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Cardamonin retards progression of autosomal dominant polycystic kidney disease via inhibiting renal cyst growth and interstitial fibrosis

Running title: Cardamonin retards progression of ADPKD

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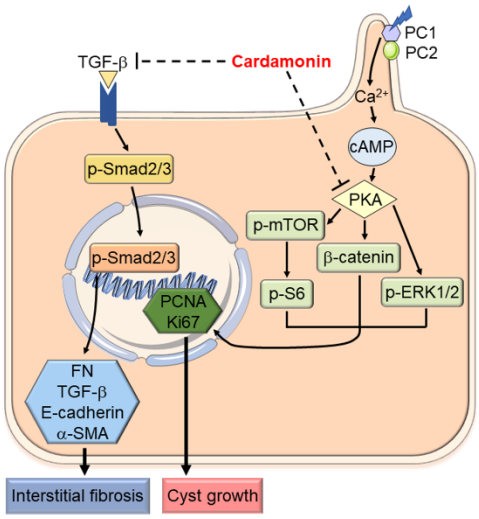
Graphical abstract



Highlights

- Cardamonin significantly retards the progression of PKD.
- Cardamonin inhibited cyst epithelial cell proliferation by repressing the MAPK, Wnt, and mTOR signaling pathways.
- Cardamonin effectively suppressed fibrosis by regulating TGF-β/Smad signaling.

Abstract

Autosomal dominant polycystic kidney disease (ADPKD) is the most common monogenetic inherited kidney disease characterized by renal progressive fluid-filled cysts and interstitial fibrosis. Inhibiting renal cyst development and interstitial fibrosis have proven effective in delaying the progression of ADPKD. The purpose of this study was to discover effective drugs from natural products for preventing and treating ADPKD. Candidate compounds were screened from a natural product library by virtual screening. A Madin-Darby canine kidney (MDCK) cyst model, embryonic kidney cyst model, and orthologous mouse model of ADPKD were utilized to determine the pharmacological activities of the candidate compounds. Western blot and morphological analysis were used to investigate underlying mechanisms. The experimental results showed that 0.625, 2.5, and 10 μM cardamonin dose-dependently reduced formation and enlargement in MDCK cyst model. Cardamonin also significantly attenuated renal cyst enlargement in *ex vitro* mouse embryonic kidneys

and PKD mouse kidneys. We found that cardamonin inhibited renal cyst development and interstitial fibrosis by downregulating the MAPK, Wnt, mTOR, and transforming growth factor-β/Smad2/3 signaling pathways. Cardamonin significantly inhibits renal cyst development and interstitial fibrosis, suggesting that cardamonin shows promise as a potential therapeutic drug for preventing and treating ADPKD.

Chemical compounds studied in this article: Cardamonin (PubChem CID: 641785), Esculetin (PubChem CID: 5281416) and 3-acetamidocoumarin (PubChem CID: 136620).

Abbreviation

ADPKD, autosomal dominant polycystic kidney disease

PC1, polycystin 1

PC2, polycystin 2

ESRD, end-stage renal disease

MDCK, Madin-Darby canine kidney

DMEM, Dulbecco's modified Eagle's medium

TGF-β, transforming growth factor-β

FBS, fetal bovine serum

FSK, forskolin

8-Br-cAMP, 8-bromoadenosine-3`,5`-cyclic monophosphate

LTL, *Lotus tetragonolobus* lectin

DBA, *Dolichos biflorus* agglutinin

HE, hematoxylin and eosin

FN, fibronectin

3D, three-dimensional

ECM, extracellular matrix

EMT, epithelial-mesenchymal transition

HIF-1α, hypoxia-inducible transcription factor 1α

Keywords: ADPKD; natural drug; cardamonin; renal cyst; fibrosis; drug discovery.

1. Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most

common hereditary diseases, affecting around 1/1000 ~ 1/400 individuals worldwide (Cornec-Le Gall, Alam, & Perrone, 2019; Lanktree et al., 2018). Mutations in the genes *PKD1* and *PKD2*, which encode polycystin 1 (PC1) and polycystin 2 (PC2) respectively, result in profound cystogenesis and are the initial causes of ADPKD (A. C. Ong, Devuyst, Knebelmann, Walz, & Diseases, 2015; V. E. Torres, Harris, & Pirson, 2007). Formation and enlargement of fluid-filled cysts characterize the pathogenesis of ADPKD, resulting in gradually compromised renal parenchymal and declined renal function (Hassane et al., 2010; Song, Zimmerman, Henke, & Yoder, 2017; V. E. Torres & Harris, 2014). ADPKD progressively reach to end-stage renal disease (ESRD) with extrarenal manifestations including liver cysts, cerebral aneurysms, and cardiovascular disorders (Kanaan, Devuyst, & Pirson, 2014). Due to a lack of effective drugs, most of patients still rely on prophylactic measures to slow the progression of ADPKD, and renal replacement therapy upon progression to ESRD. Therefore, discovering effective, safe, and satisfactory drugs has been an imperative task for ADPKD therapy.

Despite substantial efforts to combat ADPKD, only the type-2 vasopressin receptor antagonist tolvaptan has been approved by the FDA for ADPKD treatment (Ingelfinger, 2017; A. C. M. Ong, 2018; V. E. Torres et al., 2017). However, the hepatotoxicity and other side effects of tolvaptan limit its long-term use (Cornec-Le Gall et al., 2019). Over the past decade, natural products derived from fruits, vegetables, and animals have been considered as promising candidates to inhibit renal cyst development.

It is worth mentioning that compounds with anti-cancer properties, such as triptolide, *Ganoderma* triterpenes, curcumin, and ginkgolide B, showed impressive inhibitory effects on renal cyst growth (Ciolek et al., 2017; Gao et al., 2011; Leuenroth, Bencivenga, Igarashi, Somlo, & Crews, 2008; Su et al., 2017; Zhou et al., 2012). Natural products that were originally developed for treating cancer and targeting the pathologic features of ADPKD have great potential to curtail the cystogenesis. Several drugs such as lanreotide, bosutinib, and phosphatidic acid inhibitors, have shown significant benefits in ADPKD treatment by regulating intracellular calcium concentrations, increasing cAMP levels, and activating the MAPK/ERK, PI3K/Akt, Wnt/β-catenin, and mTOR signaling pathways (Harris & Torres, 2014; Lanktree & Chapman, 2017; Liu et al., 2013).

In the present study, we screened out cardamonin from a natural compound library

by virtual screening and *in vitro* and *in vivo* models as a potential drug to restrain renal cyst enlargement. Furthermore, we investigated if cardamonin could inhibit the initiation and development of cysts using a Madin-Darby canine kidney (MDCK) cyst model, embryonic cyst model, and ADPKD mouse model. Our experimental results showed that cardamonin retarded cyst growth and alleviated renal fibrosis by down-regulating the MAPK, Wnt, mTOR, and transforming growth factor β (TGF-β) signaling pathways. These data indicate that cardamonin may be developed as a promising novel drug for ADPKD treatment.

2. Materials and methods

2.1 Natural compounds

25 natural products were purchased from MedChemExpress and dissolved in 100% DMSO. Cardamonin (T2994, Targetmol), Esculetin (19286, Caymen), and 3-acetamidocoumarin (A2972, TCI) were dissolved in distilled water with 1/500 DMSO using applied ultrasound for 30 minutes.

2.2 Mouse model

Pkd1$^{loxp/loxp}$*:Ksp-Cre* mice (C57BL/6J genetic background) were generated by intercrossing *pkd1*$^{flox/+}$ mice (from the laboratory of Dr. Stefan Somlo) with *Ksp-Cre* transgenic mice (from the laboratory of Dr. Peter Igarashi). In the *Pkd1*$^{loxp/loxp}$*:Ksp-Cre* mice, exons 2-4 of the *pkd1* gene were flanked by *loxp* sites and deleted by *Ksp*-promoted *Cre* recombinase, resulting in kidney tissue-specific deletion of PC1. *Pkd1*$^{+/+}$*:Ksp-Cre* and *Pkd1*$^{loxp/loxp}$*:Ksp-Cre* mice were identified as wild-type and PKD mice, respectively. PKD mice show aggressive cyst growth and die around postnatal day 14. Genotyping was conducted on postnatal day 1.

Mice of the same genotype in the same litter were injected subcutaneously with 100 mg (kg· day)$^{-1}$ of test compound or an equivalent volume of vehicle, from postnatal day 1 to day 5. On postnatal day 5, mice were weighed and sacrificed for collection of experimental samples. All animal experiments were performed according to animal protocols approved by the Peking University Health Science Center Committee on Animal Research.

2.3 Cell culture and CCK8 assay

Type Ⅰ MDCK cells (ATCC Cat# CRL-2936, RRID:CVCL_B034) were cultured with Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal

bovine serum (FBS), 100 μg ml^{-1} streptomycin, and 100 U ml^{-1} penicillin in a 5% CO_2 and 37℃ humidified incubator. When the cells reached to 70% confluence, cells were incubated with or without 10 ng ml^{-1} TGF-β (R&D Systems) and treated with different doses of cardamonin for 1 h or 48 h. MDCK cells were seeded on a 96-well plate at a density of 5000 cells/well. After treatment with 25 μM of the test drugs for 24 h, 100 μl 5% CCK8 solution (Dojindo Molecular Technologies) was added to each well and incubated for 2 h. Absorbance values at a wavelength of 450 nm were then measured with a microplate reader (Biotek, MQX200).

2.4 MDCK cyst model

MDCK cells were digested and resuspend in type Ⅰ collagen (PureCol, Inamed Biomaterials) solution at pH ~7.2, containing ice-cold modified Eagle's medium and 10 mM HEPES. The resulting cell-gel suspensions were allocated into 24-well plates and cultured with DMEM-F12 with 10% FBS. To promote cyst growth, 10 μM forskolin (FSK, Sigma) was supplemented with or without addition of test compounds from day 5. In the presence of FSK, visible cysts progressively expanded from day 4 (control). Medium was changed every 12 h. Cysts were tracked by taking photos (20 cysts/well) every two days until day 12 to record their growth curve. Cyst diameter was measured and analyzed using Image J software (National Institutes of Health). To assay cyst formation, the numbers of cysts and colonies were counted at day 6 of incubation with the test compounds.

2.5 Embryonic kidney cyst model

Ex vivo cyst assay was performed as previously described (Su et al., 2017). Briefly, embryonic kidneys at day 13.5 were harvested from pregnant wild-type ICR mice. Embryonic kidneys were placed on 0.4 μm diameter Transwell filters (Corning) and the lower chamber was filled with DMEM-F12 supplemented with 2 mM L-glutamine, 250 U ml^{-1} penicillin, 250 μg ml^{-1} streptomycin, 10 mM HEPES, 5 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin, 2.8 nM selenium, 25 ng ml^{-1} prostaglandin E, 32 pg ml^{-1} T3, and 100 μM 8-bromoadenosine-3`,5`-cyclic monophosphate (8-Br-cAMP, Sigma). Embryonic kidneys formed cysts that developed rapidly from day 0 to day 6 in the presence of 100 μM 8-Br-cAMP (control). Test compounds were supplemented from day 0 and medium was changed every 12 h. Micrographs of the same kidney were taken on day 0, 2, 4, and 6 post-seeding. To analyze cyst area ratios, cyst area and total kidney area were measured using Image-Pro Plus 6.0 software (Rockville).

2.6 Western blot

Tissue and cell lysates were extracted as previously described (W. Wang et al., 2019). Proteins were separated based on molecular weight through electrophoresis and then transferred to polyvinylidene difluoride membranes (Amersham Biosciences). After blocking, membranes were incubated with the following antibodies respectively, anti-PCNA (Cell Signaling Technology, Cat# 2586, RRID:AB_2160343, 1:2000 dilution), anti-p-ERK (Cell Signaling Technology, Cat# 4370, RRID:AB_2315112, 1:2000 dilution), anti-ERK2 (ABclonal, Cat# A11186, RRID:AB_2814870, 1:1000 dilution), β-catenin (ABclonal, Cat# A11512, RRID:AB_2814869, 1:1000 dilution), anti-p-mTOR (Cell Signaling Technology, Cat# 2971, RRID:AB_330970, 1:1000 dilution), anti-mTOR (Cell Signaling Technology, Cat# 2972, RRID:AB_330978, 1:1000 dilution), anti-p-S6 (ABclonal, Cat# AP0537, RRID:AB_2771524, 1:1000 dilution), anti-S6 (ABclonal, Cat# A6058, RRID:AB_2766731, 1:1000 dilution), anti-β-actin (ABclonal, Cat# AC026, RRID:AB_2768234, 1:20000), anti-TGF-β (Cell Signaling Technology, Cat# 3711, RRID:AB_2063354, 1:1000 dilution), anti-fibronectin (FN) (Proteintech, Cat# 15613-1-AP, RRID:AB_2105691, 1:1000 dilution), anti-α-SMA (ABclonal, Cat# A1011, RRID:AB_2757633, 1:1000 dilution), anti-p-Smad2/3 (Abcam, Cat# ab63399, RRID:AB_1142934, 1:1000 dilution), anti-Smad2 (Cell Signaling Technology, Cat# 5339, RRID:AB_10626777, 1:1000 dilution), anti-E-cadherin (Cell Signaling Technology, Cat# 3195, RRID:AB_2291471, 1:1000 dilution). Membranes were then washed 3 times and incubated with goat anti-mouse IgG or goat anti-rabbit IgG secondary antibodies. Blots were then developed with the ECL kit (Biodragon) and detected with a chemiluminescence detection system (Syngene). The protein levels were quantified relative to β-actin expression.

2.7 Histological staining and immunofluorescence staining

Kidneys were collected and processed as previously described (W. Wang et al., 2019). Kidney sections were cut at 5 μm thickness. For hematoxylin and eosin (HE) and Masson's trichrome staining, paraffin sections were used. Cyst size, index, number, and positive fibrotic area were calculated using Image-Pro Plus 6.0 software (Rockville). Cyst numbers were counted based on the definition that a cyst was spherical structure with diameter $\geq$ 50 μm (cyst index = (total cystic area/total kidney area)*100%; fibrosis (%) = (total positive blue area/total kidney area)*100%). For immunofluorescence staining, sections were blocked with 5% (w/v) bovine serum

albumin at room temperature for 1 h and then incubated with a primary antibody for Ki-67 (Abcam, Cat# ab15580, AB_443209, 1:500 dilution) at 4℃ for overnight. After washing 3 times, sections were incubated with secondary antibody (goat-anti-rabbit, FITC, Invitrogen, Cat# A11008, AB_143165, 1:200 dilution) or *Lotus tetragonolobus* lectin (LTL, Vector Laboratories, Cat# FL-1321, AB_2336559, 1:400 dilution) and *Dolichos biflorus* agglutinin (DBA, Vector Laboratories, Cat# RL-1032, AB_2336396, 1:400 dilution) for 1 h. The unit areas of Ki-67 positive cells were calculated using Image-Pro Plus 6.0 software (Rockville). Hoechst dye 13342 (Sigma, Cat# B2883, 1:1000 dilution) was used to stain nuclei. All images were captured with a Leica fluorescence microscope.

2.8 Statistical analysis

All results were analyzed with GraphPad Prism software and expressed as mean ± SEM. Student's *t* test or one-way ANOVA followed by the Tukey's multiple comparison tests was performed in statistical analyses. $P < 0.05$ was considered statistically significant for all tests.

3. Results

3.1 Cardamonin was identified with cyst inhibition activity

Utilizing virtual screening mainly based on the effect on tumor and cell proliferation-related signaling pathways, such as MAPK, Wnt, and mTOR, 25 natural compounds (Supplementary Table 1) were selected from a natural compound library at MedChemExpress. The CCK8 assay eliminated 5 of these compounds (B3, B5, C5, C6 and C8) from the list due to cytotoxicity against MDCK cells (Figure 1A). 20 natural compounds (at 25 μM) were assayed for their effects on cyst enlargement with MDCK cyst model. Compared to the control, compound A1 significantly accelerated cyst growth. Compounds A3, A4, B1, B2, B7, C2, C4, C9, and C10 significantly impeded cyst growth (Figure 1B, Supplementary Figure 1). Figure 1C shows representative images of cyst growth following treatment with compounds A3, B1, B4, and C10. Compounds B2 and C2 quickly disrupted cyst construction and caused cell death (data not shown), indicating their toxicity in three-dimensional (3D) cell culture. Other compounds demonstrated little effect on cyst growth.

The cyst inhibition activity of compounds A3, A4, B1, B7, C4, C9, and C10 at 25 μM was also determined with an embryonic kidney cyst model. We found that compounds

A3, C9, and C10 significantly slowed renal cyst development (Figure 1D). In contrast, compound A4 showed only minor effects on cyst growth, while compounds B1, B7, and C4 severely inhibited normal embryonic kidney development.

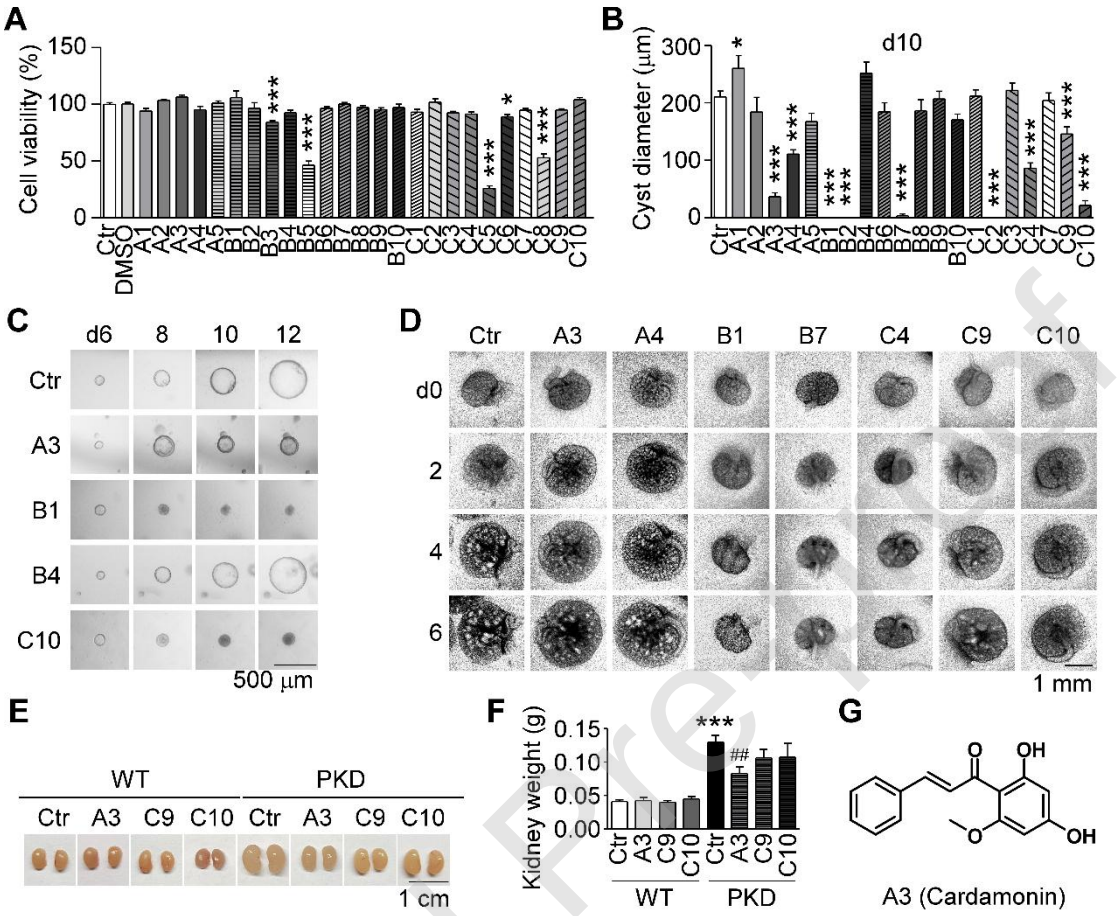


Figure 1. **Cardamonin was identified with cyst inhibition activity. (A)** Viability of MDCK cells treated with candidate compounds at 25 μM. **(B)** Cyst diameter at day 10 with or without exposure to candidate compounds at 25 μM. **(C)** Representative images of cyst growth during incubation with compounds A3, B1, B4, or C10. Bar = 500 μm. **(D)** Embryonic kidneys were cultured with or without compounds A3, A4, B1, B7, C4, C9, or C10. Bar = 1 mm. **(E)** Kidneys from wild-type (WT) and PKD mice treated with or without 100 mg (kg· d)$^{-1}$ of compounds A3, A9, or A10 for 5 days. Bar = 1 cm. **(F)** Kidney weights for the different treatment groups. N for different groups is listed in Table 2. **(G)** Chemical structure of compound A3, which is cardamonin. Data are presented as mean ± SEM. *P < 0.05, ***P < 0.001 *vs*. MDCK or WT control group. ##P < 0.01 *vs*. PKD control group.

Based on the above experimental results, compounds A3, C9, and C10 were selected for further evaluation using a PKD mouse model (Supplementary Figure 2).

All 3 compounds (at 100 mg (kg· d)$^{-1}$) had little effect on the body weight, liver index, and kidney index of wild-type mice (Supplementary Table 2). These compounds did not affect body weight or liver index in PKD mice either (Supplementary Table 2). Interestingly, compound A3 more effectively and stably decreased kidney size, weight (Figure 1E, F), and index (Supplementary Table 2) than compounds C9 and C10 in PKD mice. Compound A3 is cardamonin, whose chemical structure is showed in Figure 1G. Based on our *in vitro* and *in vivo* experimental results, we identified cardamonin as an ideal candidate compound for further pharmacological and mechanistic studies.

3.2 Cardamonin ameliorated renal cyst development in PKD mice

Cardamonin treatment (100 mg (kg· d)$^{-1}$ for 5 days) did not affect growth and physical appearance in both wild-type and PKD mice (Figure 2A). HE-stained sections showed that cardamonin significantly slowed cyst development in PKD kidneys, but did not affect wild-type kidneys (Figure 2B). The cyst index for PKD kidneys was obviously decreased following cardamonin treatment (Figure 2C). Immunofluorescence staining showed that cardamonin significantly reduced the cyst size derived from collecting duct in PKD mice (Figure 2D). These results indicate that cardamonin inhibits cyst enlargement.

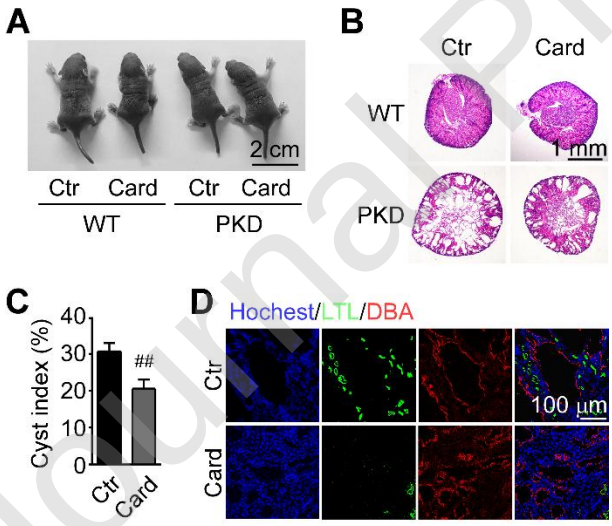


Figure 2. Cardamonin significantly inhibited renal cyst development in PKD mice. **(A)** Physical appearances of mice in wild-type (WT) and PKD groups treated without (Ctr) or with cardamonin (Card). Bar = 2 cm. **(B)** Representative HE staining images of WT and PKD mice with or without cardamonin treatment. Bar = 1 mm. **(C)** Histogram of cyst index in control and cardamonin-treated PKD mice. n = 8. **(D)** LTL

(Green), DBA (Red), and Hochest (blue) immunofluorescence staining in kidneys from control and cardamonin-treated PKD mice. Bar = 100 μm. Data are presented as mean ± SEM. ##P < 0.01 *vs*. PKD control group.

3.3 Cardamonin dose-dependently inhibited formation and enlargement of cysts in MDCK cyst model and embryonic kidney cyst model

To determine the effects of cardamonin on cyst formation, MDCK cells were cultured in a 3D collagen I gel and treated either without or with 0.625, 2.5, 10 μM cardamonin in the presence of 10 μM FSK over a 6-day time span. On day 6, the number of cysts (diameter ≥ 50 μm) and non-cyst colonies were counted. There was no significant difference in the total number of colonies (including colonies of both cells and cysts) among all groups (Figure 3A). However, there were dose dependently fewer cysts in cardamonin treated group than control group (Figure 3B).

When MDCK cysts were incubated with different doses of cardamonin from day 5 to day 12 under FSK-stimulated conditions, cardamonin significantly repressed cyst enlargement in a dose-dependent manner (Figure 3C, D). After washing out cardamonin from day 9, cyst growth curve showed slightly rise-up from day 10 to day 12, but without significant difference, implying that cardamonin persistently and irreversibly inhibited cyst enlargement.

The inhibitory effect of cardamonin on renal cysts was also tested in the embryonic kidney cyst model. In the presence of 8-Br-cAMP, numerous cystic structures developed rapidly in cultured embryonic kidneys, while the renal cyst development was significantly inhibited by 2.5 and 10 μM cardamonin treatment (Figure 3E). After 6 days of continuous photo tracking, we quantified the cyst area fraction and found that cardamonin did not affect kidney growth but remarkably decreased the cyst area (Figure 3E, F). Removing cardamonin from the embryonic kidney on day 4 did not alter the depressed cysts and showed no apparent recovery compared with the group incubated with 10 μM cardamonin from day 0 to day 6. These results suggest that cardamonin significantly and dose-dependently inhibited cyst formation and enlargement, and that this inhibition is not dependent on cytotoxicity.

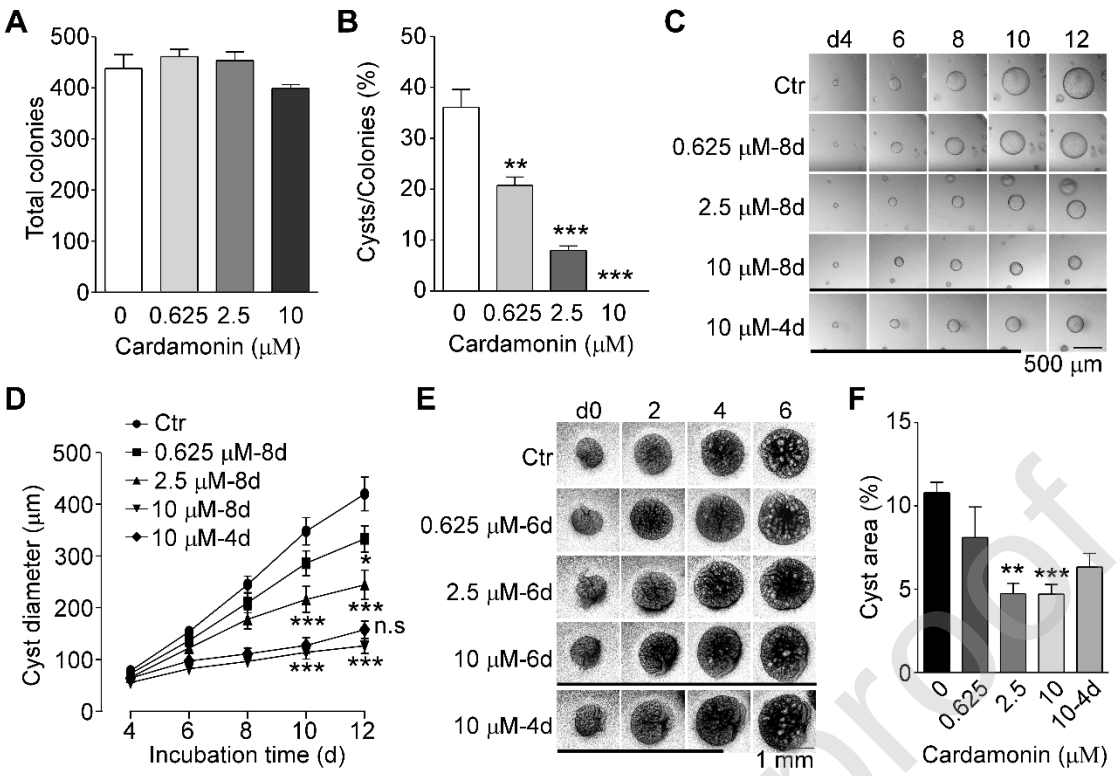


Figure 3. Cardamonin suppressed PKA-mediated cyst growth *in vitro*. (A) Histogram of total colony number in groups with or without exposure to different concentrations of cardamonin for 6 days. n = 3. **(B)** Ratio of cyst number to colony number. n = 3. **(C)** Representative images of MDCK cyst growth in gel incubated for 8 or 4 days. Bar = 500 μm. Thick black lines indicate the incubated time with cardamonin. **(D)** Cyst diameters. n = 20. **(E).** Representative images of embryonic kidney cysts treated with various concentrations of cardamonin from day 0 to day 4 or day 6. Bar = 1 mm. Thick black lines indicate the incubated time with cardamonin. **(F)** Ratios of total cyst area to whole kidney area in embryonic kidneys. n = 7. Data are presented as mean ± SEM. **P < 0.01, ***P < 0.001 *vs.* control group. n.s represents no significant difference compared with 10 μM-8d group.

3.4 Cardamonin reduced cyst epithelial proliferation by inhibiting the MAPK, Wnt, and mTOR signaling pathways in ADPKD mice

Given that abnormal epithelial cell proliferation exists throughout the process of cyst development, we hypothesized that the cyst inhibition of cardamonin resulted from slowing cell proliferation. To confirm this hypothesis and investigate the underlying protective mechanisms of cardamonin, we examined the expression levels of several proliferation indicators in PKD kidneys. PKD mice treated with cardamonin exhibited

significantly decreased Ki-67 expression, with a 53.74% reduction in renal Ki-67 expression compared with PKD mice treated with vehicle (Ctr) (Figure 4A, B). Western blots showed that cardamonin down-regulated the expression of PCNA (Figure 4C, D), implying an inhibition of cell proliferation. To further elucidate the effect of cardamonin on cell proliferation, we detected pivotal proliferation-related signaling pathways in PKD and found that the activation and expression of ERK1/2, β-catenin, S6 and its upstream molecule mTOR, were all inhibited by cardamonin (Figure 4E, F). Meanwhile, cardamonin had little influence on these proliferative signaling pathways in wild-type mice. These results suggest that cardamonin inhibited cyst epithelial cell proliferation by repressing the MAPK, Wnt, and mTOR signaling pathways.

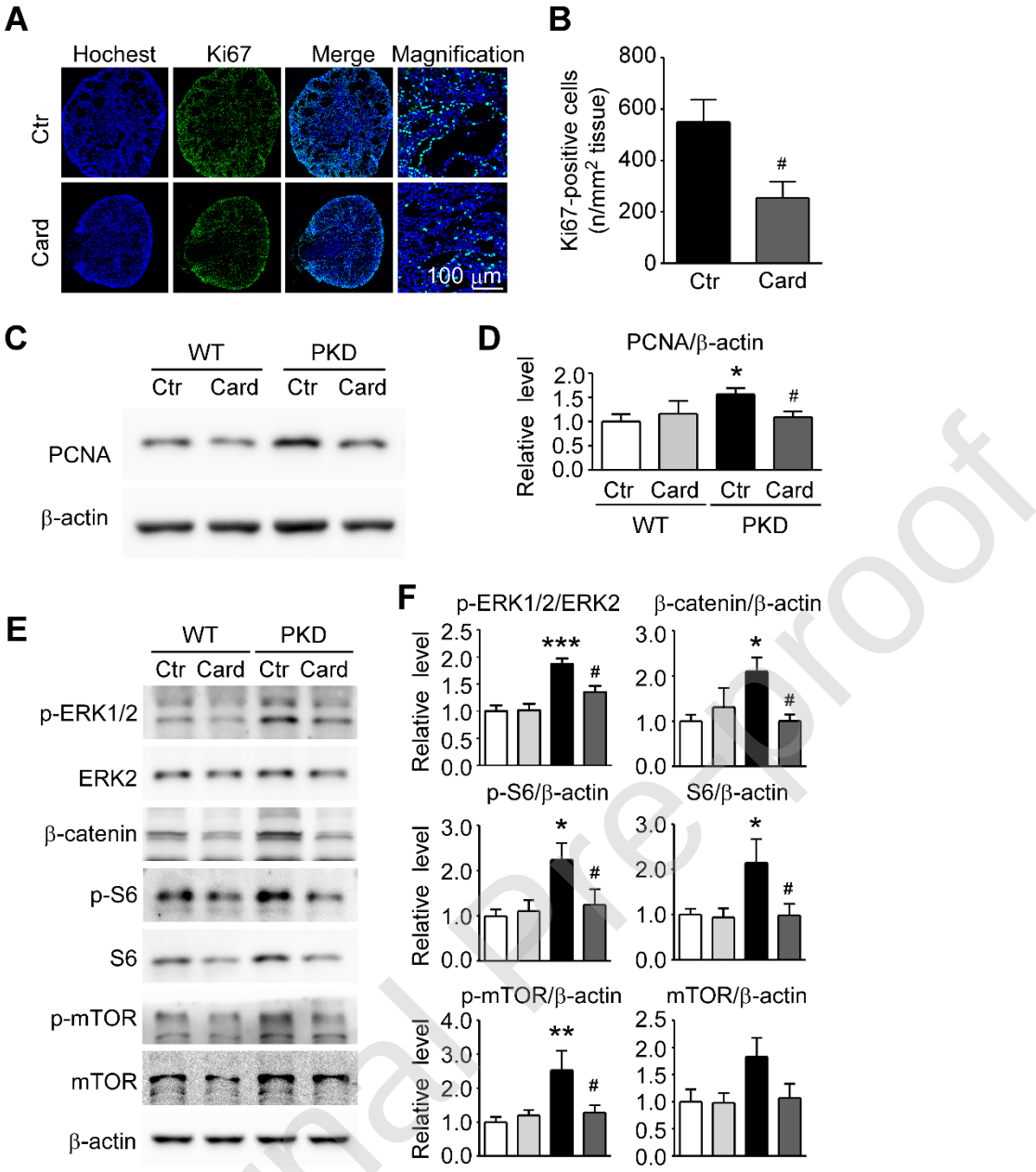


Figure 4. Cardamonin inhibited cell proliferation and related pathways in PKD kidneys. (A) Immunofluorescence staining of Ki-67 (Green) in PKD kidneys treated without (Ctr) or with cardamonin (Card). Blue represents nuclei stained with Hochest. Bar = 100 μm. **(B)** Analysis of the number of Ki-67-positive cells per mm^2 of tissue. n =5. **(C)** Western blot for PCNA in wild-type (WT) and PKD mice with or without cardamonin treatment. **(D)** Relative expression levels of PCNA to β-actin. n = 5. **(E)** Representative Western blots for cell proliferation signaling proteins in the kidneys. **(F)** Bar graph shows the relative expression of different proteins. n = 5~6. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 *vs*. WT control group.

#P < 0.05 *vs*. PKD control group.

3.5 Cardamonin inhibited fibrosis by regulating the TGF-β signaling pathway

Considering fibrosis has been recognized as a critical pathology during PKD pathogenesis (Song et al., 2017), we tested whether cardamonin affected this process. A hallmark of fibrosis is excessive extracellular matrix (ECM) deposition (Nastase, Zeng-Brouwers, Wygrecka, & Schaefer, 2018). Masson's trichrome staining showed that cardamonin treatment robustly reduced collagen deposition in PKD mouse kidneys (Figure 5A, B). Correspondingly, Western blot showed decreased FN levels in the cardamonin-treated PKD groups compared to the PKD controls. Up-regulation of α-SMA was also found in PKD kidney, implying increased fibroblast cells which is a potential producer of ECM. We found that cardamonin slightly suppressed α-SMA levels in PKD kidneys, with undetectable effects on wild-type kidneys (Figure 5C, D). Additionally, TGF-β and its downstream effector p-Smad2/3, as the major pro-fibrotic factor in PKD (Song et al., 2017), were also declined following cardamonin treatment.

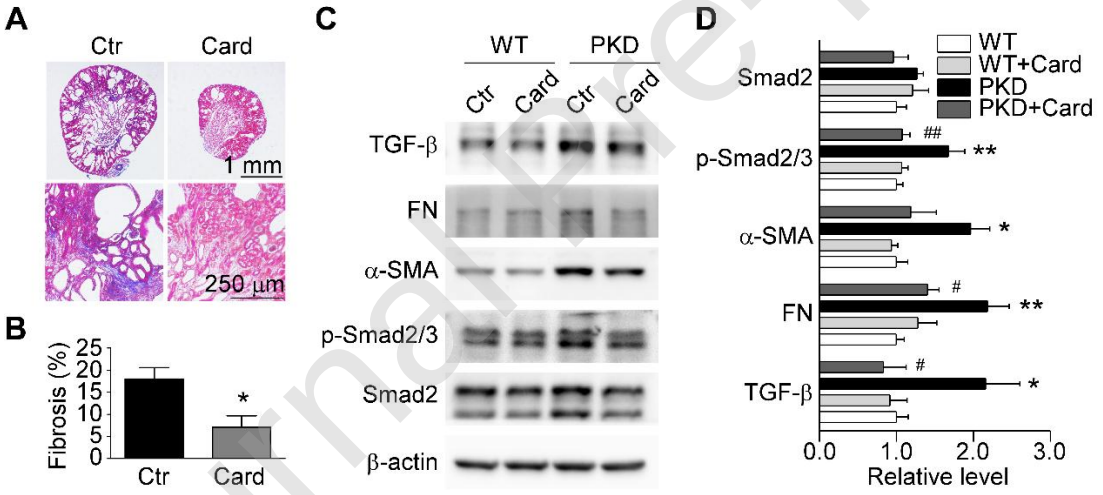


Figure 5. Cardamonin alleviated fibrosis in PKD kidneys. (A) Masson's trichrome staining of PKD mouse kidneys with or without cardamonin (Card) treatment. Bar (top) = 1 mm, Bar (bottom) = 250 μm. **(B)** Ratios of positive fibrotic area to whole kidney area in PKD mouse kidneys with or without cardamonin treatment. n = 5. **(C)** Representative Western blots for fibrosis-related proteins in wild-type (WT) and PKD kidney lysates with or without cardamonin treatment. **(D)** Quantification of fibrotic protein expression levels relative to β-actin. n = 5~6. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01 *vs*. WT control group. #P < 0.05, ##P < 0.01 *vs*. PKD

control group.

To further clarify whether TGF-β signaling is involved in cardamonin-mediated fibrosis inhibition, expression of fibrosis markers were monitored in cultured MDCK cells incubated with 10 ng ml^{-1} TGF-β in the presence of different doses of cardamonin. Cardamonin at 10 μM significantly repressed the upregulation of FN and restored the expression levels of α-SMA and E-cadherin (Figure 6A, B), indicating its inhibition of the epithelial-to-mesenchymal transition (EMT) in PKD kidneys (D. Y. Kim et al., 2019). In addition, cardamonin treatment dose-dependently down-regulated the level of p-Smad2/3 after TGF-β stimulation (Figure 6C, D). These results demonstrate that cardamonin effectively suppressed fibrosis by regulating TGF-β/Smad signaling.

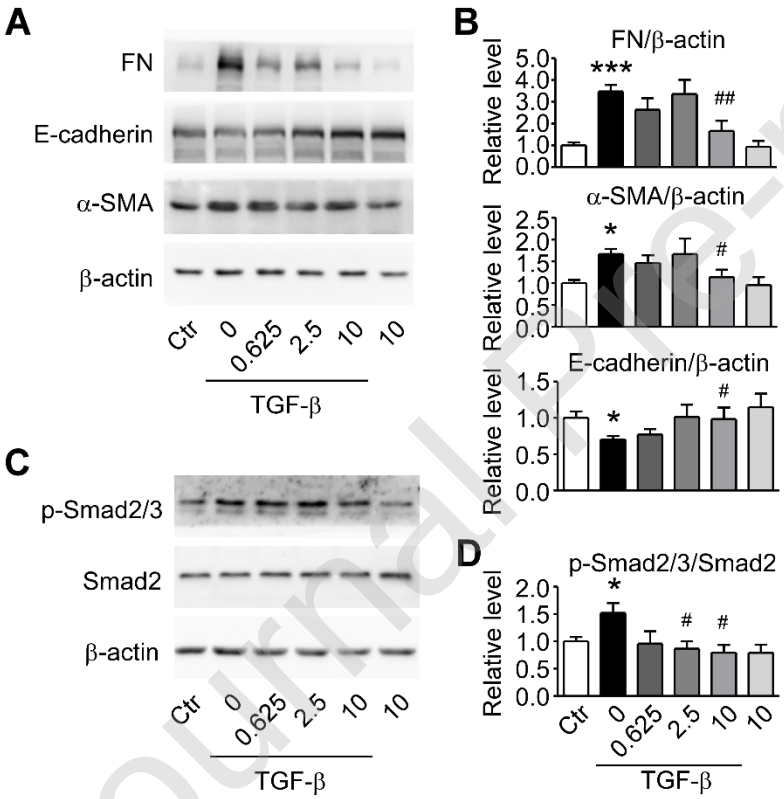


Figure 6. Cardamonin regulated TGF-β-mediated fibrotic process *in vitro*. (A) Representative Western blots for relevant TGF-β-stimulated fibrotic proteins. **(B)** Quantification of fibrotic protein relative expression levels. **(C)** Representative Western blots for TGF-β–meditated p-Smad2/3 and Smad2 expression levels. **(D)** Quantification of the relative level of p-Smad2/3 and Smad2. Data are presented as mean ± SEM. n = 5. *P < 0.05, ***P < 0.001 *vs*. control group. #P < 0.05, ##P < 0.01 *vs*. MDCK treated with TGF-β group.

4. Discussion

In this study, we identified cardamonin as an active compound for PKD treatment utilizing MDCK cyst model, embryonic cyst model and PKD mouse model. Importantly, the current study provided well-defined evidence that cardamonin alleviated PKD progression by reducing both renal cyst growth and interstitial fibrosis. Furthermore, downregulation of the MAPK, mTOR, Wnt/β-catenin, and TGF-β/Smad signaling pathways contributed to cardamonin-mediated disease mitigation (Figure 7).

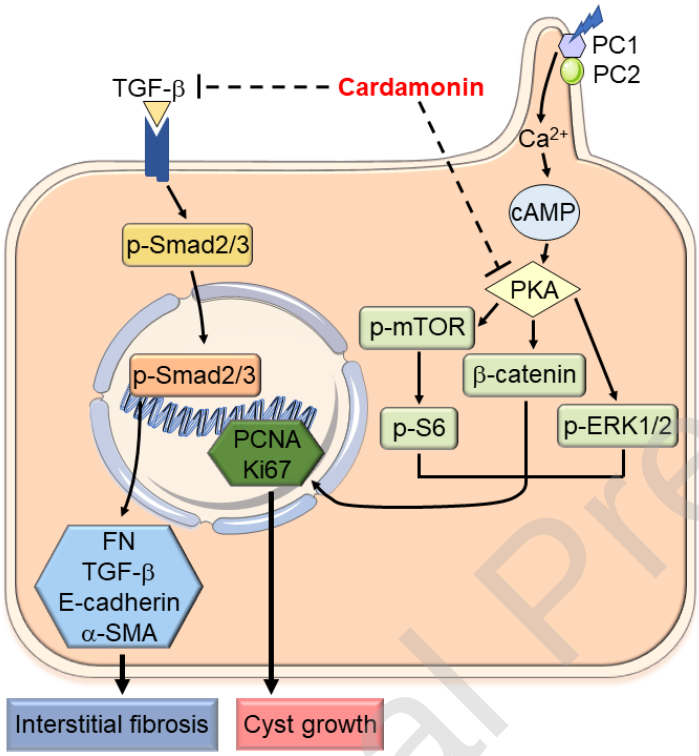


Figure 7. Tentative hypothesis for the mechanism by which cardamonin retards PKD progression. Arrows indicate an activated regulation between two molecules. Dotted lines indicate an undefined directly-inhibited relationship between cardamonin and PKA or TGF-β. Consult the text for specific details.

In ADPKD, the deficiency in PC1 or PC2, caused by mutations in *PKD1* or *PKD2* respectively, leads to sustained abnormal cytoplasmic calcium levels and elevated intracellular cAMP, which subsequently activate the MAPK, mTOR, Wnt/β-catenin, and additional downstream signaling pathways (V. E. Torres & Harris, 2014). PKA-mediated activation of the MAPK and mTOR pathways has been recognized as a major pro-proliferative signaling pathway in PKD. Therefore, targeting the MAPK and

mTOR pathways may have crucial impacts on the development of ADPKD. Several candidate drugs such as sorafenib, bosutinib, tesevatinib, and rapamycin (Cornec-Le Gall et al., 2019; Lanktree & Chapman, 2017), which target the MAPK and mTOR pathways separately, have shown benefits in preclinical studies and are undergoing clinical trials. Previously, we also found that ginkgolide B, curcumin, and *Ganoderma* triterpenes inhibited the formation and enlargement of renal cysts by regulating Ras/MAPK signaling and promoting cell differentiation, without affecting the mTOR pathway (Gao et al., 2011; V. E. Torres et al., 2017; Zhou et al., 2012).

In addition, upregulation of β-catenin contributes to the increased cell proliferation index in PKD kidneys. Pharmacologic inhibition of β-catenin also showed protective effects against PKD progression (A. Li et al., 2018; Xu et al., 2018). Sustained activation of Wnt/β-catenin blocks normal epithelial tubulogenesis (Gallegos et al., 2012). Thus, down-regulation of β-catenin in the cardamonin-treated groups may have contributed to the improvement of cell differentiation in PKD mice, which eventually alleviated renal cyst development.

Interestingly, ADPKD and some solid tumors share MAPK, mTOR and Wnt/β-catenin signaling pathways for their sustained cell proliferation (Galluzzi, Spranger, Fuchs, & Lopez-Soto, 2019; Seeger-Nukpezah, Geynisman, Nikonova, Benzing, & Golemis, 2015). MAPK signaling, which plays a pivotal role in cell proliferation, differentiation, and apoptosis, was found to be aberrantly expressed in almost one-third of all human cancers including renal cell carcinomas, pancreatic ductal adenocarcinomas, and colorectal cancers (Roskoski, 2019). Dysregulation of mTOR signaling as the major constitution contributes to abnormal epithelial cell proliferation in ADPKD and drives the progression of various cancers, including gastric cancer and renal cell carcinoma, by mediating metabolic homeostasis, cell growth, and autophagy (Ma, Yung, & Chan, 2018; X. Zhang et al., 2019). In adrenocortical, hepatocellular, pulmonary, and several other carcinomas, elevated Wnt/β-catenin signaling contributes substantially to tumor progression by stimulating cancer cell proliferation and metastasis (Galluzzi et al., 2019). Furthermore, atypical fibrotic changes have been recognized as the most crucial ECM remodeling manifestation in cancer. Although TGF-β signaling serves as a “double edged sword” in cancer progression, TGF-β/Smad-mediated EMT and immune evasion are the crucial forces enabling the development of various late-stage cancers (Batlle & Massague, 2019). These data

indicate that ADPKD might have the same therapeutic targets as some tumors, which sheds light on potential novel therapies (Seeger-Nukpezah et al., 2015).

It has been found that certain natural products derived from fruits, vegetables, and animals show promising potential to inhibit cancer have been identified potential to slow down PKD disease. Triptolide, a traditional Chinese medicine, slows carcinogenesis via impairing tumor growth and metastasis, and inducing apoptosis (Noel et al., 2019), It also reduces cyst growth by restoring calcium levels in cells (Leuenroth et al., 2007). Quercetin, which is derived from certain fruits and vegetables, exerts anti-tumor effects by inducing cell death, and also suppresses renal cyst development *in vitro* and *in vivo* by reducing the activation of the ERK and Akt signaling pathways (Lan, Chen, Kuo, Lu, & Yen, 2019; Zhu et al., 2018). Besides, other cancer therapy interventions also exert beneficial effects in PKD treatment, indicating the mechanistic and therapeutic overlap between cancer and PKD. Ketogenic diets, which are high-fat, low-carbohydrate, and adequate-protein diet, can counteract the metabolic alterations in cancer cells and facilitate tumor repression in several cancer types (Weber et al., 2019). A recent study showed that feeding animals with ketogenic diet robustly ameliorated the progression of PKD by strongly inhibiting mTOR signaling, proliferation, and fibrosis (J. A. Torres et al., 2019). These previous studies reinforce our strategy to screen for novel PKD drugs from anti-tumor compounds.

From non-cytotoxic anti-tumor active compounds, we found that cardamonin suppressed renal cyst development in both *in vivo* and *in vitro* experimental models. Cardamonin is a natural chalcone extracted from *Alpiniae Katsumadai* semen, which is a common nutraceutical identified from dietary agents (Rajagopal, Lankadasari, Aranjani, & Harikumar, 2018). In recent years cardamonin has been studied extensively for its pleiotropic bioactivity, including anti-tumor, anti-inflammation, anti-oxidation, and EMT-inhibition effects (Hou, Yuan, Li, Hou, & Liu, 2019; James et al., 2017; Jia et al., 2015; K. Wang et al., 2018). A constellation of cell and animal experiments have suggested that cardamonin could regulate proliferation-related signaling pathways (Chen, Shi, Niu, Zhu, & Zhou, 2018; Hou et al., 2019; Jia et al., 2015; J. Zhang et al., 2017), most of which, such as the MAPK signaling pathway, are relevant with renal cyst development (Y. J. Kim, Kang, Choi, & Ko, 2015). Additionally, cardamonin has been elucidated with capability of blocking β-catenin signaling in breast cancer cell line (Chen et al., 2018; Jia et al., 2015), implying that cardamonin may be able to target

those pathological process that overlap with PKD.

Utilizing FSK and 8-Br-cAMP-driven cyst formation and growth models, we showed that cardamonin can interfere with PKA-elicited cell proliferation. Moreover, our results indicated that cardamonin reduced the expression of p-ERK1/2, p-mTOR, and p-S6 in PKD mice, which are key components in the MAPK and mTOR pathways. Cardamonin also decreased the expression of total mTOR and S6, which were also abnormal in PKD mice, demonstrating its inhibition of these pathways. Cardamonin is capable of inhibiting cyst growth as well as reducing cyst formation in MDCK cyst model.

Previous studies have shown that cardamonin exerts notable anti-cancer effects by inhibiting EMT (E. J. Kim et al., 2015; Y. J. Kim et al., 2015; Shrivastava et al., 2017; J. Zhang et al., 2017). However, the specific relationship between cardamonin and EMT or fibrosis is not clear. Surprisingly, by detecting for TGF-β/Smad signaling *in vitro* and *in vivo*, we found that cardamonin ameliorated renal fibrosis and restored the expression level of FN, E-cadherin, and α-SMA by down-regulating TGF-β/Smad signaling. These results establish a plausible relationship between cardamonin treatment and TGF-β-mediated fibrosis mitigation.

Fibrosis is a critical and irreversible process throughout the course of ADPKD progression, in which TGF-β/Smad-mediated ECM deposition and EMT are highly upregulated (Song et al., 2017). TGF-β is a major fibrogenic driver and highly expressed in cystic epithelia in human, rat, and mouse PKD (Hassane et al., 2010). Inhibition of renal fibrosis may be a viable strategy for PKD treatment. Recent studies have shown that knock-down of integrin-linked kinase slows the progression of PKD by reducing renal fibrosis (Raman et al., 2017). Smad is a central downstream effector, with increased nuclear accumulation of phosphorylated Smad2 present in cyst-lining cells. Additionally, the Smad-specific inhibitor AZ505 delayed PKD progression in animal models, indicating that TGF-β/Smad signaling accounts for the development of cysts (L. X. Li et al., 2017). It has been demonstrated that inhibition of the TGF-β superfamily activin slowed cyst growth associated with reduced Smad2/3 signaling (Leonhard et al., 2016). The inhibitory effects of cardamonin on cAMP-mediated cyst growth and TGF-β-mediated fibrosis suggest it may be a promising candidate for PKD treatment.

In addition to the abnormal proliferation and interstitial fibrosis, fluid secretion

into cyst lumen, especially the chloride ion secretion, also has been recognized as a major driving force of cyst enlargement (Nantavishit, Chatsudthipong, & Soodvilai, 2018). The effect of cardamonin on fluid secretion has not been defined. Cystic fibrosis transmembrane conductance regulator mediates chloride secretion and abnormal Na^+/K^+-ATPase expression play crucial role in water handling in the formation of PKD cysts (Ghata & Cowley, 2017). Thus, the impact of cardamonin on ion channel and transporters will be further explored in our subsequent studies.

Additionally, hypoxia has been characterized as an universal phenomenon in PKD, in which hypoxia-inducible transcription factor 1α (HIF-1α) might promote the expansion of cyst *in vivo* and *in vitro* (Buchholz et al., 2014; Kraus et al., 2018). Meanwhile, cardamonin exerts inhibitory effect on HIF-1α expression. We speculate that cardamonin might suppress the HIF-1α level in PKD cysts. However, the expression level of HIF-1α in PKD kidney depends on the progression degree of PKD (Kraus et al., 2018). Due to the limitation of the PKD mice we used in this study, we look forward to study this effect with an adult inducible PKD animal model.

In summary, our study elucidated that cardamonin attenuated renal cyst development by inhibiting MAPK, Wnt, and mTOR-mediated cyst epithelial cell proliferation, and reduced TGF-β-regulated fibrosis. The experimental results showed that cardamonin retards the progression of ADPKD, which provides a clue that cardamonin could be applied to prevent and/or treat ADPKD or other fibrotic diseases. However, all data in this study were obtained from cell and animal models. To develop cardamonin into a novel therapeutic drug for ADPKD treatment, the pharmacological characteristics of cardamonin should be further elucidated in preclinical tests and clinical trials.

Conflict of interest statement

The authors declare no competing financial interests.

Author contributions

B.Y. is the corresponding author. J.H., H.Z., and B.Y. designed the experiments. J.H. performed the research and analyzed the data. J.H., J.M., S.Z., S.W, X.L, S.Z., X.G., H.Z., and B.Y. interpreted the results. J.H., H.Z., W.J., and B.Y. drafted and edited the manuscript. All authors commented on and approved the final manuscript.

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