 or fasudil-D-6h significantly downregulated ROCK1/ROCK2 expression in MCF-7 cells and that the inhibition of ROCK1/ROCK2 expression by fasudil-D-6 h was significant. To further investigate the effects of HA-1100 and fasudil-D-6h on the functions of the TNBC cell lines MCF-7 and MDA-MB-231, we treated MCF-7 cells and MDA-MB-231 cells with equal concentrations of HA-1100 and fasudil-D-6h, respectively, and observed changes in cell proliferation, migration, invasion, and apoptosis. The function of the Rho kinase pathway in cell migration and invasion has been confirmed by several studies. These results are consistent with those of published studies, and our study further supports the critical role of the Rho/Rho kinase pathway in regulating cell proliferation, migration, invasion and apoptosis. The Rho-kinase inhibitor Wf-536 reduces the invasion and migration of Lewis lung cancer cells and inhibits endothelial cell invasion, migration and capillary-like tube formation in Matrigel.¹⁵ In another study, Wf-536 inhibited the migration of mouse melanoma B16BL6 cells and exhibited anti-invasive effects under chemotaxis and chemotaxis conditions.¹⁵ Similar results were reported for Y-27632, which inhibits the lysophosphatidic acid-induced migration and invasiveness of Caov-3 and OVCAR-3 ovarian cancer cells.¹⁶ Our data position fasudil-D-6h not merely as another ROCK inhibitor, but as a candidate with distinct advantages over existing agents. First, it demonstrates superior potency in vitro and enhanced

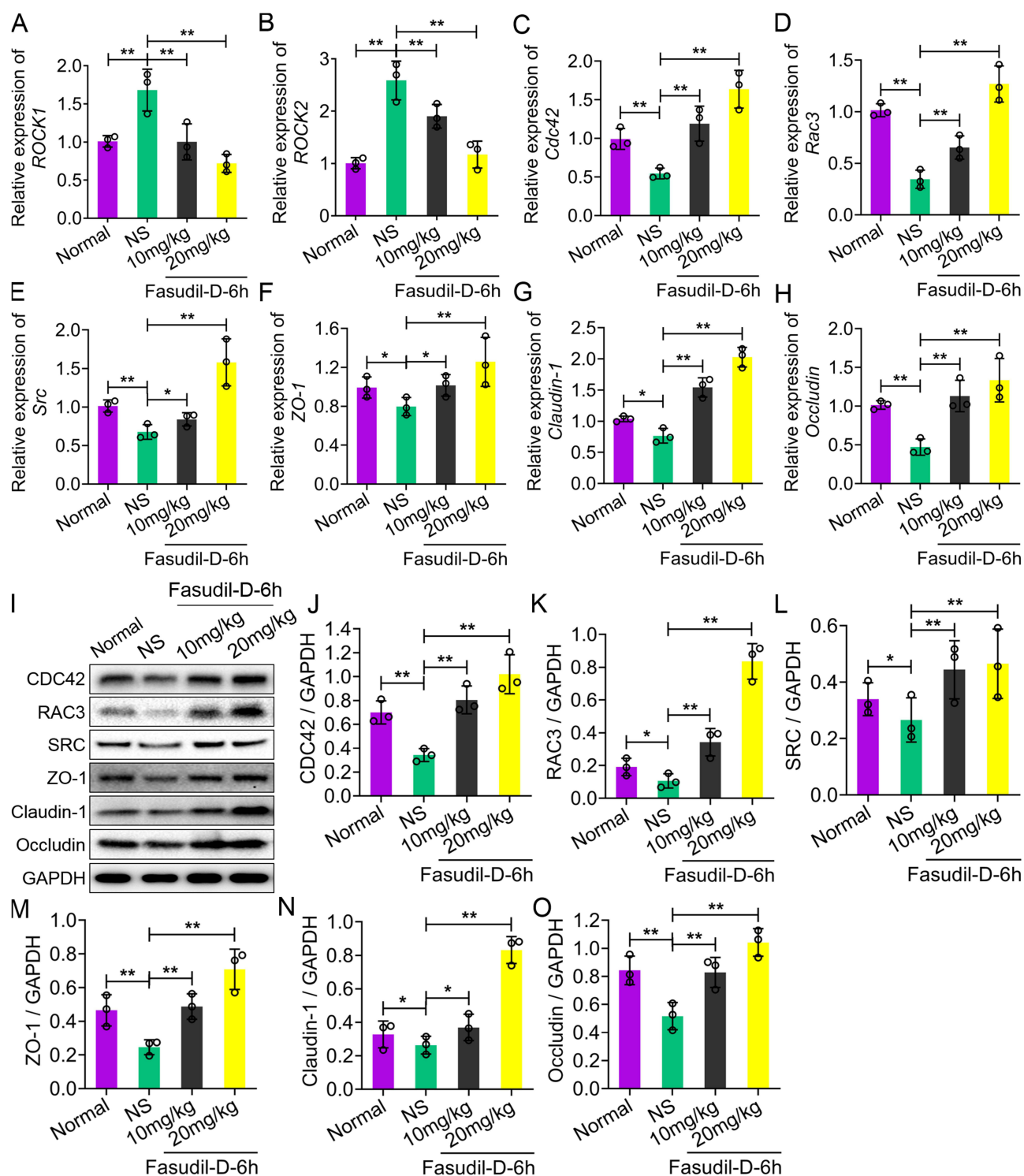


Figure 6 Fasudil-D-6h altered the expression of genes related to the adherens junction signalling pathway in subcutaneous breast tumours in nude mice. **(A–H)** The expression levels of ROCK1, ROCK2 and adherens junction signalling pathway-related genes (Cdc42, Rac3, Src, ZO-1, Occludin and Claudin-1) were analysed via qRT-PCR in subcutaneous breast tumours in nude mice injected with MDA-MB-231 cells and treated with fasudil-D-6h. **(I)** The protein levels of CDC42, RAC3, SRC, ZO-1, Occludin and Claudin-1 were detected by Western blotting. **(J–O)** For the quantitative analysis of the protein levels, target protein expression was normalised to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). * $P < 0.05$, ** $P < 0.01$. The bars represent the means $\pm$ SEMs.

efficacy *in vivo* compared to its parent compound, fasudil, and the research tool Y-27632. Second, and more importantly, its mechanism of action appears uniquely focused on the comprehensive restoration of the epithelial barrier. While previous studies with Y-27632 have primarily focused on preventing EMT and cell migration,¹³ our transcriptomic and validation data reveal that fasudil-D-6h actively promotes an epithelial phenotype by robustly upregulating a suite of adherens and tight junction genes. This “junction-stabilizing” effect may provide a dual therapeutic advantage: directly reinforcing tissue architecture to suppress invasion and potentially re-sensitizing tumors to therapies that require intact cell-cell contact. The structural modifications in fasudil-D-6h are likely responsible for this enhanced profile, potentially conferring better target engagement, altered subcellular localization, or influence on specific ROCK isoforms or co-factors.

Furthermore, there might be a relationship between the degree of invasiveness and the dynamic behaviour of the key Rho GTPase within colorectal cancer cell lines.¹⁷ It is thought that the aggressive nature of these cell lines is linked to noticeable decreases in Cdc42 and Rac1 function, together with an increase in ROCK function, although RhoA function does not exhibit this association.¹⁸ This cellular profile is connected to a rounded, bubble-like cell shape. Certainly, when the aggressive SW620 cell line was subjected to simultaneous administration of PDGF and Y27632, it inhibited the formation of blisters, leading to the cells adopting a spread out and flatter shape.¹⁹ Concurrent with this morphology change, the integrity of cell–cell adhesion was re-established, as evidenced by the reappearance of E-cadherin at the junctions between cells. The observed change in form correlated with a notable decrease in the cellular capacity for Matrigel penetration, given that the characteristics of cellular interconnectivity and shape are closely connected to invasive potential.

Research has shown that a marked decrease in Cdc42 and Rac1 activity is significantly correlated with an increase in TNBC invasiveness. These findings confirm the results obtained in other epithelial cell lines. In Madin Darby canine kidney (MDCK) epithelial cells, the activation of Cdc42 and Rac1 is associated with the formation of adhesion junctions. Furthermore, the constitutively activated forms of Cdc42 and Rac1 can block hepatocyte growth factor (HGF)-induced cell scatter by increasing E-cadherin-mediated cell–cell adhesion.²⁰ Our results extend these observations to TNBC cells. Specifically, diffusion occurs when PDGF treatment restores the formation of the GTP component. Furthermore, the homeostatic activity of Cdc42 or Rac1 inhibits the invasiveness of TNBC cells. Moreover, PDGF promotes the reconstitution of adhesion protein-dependent adhesion junctions. Other studies have shown that Rac1 activation leads to E-cadherin-mediated cell–cell adhesion, which in turn inhibits epithelial cell migration and invasion.²¹ In contrast, in liver tumours, PDGF is involved in the maintenance of EMT.²² In addition, Cdc42 and Rac1 may also play key roles in cell migration and invasion through WAVE2 signalling in breast cancer.²³ Thus, controlling cell contacts to maintain cells as cohesive epithelia and regulating cell migration so that invasion is favoured can be considered two antagonistic effects of Cdc42 and Rac1 on tumour progression. In one study, PDGF treatment restored E-cadherin-dependent cell–cell contact but was insufficient to prevent invasion when it was used alone. ROCK also plays a central role, and recent studies have identified FilGAP, a filament protein-binding protein with Rac1-specific Rho GTPase activation, as an important mediator of ROCK-induced Rac1 inhibition.⁵ In addition, ROCK signalling also activates ARHGAP22 (another RacGAP), which in turn inhibits Rac1 activation to avoid the inhibition of vesicle-dependent amoeboid motility.²⁴ However, RhoA can also mediate Rac1 activation and subsequent plate foot formation by recruiting mediators associated with membrane ruffles, and given this dual role, the lack of correlation between RhoA activation and cell invasiveness is not surprising. ROCK activity is more likely to be associated with invasiveness, whereas the effect of RhoA oscillates between mediator and ROCK activation, depending on the cell type. Thus, exoenzyme C3 (Rho inhibitor) and Y27632 have very different roles.^{25,26} In LPA-stimulated Swiss 3T3 cells, treatment with Y27632 but not C3 increases Rac1 activity and induces the formation of membrane ruffles.²⁷

Despite the promising findings, this study has limitations including a relatively small *in vivo* sample size, the lack of comprehensive pharmacokinetic studies on fasudil-D-6h, and validation limited to two TNBC cell lines. Future studies should further elucidate the detailed mechanisms of fasudil-D-6h in modulating the adherens junction pathway, explore its efficacy in combination with standard therapies, and rigorously assess its translational potential through preclinical and clinical validation.

In conclusion, our study provides comprehensive evidence that the novel ROCK inhibitor fasudil-D-6h is a potent anti-tumor agent against triple-negative breast cancer. The principal findings can be summarized as follows: First, fasudil-D-6h effectively suppresses TNBC cell proliferation and induces apoptosis *in vitro*. Second, an unbiased

transcriptomic approach identified the adherens junction pathway as a primary target, a finding that was robustly validated by the consistent upregulation of key junctional proteins (Cdc42, Rac3, ZO-1, Occludin, Claudin-1) upon treatment, both in cultured cells and in vivo. Third, fasudil-D-6h demonstrated significant efficacy in reducing tumor growth in a preclinical TNBC mouse model.

The broader impact of these findings is twofold. Mechanistically, we elucidate a previously underexplored anti-tumor mechanism of ROCK inhibition in TNBC—the active restoration of epithelial junctional integrity. This moves beyond the conventional focus on inhibiting EMT and positions the reinforcement of cell-cell contacts as a viable therapeutic strategy. Translationally, the derivation of fasudil-D-6h from the clinically approved drug fasudil de-risks its development path and highlights its strong immediate potential. Our work not only characterizes a promising drug candidate but also provides a compelling rationale for targeting junctional stability as a novel therapeutic paradigm in aggressive carcinomas. Future studies focusing on the pharmacokinetic profile of fasudil-D-6h and its efficacy in combination with standard chemotherapy are warranted to advance this compound toward clinical evaluation.

Data Sharing Statement

All data used to support the findings of this study are available from the corresponding author upon request.

Ethics Approval

All experimental protocols were approved the Ethics Committee of the Jiangxi Cancer Hospital. All methods were performed in accordance with ARRIVE guidelines.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no competing interests with this manuscript.

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