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Resveratrol inhibits calcium oxalate crystal growth, reduces adhesion to renal cells and induces crystal internalization into the cells, but promotes crystal aggregation

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ABSTRACT

Resveratrol is a natural phenolic compound that belongs to stilbenoid group found in diverse plants. Health benefits and therapeutic potentials of resveratrol have been widely recognized in various diseases. In kidney stone disease, it can alleviate oxalate-induced hyperproduction of free radicals in renal epithelial cells. Nevertheless, its direct effects on calcium oxalate (CaOx) crystal, which is the major stone component, remained unclear. This study therefore addressed the direct effects of resveratrol (at 1, 10 or 100 μM) on each step of CaOx kidney stone formation. The results revealed that resveratrol had no significant effects on CaOx crystallization. However, resveratrol significantly decreased CaOx crystal growth and adhesion to renal epithelial cells at all concentrations, and induced crystal internalization into the cells (a process related to crystal degradation by endolysosomes) in a concentration-dependent manner. On the other hand, resveratrol promoted crystal aggregation. These data indicate that resveratrol serves as a dual modulator on CaOx stone formation. While it inhibits CaOx stone development by reducing crystal growth and adhesion to renal cells and by inducing crystal internalization into the cells, resveratrol promotes crystal aggregation, which is one of the mechanisms leading to kidney stone formation.

1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene) is a natural phenolic compound and a member of the stilbenoid group found in diverse plants, e.g., peanuts, grapes, blueberries and cranberries (Li et al., 2024). It is also known as a phytoalexin that serves as a plant-derived anti-oxidant and antibiotic (Recalde et al., 2020; Song et al., 2021; Wang et al., 2022a). Other health benefits and therapeutic potentials of resveratrol and its derivatives have been widely recognized in various diseases, including diabetes (Park et al., 2016; Xu et al., 2022), cardiovascular diseases (Lu et al., 2019; Xia et al., 2017), Alzheimer's disease (Han et al., 2023; Khan et al., 2023), and cancers (Arif et al., 2024; Dariya et al., 2023). Resveratrol also shows anti-inflammatory effects in mice with colitis and mucosal damage (Zhang et al., 2023). Additionally, resveratrol ameliorates high glucose-induced endoplasmic reticulum stress, cellular apoptosis and hyperuricemia-induced renal injury (Xiao et al., 2021; Zhang et al., 2020). Moreover, the anti-fibrotic effects of resveratrol have been well documented in several organ systems, including kidney (Wang et al., 2022b; Zhang et al., 2019), liver (Anapali et al., 2022; Mostafa et al., 2023), lung (Liu et al., 2023; Singh et al., 2023) and prostate (Zeng et al., 2018).

Kidney stone disease is a common medical problem caused by deposition of precipitated solid salts (in crystalline forms) within the kidney. With rises in its incidence and prevalence as well as its high recurrence rate, this disease is considered a global healthcare burden (Lang et al., 2022; Tasian et al., 2017). Dietary management using natural products and nutritional supplements is getting more attractive to reduce the risk of this disease (Peerapen and Thongboonkerd, 2023). For example, red wine intake is associated with a decrease in the prevalence of kidney stone disease (Ferraro et al., 2013; Legay et al., 2023). In addition, a previous study has reported that polyphenols extracted from grape seeds can rescue renal injury by mitigating intrarenal crystalline deposition in ethylene glycol-induced kidney stone rats (Grases et al., 2015). Interestingly, resveratrol can be detected in human urine and serves as a potential biomarker for wine intake (Urpi-Sarda et al., 2015; Zamora-Ros et al., 2009, 2017). A few studies have reported the

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anti-lithiatic properties of resveratrol to prevent kidney stone disease (Hong et al., 2013; Oksay et al., 2017; Wu et al., 2021; Ye et al., 2021). Resveratrol alleviates oxalate-induced hyperproduction of free radicals in renal epithelial cells (Hong et al., 2013). It also increases the level of intracellular malondialdehyde, an oxidative stress marker, in renal epithelial cells (Hong et al., 2013). Consistently, the study in rats with hyperoxaluria, one of the major risks for calcium oxalate (CaOx) stone, has revealed that resveratrol ameliorates hyperoxaluria-induced increase of serum malondialdehyde level (Hong et al., 2013; Oksay et al., 2017).

However, the direct effects of resveratrol on CaOx crystals remained unclear. We therefore hypothesized that resveratrol has some direct modulatory activities on CaOx crystal, which is a major stone component. To verify our hypothesis, a set of crystal assays representing various steps of kidney stone formation were performed and the direct effects of resveratrol on the CaOx crystal, if any, were examined.

2. Materials & methods

2.1. Chemical preparation

A crystallization buffer containing 10 mM Tris (Affymetrix Inc.; Cleveland, OH) and 90 mM sodium chloride (Bio Basic Inc.; Toronto, Canada) was prepared at a final pH of 7.4. Stock solutions of 10 mM calcium chloride (Merck; Branchburg, NJ) and 1 mM sodium oxalate (Sigma-Aldrich; St. Louis, MO) were then prepared in the crystallization buffer. Resveratrol (Sigma-Aldrich) was solubilized in absolute ethanol at 0.1, 1 and 10 mM.

2.2. Preparation of plain and fluorescein isothiocyanate (FITC)-labeled CaOx crystals

Plain CaOx crystals (for crystal aggregation and crystal-cell adhesion assays) were prepared following a previously described protocol (Thongboonkerd et al., 2006, 2008). Briefly, 500 ml of 1 mM sodium oxalate in crystallization buffer was slowly poured into 500 ml of 10 mM calcium chloride in crystallization buffer. Following overnight incubation at 25 °C, CaOx crystals were harvested by centrifugation at 2000 *g* and 25 °C for 5 min. After washing with methanol, the crystals were air-dried.

FITC-labeled CaOx crystals (for crystal internalization assay) were prepared following the previously described protocol (Chaiyarit et al., 2010, 2016). Briefly, 0.1 μg/ml FITC (Thermo Scientific Pierce; Rockford, IL) was added to 10 mM calcium chloride in crystallization buffer before being mixed gently with 1 mM sodium oxalate in crystallization buffer as described for the plain crystals. Following overnight incubation at 25 °C in the dark, FITC-labeled CaOx crystals were harvested by centrifugation at 2000 *g* and 25 °C for 5 min. After washing with methanol, the crystals were air-dried and kept in the dark until used.

2.3. Preparation of renal cell monolayer

The renal cell monolayer (for crystal-cell adhesion and internalization assays) was prepared from MDCK renal cell line (ATCC; Manassas, VA). The cells were cultivated in Eagle's minimum essential medium (Gibco; Grand Island, NY) containing 10% fetal bovine serum (Gibco), 60 U/ml penicillin G (Sigma-Aldrich) and 60 μg/ml streptomycin (Sigma-Aldrich) in each well of 6-well culture plate (Corning Costar; Cambridge, MA). After overnight maintenance in a CO_2 incubator at 37 °C with 5% CO_2 and 95% relative humidity, the medium was removed and the cell monolayer was washed twice with PBS before use.

2.4. Crystallization assay

This assay was done following a previously described protocol (Thongboonkerd et al., 2006, 2008). Briefly, 500 μl of 10 mM calcium chloride in crystallization buffer was added with 10 μl of 100 μM, 1 mM or 10 mM resveratrol (Sigma-Aldrich) or ethanol (the solvent used to solubilize resveratrol) in each well of 24-well plate (Corning Costar). Thereafter, 500 μl of 1 mM sodium oxalate in crystallization buffer was gently added into each well. The final concentration of resveratrol in each well was 1, 10 or 100 μM, whereas the well added with ethanol served as the blank control. After 1-h incubation at 25 °C, an inverted phase-contrast microscope (Eclipse Ti–S) (Nikon; Tokyo, Japan) was employed to capture the images of newly generated crystals (neocrystals). Crystal size was then measured from 100 random crystals per condition using NIS-Elements D version 4.11 (Nikon). Number of the crystals was counted from 10 random fields per condition. Crystal mass was then calculated by using the following formula.

$$\text{Crystal mass } (\mu m^2/\text{field}) = \text{Average crystal size in each field } (\mu m^2) \times \text{Average crystal number in each field (/field)} \quad \text{Formula I}$$

2.5. Crystal growth assay

This assay was done following a previously described protocol (Amimanan et al., 2017; Khamchun et al., 2019). Briefly, 500 μl of 1 mM sodium oxalate in crystallization buffer was gently added into each well of 24-well plate containing 500 μl of 10 mM calcium chloride in crystallization buffer. After 1-h incubation at 25 °C (termed as "T_0"), when the neocrystals completely formed, the Eclipse Ti–S inverted phase-contrast microscope was employed to capture the images of these neocrystals. Then, 10 μl of 100 μM, 1 mM or 10 mM resveratrol or ethanol (the solvent used to solubilize resveratrol) was added into each well. The final concentration of resveratrol in each well was 1, 10 or 100 μM, whereas the well added with ethanol served as the blank control. After 60-min incubation at 25 °C (termed as "T_{60}"), the images of these growing crystals were taken again by using the Eclipse Ti–S inverted phase-contrast microscope. Crystal sizes at T_0 and T_{60} were then measured from 100 random crystals per condition by using the NIS-Elements D version 4.11 (Nikon). Crystal growth (represented by Δ crystal size) was calculated as follows.

$$\Delta \text{ Crystal size } (\mu m^2) = \text{Crystal size at } T_{60} \ (\mu m^2) - \text{Crystal size at } T_0 \ (\mu m^2) \quad \text{Formula II}$$

2.6. Crystal aggregation assay

This assay was done using a previously described protocol (Chaiyarit and Thongboonkerd, 2017; Kanlaya et al., 2019). Briefly, 1000 μg of the dried plain CaOx crystals prepared as described above was resuspended in 1 ml crystallization buffer in each well of 6-well plate. Subsequently, 10 μl of 100 μM, 1 mM or 10 mM resveratrol or ethanol (the solvent used to solubilize resveratrol) was added into each well. The final concentration of resveratrol in each well was 1, 10 or 100 μM, whereas the well added with ethanol served as the blank control. The mixtures were then shaken at 300 rpm in a ThermoMixer C (Eppendorf; Hauppauge, NY) at 25 °C for 1 h. Thereafter, the Eclipse Ti–S inverted phase-contrast microscope was employed to capture the images of the crystal aggregates, each of which was defined as a tight assembly of at least 3 crystals (Chaiyarit and Thongboonkerd, 2017). The number of these crystal aggregates was counted from 10 random fields per condition.

2.7. Crystal-cell adhesion assay

This assay was done using a previously described protocol (Fong-ngern et al., 2016; Peerapen and Thongboonkerd, 2016). MDCK cell monolayer was prepared as described above. After washing with PBS, the fresh medium containing 100 μg/ml plain CaOx crystals and 10 μl of

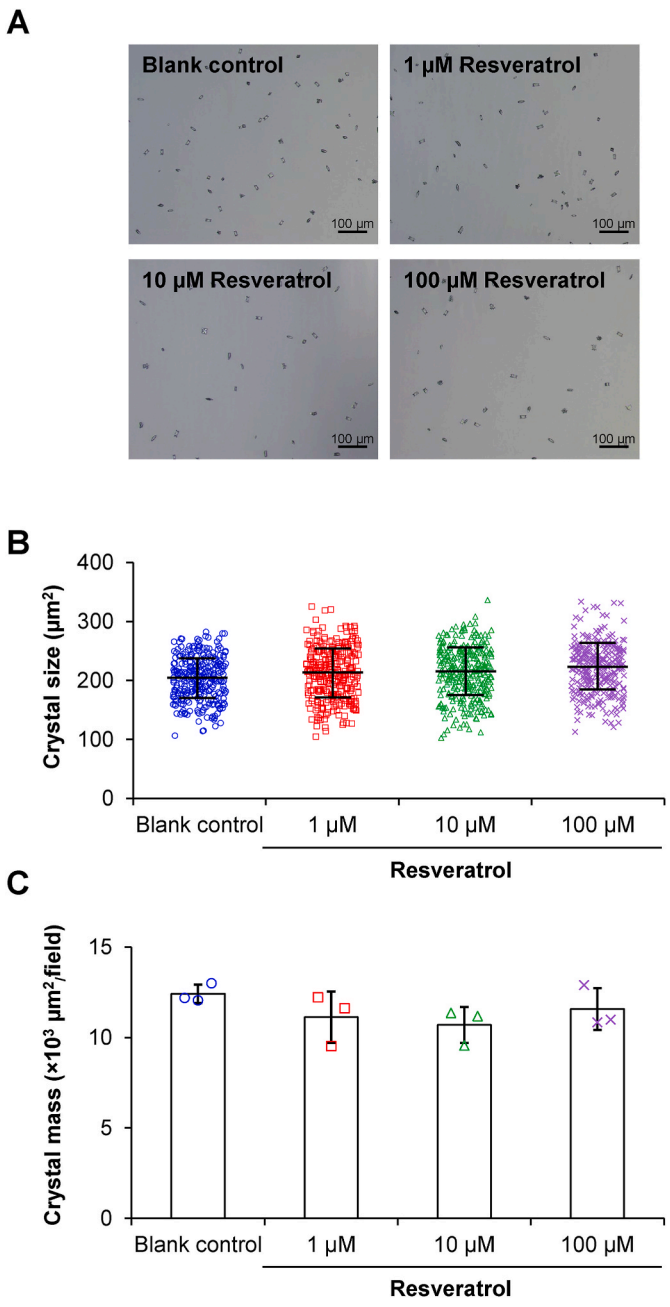


Fig. 1. Effects of resveratrol on CaOx crystallization. Crystallization assay was performed in the absence (blank control) or presence of resveratrol (at a final concentration of 1, 10 or 100 μM). **(A):** Microscopic images of newly generated CaOx crystals (neocrystals) after 1-h incubation. **(B):** Crystal size was measured from 100 random crystals per condition. **(C):** Crystal mass was calculated from 10 random fields according to *Formula I* (see **Materials and Methods**). The data are presented as mean ± SD of measurements from three independent experiments.

100 μM, 1 mM or 10 mM resveratrol or ethanol (the solvent used to solubilize resveratrol) were added into each well of 6-well culture plate. The final concentration of resveratrol in each well was 1, 10 or 100 μM, whereas the well added with ethanol served as the blank control. After 1-h incubation, the culture supernatant was discarded followed by 5 vigorous washes with PBS to remove the non-adhered crystals. The adhered crystals were then observed and counted from 10 random fields per condition under an inverted microscope.

2.8. Crystal internalization assay

This assay was done using a previously described protocol (Kanlaya et al., 2013; Khamchun et al., 2021). MDCK cell monolayer was prepared as described above. After washing with PBS, the fresh medium containing 1000 μg/ml FITC-labeled CaOx crystals and 10 μl of 100 μM, 1 mM or 10 mM resveratrol or ethanol (the solvent used to solubilize resveratrol) were added into each well of 6-well culture plate. The final concentration of resveratrol in each well was 1, 10 or 100 μM, whereas the well added with ethanol served as the blank control. After 1-h incubation, the culture supernatant was discarded. The cells were then detached by trypsinization using trypsin-EDTA solution (Gibco). Note that the non-internalized crystals were also removed during this trypsinization step. The cells with internalized FITC-labeled crystals were then quantified from a total of 10,000 cells per condition by flow cytometry using BD Accuri C6 flow cytometer (BD Biosciences; San Jose, CA).

2.9. Statistical analysis

All assays were performed in three independent experiments. The data are reported as mean ± SD. One-way ANOVA and Tukey's post-hoc multiple comparisons were used to determine differences among groups. $P < 0.05$ was considered statistically significant.

3. Results

CaOx crystals were generated using a specific protocol used for each assay (see details in **Materials and Methods**). However, the sizes of the plain (non-labeled) and FITC-labeled CaOx crystals generated in our present study were consistent among different assays with an average size of 200 μm^2 or length of 20 μm.

3.1. Effects of resveratrol on CaOx crystallization

Crystallization assay was executed to investigate the modulatory activity of resveratrol on the formation of neocrystals (newly generated CaOx crystals) that is a common initial step of kidney stone formation. Crystal size and mass (see *Formula I*) were measured from 100 crystals per condition and calculated from 10 random fields per condition, respectively (Fig. 1A). The results showed that resveratrol at all concentrations had no significant effects on both size and mass of the neocrystals as compared with the blank control (Fig. 1B and C).

3.2. Effect of resveratrol on CaOx crystal growth

The effect of resveratrol on the ability of the already-formed crystals to further grow was examined by crystal growth assay. Crystal sizes were measured from 100 crystals per condition at two time-points, at T_0 (when the neocrystals completely formed) and T_{60} (when the already-formed crystals were allowed to further grow for 60 min) (Fig. 2A). Δ crystal size representing the crystal enlargement was then calculated (see *Formula II*). The analysis revealed that resveratrol at all concentrations markedly reduced Δ crystal size as compared with the blank control (Fig. 2B). However, there was no significant difference in the effect from different concentrations of resveratrol used (Fig. 2B).

3.3. Effect of resveratrol on CaOx crystal aggregation

Crystal aggregation assay was performed to determine the effect of resveratrol on the self-clumping ability of the CaOx crystals. Number of the crystal aggregates (each was defined as a clump of at least three crystals (Chaiyarit and Thongboonkerd, 2017)) was counted from 10 random fields per condition (Fig. 3A). The findings demonstrated that resveratrol at all concentrations increased CaOx crystal aggregation as compared with the blank control (Fig. 3B). Nevertheless, there was no

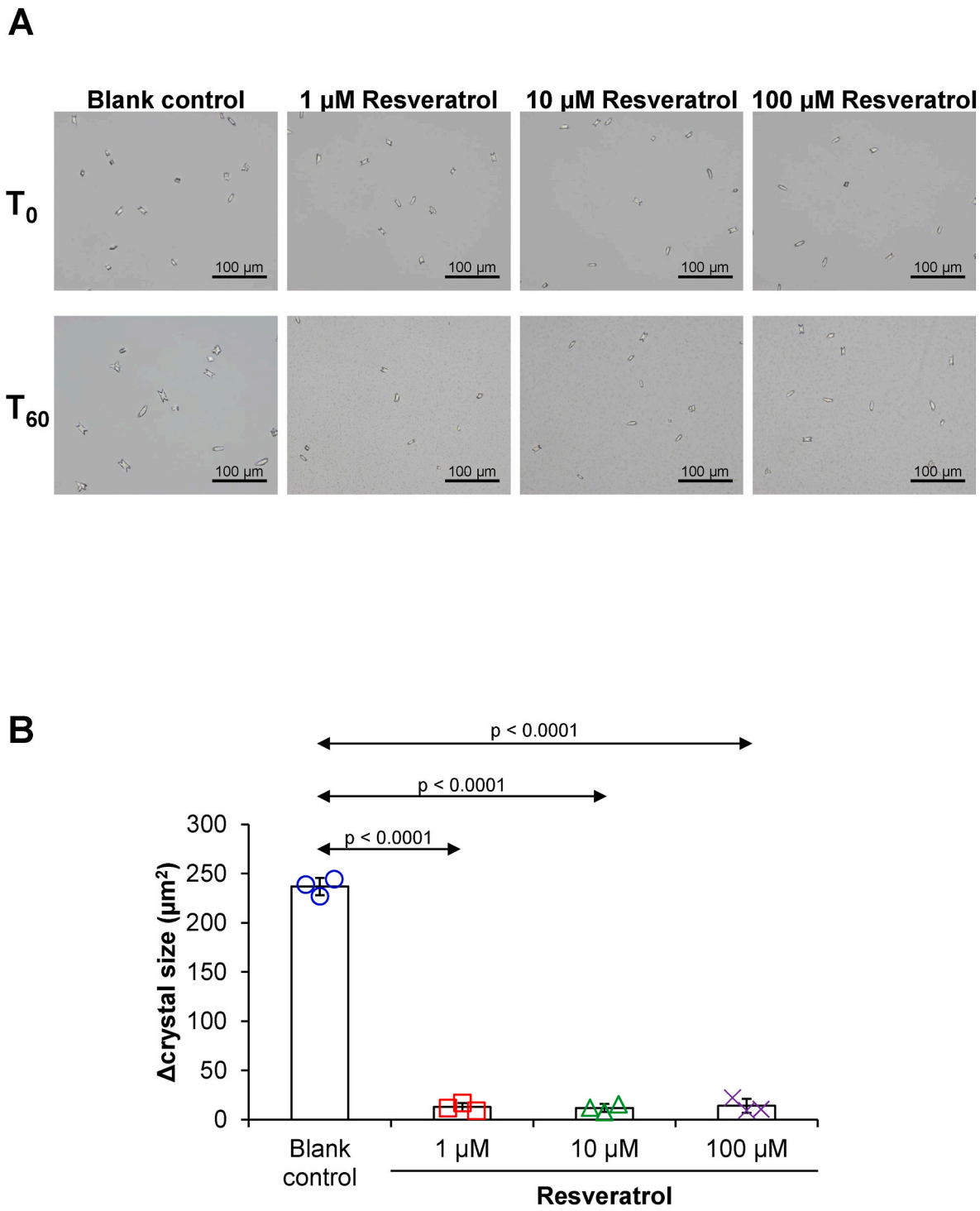


Fig. 2. Effect of resveratrol on CaOx crystal growth. Crystal growth assay was performed in the absence (blank control) or presence of resveratrol (at a final concentration of 1, 10 or 100 μM). **(A):** Microscopic images of the CaOx crystals at T_0 (after 1-h of initial crystallization) and T_{60} (after 60-min incubation of the already-formed crystals to allow them to further enlarge). **(B):** Δ crystal size was calculated from 100 random crystals according to *Formula II* (see **Materials and Methods**). The data are presented as mean ± SD of measurements from three independent experiments, and significant p values are labeled.

significant difference in the effect from different concentrations of resveratrol used (Fig. 3B).

3.4. Effect of resveratrol on CaOx crystal-cell adhesion

Crystal-cell adhesion assay was employed to determine the modulatory activity of resveratrol on the ability of the crystals to retain on renal epithelial cell surface lining along renal tubules. After 1-h incubation followed by vigorous washes to eliminate the non-adhered crystals, number of the adhered crystals that remained on the cell monolayer was counted from 10 random fields per condition (Fig. 4A). The results revealed that resveratrol at all concentrations significantly reduced number of the adhered crystals as compared with the blank control (Fig. 4B). However, there was no significant difference in the effect from different concentrations of resveratrol used (Fig. 4B).

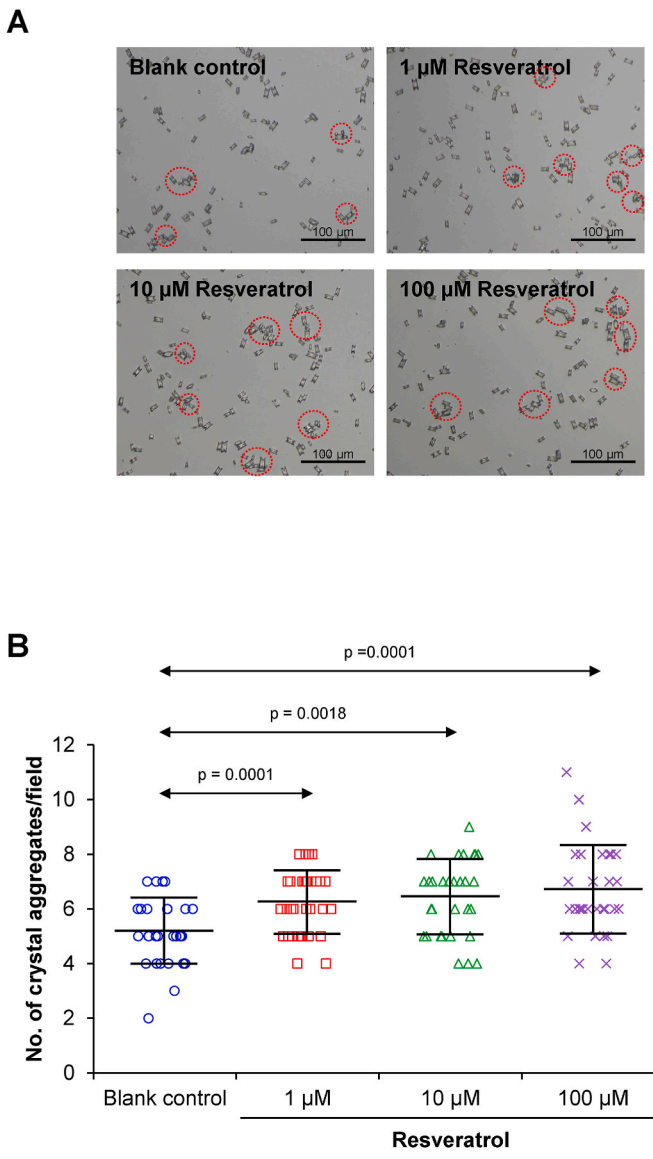


Fig. 3. Effect of resveratrol on CaOx crystal aggregation. Crystal aggregation assay was performed in the absence (blank control) or presence of resveratrol (at a final concentration of 1, 10 or 100 μM). **(A):** Microscopic images of the CaOx crystal aggregates (labeled with red dotted circles). **(B):** Number of the crystal aggregates was counted from 10 random fields per condition. The data are presented as mean ± SD of measurements from three independent experiments, and significant p values are labeled.

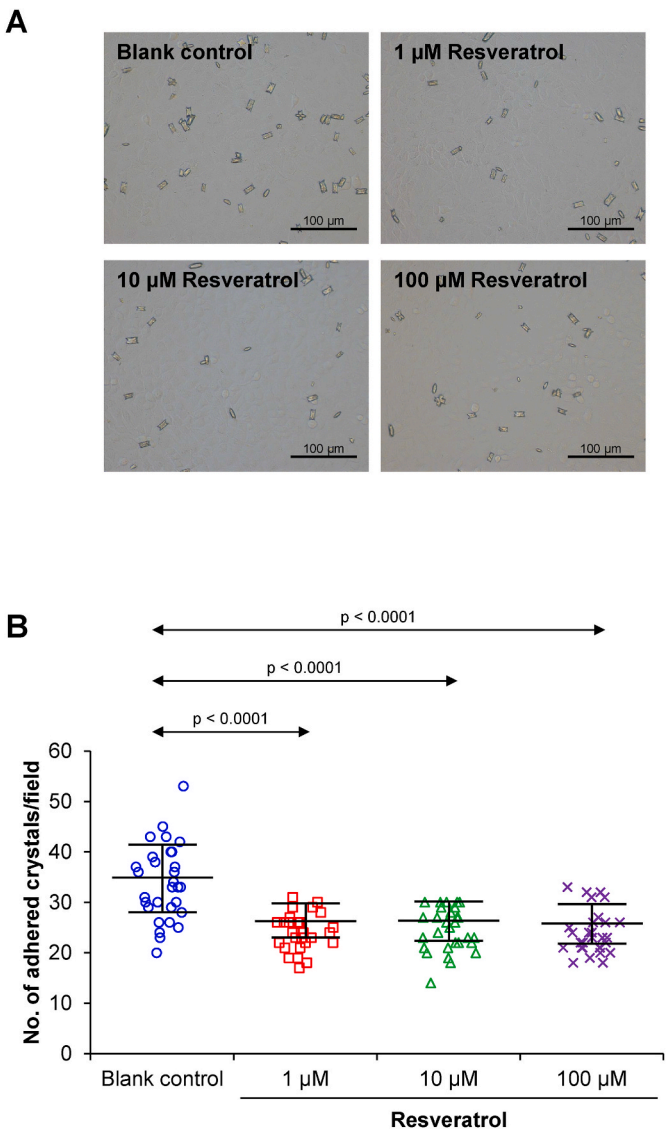


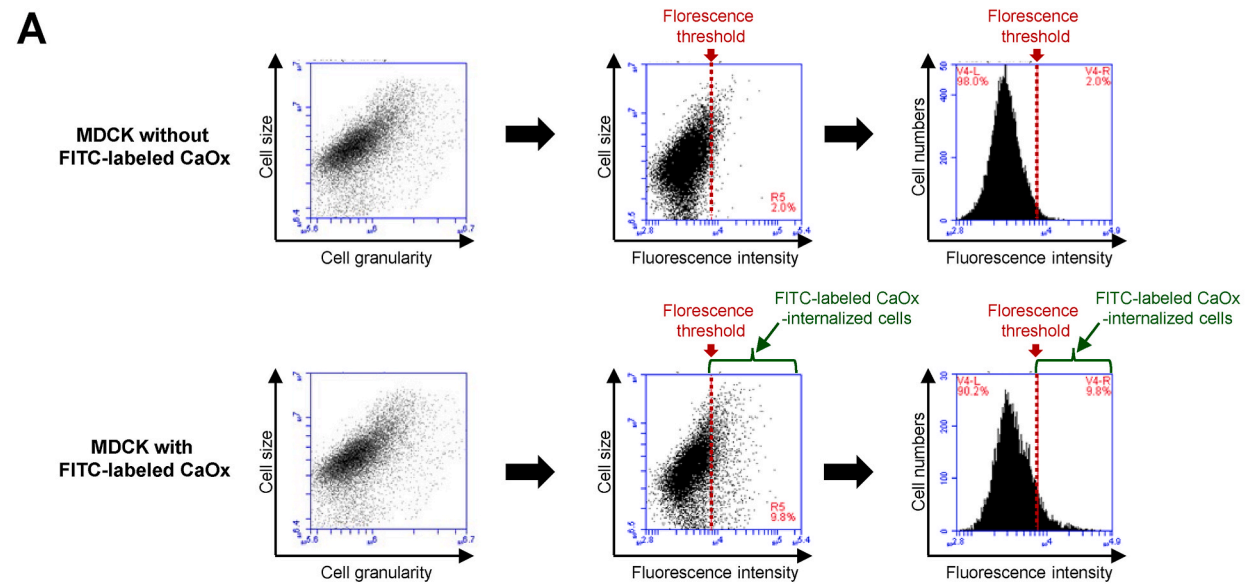
Fig. 4. Effect of resveratrol on CaOx crystal-cell adhesion. Crystal-cell adhesion assay was performed in the absence (blank control) or presence of resveratrol (at a final concentration of 1, 10 or 100 μM). **(A):** Microscopic images of the adhered CaOx crystals remaining on MDCK renal cell monolayer. **(B):** Number of the adhered crystals was counted from 10 random fields per condition. The data are presented as mean ± SD of measurements from three independent experiments, and significant p values are labeled.

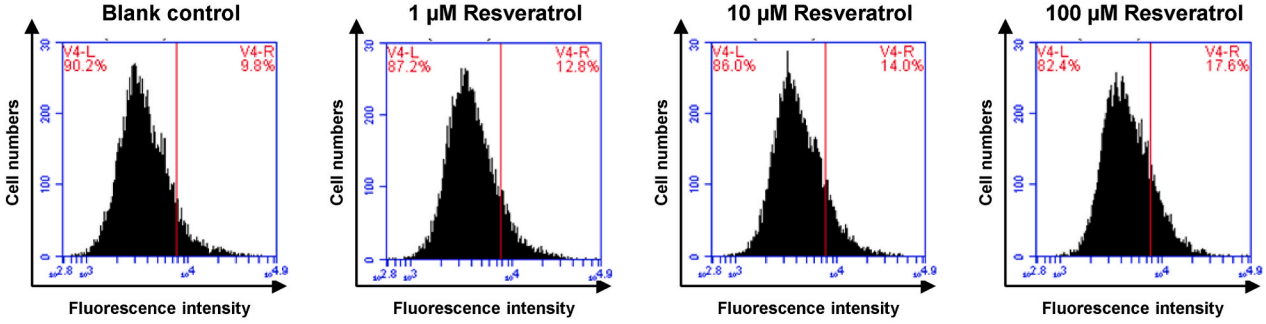
3.5. *Effect of resveratrol on CaOx crystal internalization into renal cells*

Internalization of CaOx crystals into the renal cells (a process related to crystal degradation by endolysosomes (Chaiyarit et al., 2016)) was assessed by crystal internalization assay using the FITC-labeled CaOx crystals to track the internalized crystals. After 1-h incubation followed by removal of the excess and non-internalized crystals, number of the cells with internalized crystals was quantified from a total of 10,000 cells per condition by flow cytometry (Fig. 5A and B). Quantitative analysis revealed that resveratrol at all concentrations significantly increased percentage of the cells with internalized crystals in a concentration-dependent manner (Fig. 5C).

4. Discussion

Kidney stones comprise crystalline particles tightly packed together with organic compounds and urinary macromolecules. CaOx is the major type of crystalline particles found in stones and thus is the main focus of current kidney stone research (Aizezi et al., 2022; Grant et al., 2018; Singh et al., 2015). Herein, we have addressed the direct modulatory effects of resveratrol on various steps in which CaOx crystals are involved during kidney stone formation. These stone development steps include the formation of newly generated crystals (or neocrystals) as a consequence of supersaturation of crystalline ions (i.e., calcium and oxalate), increase in size or growth of the already-formed crystals, clumping of the crystals, crystal retention on the surface of renal epithelial cells, and engulfment of crystals by the cells to be eliminated by endolysosomes (Chaiyarit et al., 2016; Chaiyarit and Thongboonkerd, 2020). Each of these stone development steps can be influenced by a variety of factors, such as urinary supersaturation of ions (Ibis et al., 2021), urine pH (Manissorn et al., 2017), and the presence of stone modulatory molecules (Peerapen and Thongboonkerd, 2021; Thongboonkerd, 2019; Vinaiphat and Thongboonkerd, 2017; Yoodee and





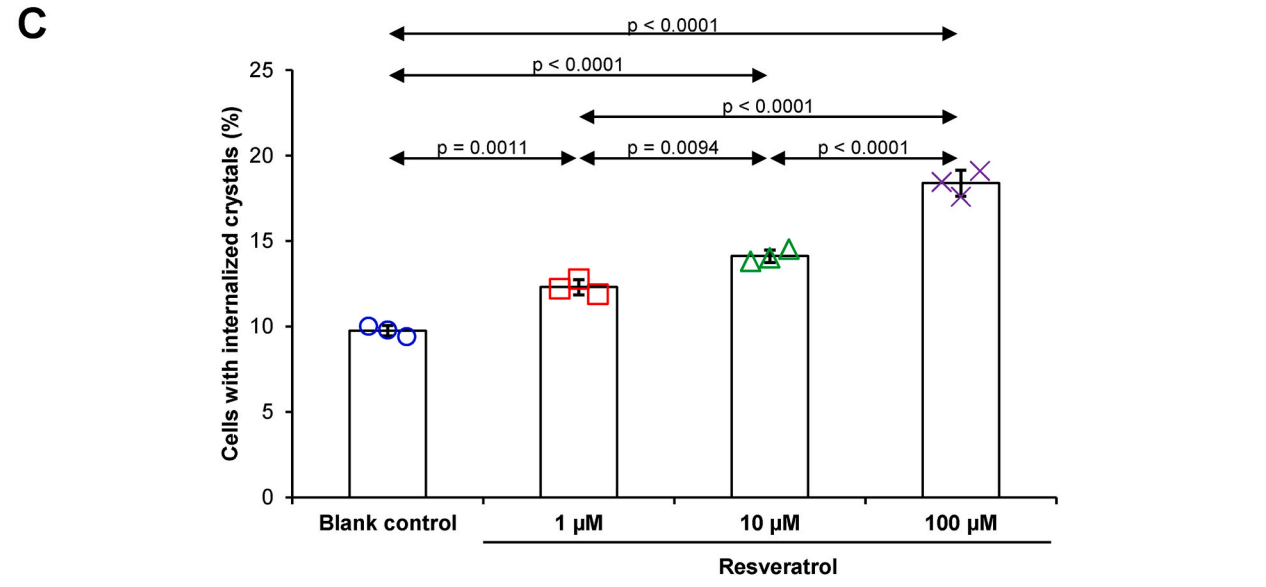


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Fig. 5. Effect of resveratrol on crystal internalization into renal cells. Crystal internalization assay was performed in the absence (blank control) or presence of resveratrol (at a final concentration of 1, 10 or 100 μM) using FITC-labeled CaOx crystals. **(A):** Determination of the threshold of fluorescence signal to detect the cells with internalized FITC-labeled CaOx crystals. The noise from the cells parallelly incubated without FITC-labeled CaOx crystals was used as the background to subtract. The cells with fluorescence signals greater than this threshold were counted as the ones with the internalized FITC-labeled CaOx crystals. **(B):** Histograms of the fluorescence signals obtained by flow cytometry (the fluorescence threshold is indicated as a vertical red line). **(C):** Percentage of the cells with internalized crystals was quantified from a total of 10,000 cells per condition. The data are presented as mean ± SD of measurements from three independent experiments, and significant p values are labeled.

Thongboonkerd, 2023).

Numerous lines of evidence have documented various kinds of stone modulatory molecules that can either inhibit or promote each stone development step. The endorsed stone modulatory molecules include proteins (Peerapen and Thongboonkerd, 2021; Sassanarakkit et al., 2020; Thongboonkerd, 2019; Vinaiphat and Thongboonkerd, 2017; Yoodee and Thongboonkerd, 2023), glycosaminoglycans (Chanthick and Thongboonkerd, 2022), plant extracts (Khan et al., 2021) and bioactive compounds (Chaiyarit et al., 2024; Kanlaya et al., 2023; Khamchun et al., 2021; Peerapen et al., 2022; Peerapen and Thongboonkerd, 2016). Some bioactive compounds, such as diosmin and quercetin, exhibited dual modulatory activities (Chaiyarit et al., 2024; Khamchun et al., 2021). Diosmin increases CaOx crystal mass but reduces size and enlargement of the crystals (Khamchun et al., 2021). Diosmin also inhibits adhesion of crystals to the cells and internalization of the crystals into the cells, but on the other hand, induces crystal aggregation and invasion through extracellular matrix (Khamchun et al., 2021). Similarly, quercetin inhibits CaOx crystallization and enlargement, but on the other hand, induces crystal aggregation and invasion through extracellular matrix (Chaiyarit et al., 2024). In this study, we also found the dual modulatory effects of resveratrol on CaOx stone development steps as follows.

After oral intake of high-dose resveratrol (5 g daily), the maximum plasma concentration of resveratrol is 4.2 μM (Brown et al., 2010). However, higher concentrations have been widely used for *in vitro* studies of the pharmacologic effects of resveratrol (Tome-Carneiro et al., 2013). We therefore used resveratrol at a wide μM range (1–100 μM) in the present study to cover both physiologic plasma levels and commonly used *in vitro* concentrations. We initially investigated the modulatory activity of resveratrol on nucleation of CaOx crystals within a clear calcium/oxalate-supersaturated solution. Supersaturation of calcium and/or oxalate ions is the major driving force for crystalline precipitation. However, resveratrol at all concentrations tested had no significant effects on CaOx crystallization.

Thereafter, enlargement of the already-formed CaOx crystals in the environment with the presence or absence of resveratrol was measured to indicate the crystal growth. Strikingly, the potent inhibitory effect of resveratrol against crystal enlargement was exhibited at all concentrations tested (1, 10 and 100 μM). Although resveratrol had no significant effect on crystal size during the initial nucleation process of the neocrystals, it exhibited a powerful inhibitory effect during the enlargement step of the already-formed crystals. This data indicates that a modulator may have a specific or selective effect on each step of the stone development, consistent with the aforementioned studies that reveal dual modulatory activities of some modulators that have different effects on various stone development steps (Chaiyarit et al., 2024; Khamchun et al., 2021). Previous studies have reported that some modulatory molecules can increase CaOx solubility (Frackowiak et al., 2010; Ibis et al., 2021) and decrease the supersaturation of calcium and oxalate ions (Chow et al., 2004). Therefore, resveratrol might also inhibit CaOx crystal growth in the same way. Additionally, resveratrol may bind free calcium ions and/or calcium on CaOx crystal surfaces, thereby handicapping the addition of free calcium ions to the CaOx crystal and inhibiting crystal growth (Fig. 6A).

Resveratrol also showed inhibitory activity on crystal-cell adhesion. The retention of crystals on renal cell surface is another crucial step for crystal deposition in the kidney. Several studies have revealed that renal injury is one of the main aggravating factors for intrarenal crystal retention (Cao et al., 2016; Davalos et al., 2010; Khamchun and Thongboonkerd, 2018). Plant extracts and plant-derived bioactive compounds have been reported to reduce renal cell injury and ameliorate crystal adhesion to the cells (Kanlaya et al., 2016; Peerapen et al., 2022; Wu et al., 2021). The reduction of crystal-cell adhesion is partly a consequence of the decreased expression of crystal-binding proteins on the cell surface (Kanlaya et al., 2016; Peerapen and Thongboonkerd, 2016). Blocking the crystal receptors (Sutthimethakorn and Thongboonkerd, 2020) and occupying the crystal surfaces (Kumar et al., 2003) can result in the reduction of crystal-cell adhesion and intrarenal retention. In this study, resveratrol also showed an inhibitory effect against crystal-cell adhesion. Interestingly, resveratrol has a potent adsorptive ability to bind calcium (Oksay et al., 2017). It is thus possible that resveratrol tends to bind calcium on the crystals, thereby occupying the crystal surfaces and reducing crystal-cell adhesion (Fig. 6C).

Nevertheless, we found the promoting activity of resveratrol on crystal aggregation. Assembly of crystals to form the self-aggregates is considered another important step for kidney stone development. A previous study has demonstrated that CaOx crystals can be found in the urine of healthy individuals, but with less degree of crystal aggregation as compared with those found in the stone formers' urine (He et al., 2010). In this study, we used an *in vitro* method with a reliable aggregation index previously established (Chaiyarit and Thongboonkerd, 2017) to investigate CaOx crystal aggregation. Our finding is consistent with previous studies reporting the promoting effects of diosmin (Khamchun et al., 2021), quercetin (Chaiyarit et al., 2024), heat shock protein 90 (Yoodee et al., 2022), and desialylated uromodulin (Viswanathan et al., 2011). The promoting activity of these molecules on CaOx crystal aggregation has been thought to occur via the bridge-like activity of the molecules that can bind to the surfaces of adjacent CaOx crystals, thereby forming the crystal clusters, clumps or aggregates (Fig. 6B).

Several previous studies have investigated the ability of renal cells to engulf or endocytose CaOx crystals (Chaiyarit et al., 2016; Han et al., 2019; Kanlaya et al., 2013; Lieske and Deganello, 1999; Lieske et al., 1997; Schepers et al., 2003). Among various endocytic mechanisms, previous evidence has revealed that macropinocytosis is the major mechanism for CaOx internalization into renal epithelial cells (Kanlaya et al., 2013). Macropinocytosis is a distinct type of endocytosis that is actin-driven (Kerr and Teasdale, 2009). The internalized crystals are then degraded and eliminated within endolysosomes inside the cells (Chaiyarit et al., 2016). Unfortunately, the effects of modulators on this mechanism have been rarely examined. Our data have shown the effect of resveratrol on inducing CaOx crystal internalization into the cells, thereby favoring the crystal degradation process. In consistent, a previous study has found that resveratrol administration in dogs increases phagocytic activity of polymorphonuclear cells (Mathew et al., 2018). Additionally, the activation of phagocytosis by resveratrol has also been demonstrated in many other studies (Bertelli et al., 1999; Kowalska et al., 2017; Sheu et al., 2010; Sheu and Wu, 2009). Likewise, other phenolic compounds, such as quercetin, can also enhance the phagocytic activity of leukocytes (Boonlaos et al., 2021). Additionally, genipin (another type of plant-derived bioactive compound) can aggravate the endocytic activity of yeast cells to prevent α-synuclein-induced cytotoxicity (Rosado-Ramos et al., 2023). Therefore, resveratrol may activate macropinocytosis or endocytosis of renal epithelial cells, thereby promoting crystal internalization to prompt the crystals for degradation by endolysosomes (Fig. 6D). Accordingly, endocytosis of particles seems to be a defense mechanism of the cells to cope with various cytotoxic

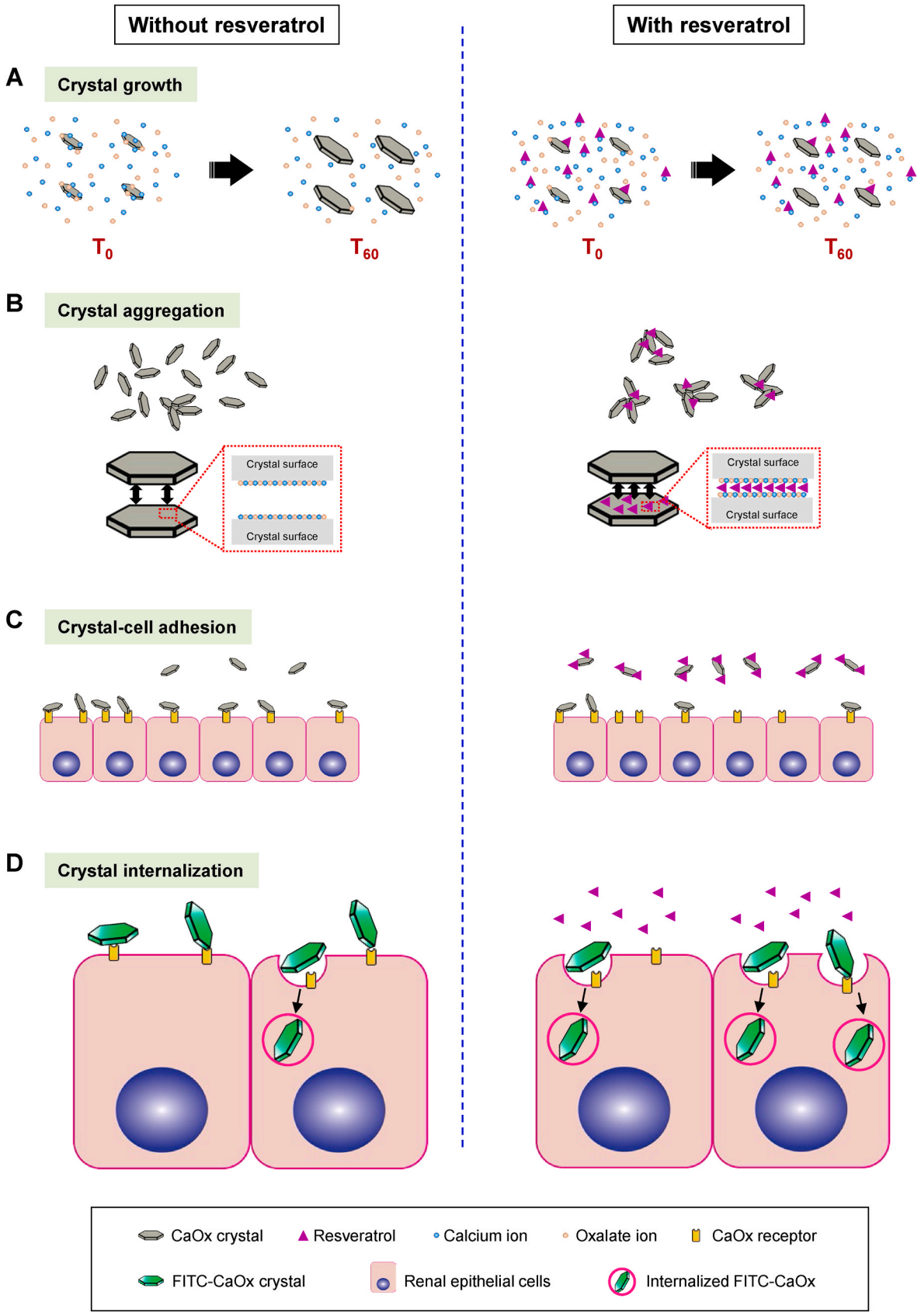

Fig. 6. Schematic illustrations of the proposed mechanisms of action of resveratrol on CaOx kidney stone formation. (A): Resveratrol binds free calcium ions and/or calcium on CaOx crystal surfaces, thereby handicapping the addition of free calcium ions to the CaOx crystal and inhibiting crystal growth. **(B):** Resveratrol binds the crystal surfaces and acts as a bridge of adjacent CaOx crystals to join together as the crystal clusters, clumps or aggregates. **(C):** Resveratrol binds calcium on the crystals, thereby occupying the crystal surfaces and reducing crystal-cell adhesion. **(D)** Resveratrol activates macropinocytosis or endocytosis of renal epithelial cells, thereby promoting crystal internalization to prompt the crystals for degradation by endolysosomes.

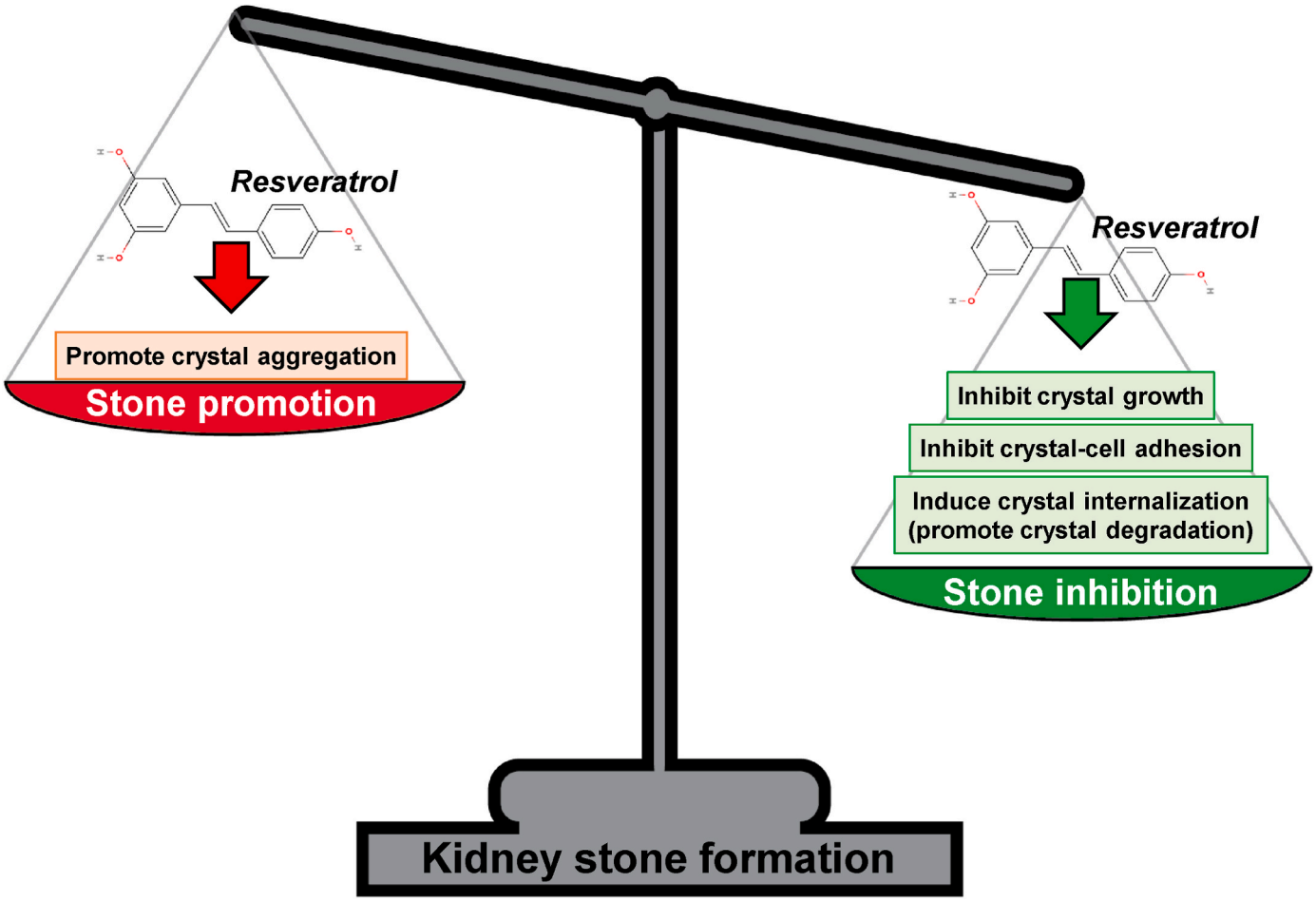

Fig. 7. Summary of the dual modulatory activities of resveratrol on CaOx kidney stone formation. Resveratrol inhibits CaOx stone development steps by reducing crystal growth and adhesion to renal cells and by inducing crystal internalization into the cells (for crystal degradation). On the other hand, resveratrol promotes crystal aggregation, which is one of the mechanisms leading to kidney stone formation.

inducers.

In summary, resveratrol has various direct modulatory effects on CaOx crystals. The proposed mechanisms of action of resveratrol are illustrated in Fig. 6. Evidence in our present study also demonstrates that resveratrol has dual modulatory effects on CaOx crystals (Fig. 7). While it inhibits CaOx stone development steps by reducing crystal growth and adhesion to renal cells and by inducing crystal internalization into the cells (for crystal degradation), resveratrol promotes crystal aggregation, which is one of the mechanisms leading to kidney stone formation (Fig. 7).

CRediT authorship contribution statement

Paleerath Peerapen: designed research, performed experiments, analyzed data, wrote the manuscript. **Pattaranit Putpeerawit:** designed research, performed experiments, analyzed data. **Wanida Boonmark:** designed research, performed experiments, analyzed data. **Visith Thongboonkerd:** designed research, analyzed data, wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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