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Research article

Drug repurposing screens to identify potential drugs for chronic kidney disease by targeting prostaglandin E2 receptor

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ABSTRACT

Renal inflammation and fibrosis are significantly correlated with the deterioration of kidney function and result in chronic kidney disease (CKD). However, current therapies only delay disease progression and have limited treatment effects. Hence, the development of innovative therapeutic approaches to mitigate the progression of CKD has become an attractive issue. To date, the incidence of CKD is still increasing, and the biomarkers of the pathophysiologic processes of CKD are not clear. Therefore, the identification of novel therapeutic targets associated with the progression of CKD is an attractive issue. It is a critical necessity to discover new therapeutics as nephroprotective strategies to stop CKD progression. In this research, we focus on targeting a prostaglandin E_2 receptor (EP2) as a nephroprotective strategy for the development of additional anti-inflammatory or antifibrotic strategies for CKD. The *in silico* study identified that ritodrine, dofetilide, dobutamine, and citalopram are highly related to EP2 from the results of chemical database virtual screening. Furthermore, we found that the above four candidate drugs increased the activation of autophagy in human kidney cells, which also reduced the expression level of fibrosis and NLRP3 inflammasome activation. It is hoped that these findings of the four candidates with anti-NLRP3 inflammasome activation and antifibrotic effects will lead to the development of novel therapies for patients with CKD in the future.

1. Introduction

Inflammation is one of the significant parts of chronic kidney disease (CKD) and has been recognized since the 1990s [1]. The incidence of patients with CKD and end-stage renal disease (ESRD) is increasing worldwide, and the population of such patients remains exceptionally high. However, the precise mechanism of chronic inflammation in kidney cells is not yet completely understood [2]. There is evidence that chronic inflammation may be involved in CKD development. Patients with CKD display chronic microinflammation that is related to premature aging of the vascular system [3]. For instance, impaired kidney function in inflammatory environments is involved in the pathogenesis of the pathological fibrosis of chronic kidney disease [4]. Importantly, recent evidence shows that the inflammatory response in CKD is closely related to the production of proinflammatory cytokines, including interleukin (IL)-1β, interleukin-6 (IL-6), and tumor necrosis factor (TNF)-α [5]. Thus,

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inflammation is a direct pathogenic problem and contributes to the progression of CKD in the uremic syndrome.

So far, the role of endothelin (ET) has been under study and become a new perspective in kidney disease [6]. The ET family is composed of 21-amino-acid-long peptides with many functions, including vasoconstriction, cell hypertrophy, promotion of inflammation, and fibrosis. Three different isoforms of ETs, termed endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3), have been found in mammals [7]. Early studies hypothesized that overexpression of ET-1 and ET-2 was significantly upregulated in kidney tissues and further caused renal cellular injury associated with the progression of CKD [8]. Regarding inflammation in renal pathophysiology, both ET-1 and ET-2 promote endoplasmic reticulum (ER) stress [9] and NLR family pyrin domain containing 3 (NLRP3) inflammasome activation [10] in proximal tubular cells of the human kidney. Thus, CKD mainly leads to a persistent inflammatory response. According to our previous report, we found a new strategy to reduce the inflammatory response by inhibiting endothelin-converting enzymes (ECEs) in the progression of CKD [11]. The ECE protease is the key enzyme in the process of ET biosynthesis [12], which could involve the generation of ET-1 via cleavage of preproendothelins or large ETs in the final processing step. Therefore, ETs and ECEs are potential targets for the development of a new strategy of renal protection to prevent CKD progression.

Prostanoids are potential proinflammatory mediators that contribute to regulating the inflammatory response and play a vital role in accomplishing kidney function [13,14]. Most research has focused on prostanoids, cytokines, and chemokines as inflammatory factors in regulating the mechanism of chronic inflammation [15]. Prostanoids belong to the eicosanoid family of lipid mediators that are derived from twenty-carbon essential fatty acids [16]. Prostanoids are initially synthesized from the conversion of arachidonic acid (AA) to prostaglandin H2 (PGH2) via the action of cyclooxygenases (COXs) [17]. The production of prostanoids is one of the mediators associated with chronic inflammation in early studies [18]. The prostanoids interact with G protein-coupled receptors of prostaglandin receptor (EP), impacting their cellular functions on the cell surface. The prostaglandin E2 receptor (EP2) is encoded by the PTGER2 gene of humans, which is a prostaglandin receptor in the prostaglandin E2 (PGE2) signaling pathway [19]. Notably, PGE2 is a known prostanoid that has potent anti-inflammatory effects and link to attenuating kidney fibrosis when acting on the EP2 [20]. Accumulating evidence has shown that PGE2 can interact with the EP2 to enhance anti-inflammatory efficiency by polarizing macrophages from the M1 to M2 type [21].

Increasing evidence indicates that activation of autophagy regulates inflammasomes in inflammatory diseases [22]. Autophagy is affected by many factors, including immune stimulation, nutritional deficiency, the concentration of calcium cations, ER stress, and organelle damage [23]. Inflammasomes are large intracellular multimeric complexes that can be activated by various stresses, such as damage-associated molecular patterns (DAMPs) [24] and pathogen-associated molecular patterns (PAMPs) [25]. Recently, autophagy has been reported to be associated with renal fibrosis [26], but the molecular mechanism in renal fibrosis is still unknown. Autophagy can be triggered by an ECE inhibitor to ameliorate kidney fibrosis and inflammation. Hence, a study on inflammasomes in kidney disease will help to discover novel therapeutic targets through understanding the mechanisms of nephropathy. Hence, the precise targets of fibrosis and inflammation need further investigation for CKD treatment. The purpose of this project is to develop potential therapeutic drugs. The therapeutic targets might be identified from cellular signaling induced by the ECE inhibitor phosphoramidon in the human kidney proximal tubular epithelial cell line. Until now, current techniques of renal therapy produce no effective treatment and have only partially slowed the symptoms of CKD. Thus, the development of effective therapeutic methods, rather than inhibition of progression, is urgently needed for CKD patients [27].

Computational drug design is an efficient and rapid tool to discover or develop potent chemicals to target specific diseases. The in silico method can help researchers accelerate the process of new pharmaceutical drug discovery or optimize therapeutic compounds before preclinical research and clinical trials. Structure-based drug design is based on knowledge of the target protein structure to implement the design of lead compounds. Until now, structure-based approaches such as homology modeling, molecular docking, and database virtual screening have been applied to identify hits as potential lead compounds from large chemical libraries with millions of small molecules, such as the ZINC database [28], DrugBank [29], and e-Drug3D [30]. In this study, homology modeling and structure-based virtual screening were carried out to investigate potential clinical drugs with binding affinity at the active site domain of the EP2 protein from a large chemical library. Furthermore, we performed a series of experimental tests to understand the anti-inflammatory or anti-fibrosis mechanism of the candidate compounds via renal cell assessments to study their biological functions.

2. Materials and methods

2.1. Cell culture and drug treatment

The proximal tubular cell line HK-2 (human kidney 2, CRL-2190) of the normal adult human kidney was purchased from the American Type Culture Collection (ATCC). Cells were maintained in keratinocyte serum-free (KCSF) medium with 40 μg/mL bovine pituitary extract (Gibco BRL, Grand Island, NY, USA) and 5 ng/mL recombinant epidermal growth factor and incubated at 37℃ and 5% CO_2 in a humidified chamber. The cultured medium was replaced every 2–3 d for the cultured cells. A density of approximately 1×10^5 HK-2 cells was seeded separately in each well of 96-well plates before treatment with test compounds and then maintained at 37℃ and 5% CO_2 in a humidified incubator for 1 d. All test chemicals, including dobutamine, dofetilide, citalopram, and ritodrine (MedChemExpress, Monmouth Junction, NJ, USA), were dissolved in dimethyl sulfoxide (DMSO) before the cell viability test.

2.2. RNA extraction and sequencing libraries

After treatment with or without phosphoramidon for 24 h, the total RNA of HK-2 cells was extracted with the EasyPrep Total RNA Kit (Biotools, Cat No. DPT-BD19) according to the manufacturer's protocol. The purity and quantification of collected RNA were checked using Biochrom's SimpliNano™ Spectrophotometers (Biochrom, MA, USA). The degradation of extracted RNA was quantified using a Qsep100 Analyzer (BiOptic Inc., Taiwan). A total of RNA (1 μl) of each sample was subjected to library preparation using a KAPA RNA HyperPrep Kit (KAPA Biosystems, Roche, Basel, Switzerland). The magnetic oligo-dT beads were utilized to isolate the purified mRNA from total RNA, and then the fragmented mRNA was captured in the presence of magnesium in KAPA buffer in a high-temperature environment. The KAPA Pure Beads system (KAPA Biosystems, Roche, Basel, Switzerland) was used to purify the library fragments and then select cDNA fragments with a length of 300–400 bp. To choose library fragments of 300–400 bp, the desired cDNA fragments were isolated via the KAPA Pure Beads system (KAPA Biosystems, Roche, Basel, Switzerland). The library was subjected to amplification with appropriate adapter sequences through KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Roche, Basel, Switzerland). In the final step, the purified PCR products were obtained with a KAPA Pure Beads system and assessed on a Qsep100 Analyzer (BiOptic Inc., Taiwan).

2.3. Next-generation sequencing (NGS) analysis

The data of the RNA-seq library were obtained by high-throughput sequencing (Illumina NovaSeq 6000 platform). The library is subjected to base calling to generate raw sequenced reads and then stored in FASTQ files using CASAVA software. The low-quality reads, trimmed adaptor sequences, and eliminated poor-quality bases were removed by Trimmomatic (v0.38). In the following analysis, the clean reads (high-quality data) obtained from each sample were aligned to the reference genome (e.g., *H. sapiens*, GRCh38) by HISAT2 software (v2.1.0). For gene expression, "trimmed mean of M-values" normalization (TMM) was analyzed by DEGseq (v1.36.1) with biological duplicates, while "relative log expression" normalization (RLE) was conducted by DESeq2 (v1.22.1) with biological duplicates. Based on the Poisson distribution model and negative binomial distribution, differentially expressed gene (DEG) analysis of the two conditions was performed by R software. The p values of the analyzed results were modulated by the Benjamini–Hochberg procedure to control the FDR value. The enrichment analysis of DEGs was conducted by clusterProfiler (v3.10.1) software. The RNA series dataset was uploaded to the Gene Expression Omnibus (Accession: GSE203181).

2.4. Enriched biological pathways and interaction network

We used the ingenuity pathway analysis (IPA) tool (QIAGEN, German) to examine the relationship between these highly significant genes from NGS data [genes with adjusted P value (FDR) < 0.05, absolute fold-change (FC) < 0.58 or > 0.58] to study biological networks and significant pathways with or without phosphoramidon treatment. According to the number of genes participating in any particular network, these networks were scored based on associated functions. IPA also analyzed significant networks related to the expressed genes differentially in HK-2 cells, including cellular proliferation, cellular movement, cell death, and inflammatory response.

2.5. Homology modeling

The sequence of EP4 structure (PDB: 5YHL) [31] was used as the template to identify regions of similarity for the EP2 protein sequence (P43116) using a sequence alignment method. A progressive pairwise alignment algorithm was selected to perform sequence alignment under ClustalW software. The homology model of the EP2 protein was built by the I-TASSER web server. Multiple sequence alignment of templates and target proteins was used to obtain the score of sequence identity and similarity for homology modeling. The reliability and compatibility of the homology model of EP2 protein were analyzed by the Ramachandran plot of BIOVIA Discovery Studio (BIOVIA, USA).

2.6. Docking study

The docking simulation was performed with the force field of CHARMM [32] using the LibDock protocol [33] of BIOVIA Discovery Studio (BIOVIA, USA). The clinical drugs used for docking were downloaded from the DrugBank database [29]. The conformation search included electrostatic energy. A smart minimizer protocol was used to perform 1000 minimization steps of 'steepest descent' followed by a conjugate gradient. Ligands were ranked using the 'LibDock score' as the primary criterion. The smart minimizer option was used to execute the minimization for the in situ ligand.

2.7. Cell viability analysis

The cell viability of HK-2 cells was evaluated by the sulforhodamine B (SRB) assay for all test chemicals. Each test compound was incubated with HK-2 cells in 96-well plates for 24 h. After the incubation process, the cells were washed twice with phosphate-buffered saline (PBS). A trichloroacetic acid solution was used to fix the cells for 1 h. Before SRB staining (Sigma–Aldrich Corp.), the supernatant of each well was removed and then washed twice with deionized water. Subsequently, the residual dye was removed after 1 h of SRB staining and then washed twice with 1% acetic acid. Finally, Tris-buffer (20 mM) was added to each well and shaken for 30 min to dissolve the protein-bound dye. The absorbance of the colored solution was measured at 562 nm in an absorbance microplate reader (Molecular Devices, Sunnyvale, CA, USA).

The calculation of cell viability was using the following equation:

$$\text{Cell viability\%} = \frac{AbsSample - AbeBlank}{AbsControl - AbsBlank} \times 100\% \quad (1)$$

where AbsSample means the optical density of drug-treated cells, AbsControl is the optical density of untreated cells, and AbsBlank indicates the absorbance of PBS.

2.8. Western blot analyses

The drug-treated cells were lysed with RIPA lysis buffer (Genestar, Taipei, Taiwan) and then separated by SDS–PAGE before transfer to polyvinylidene difluoride (PVDF) membranes. The membranes were immersed in a blocking buffer (5% nonfat milk in TBS containing 0.1% Tween-20) for 2 h at room temperature with gentle agitation. Sequentially, the membranes incubation with primary antibodies against Collagen Type I (Proteintech, Rosemont, USA), PAI-1 (Cell Signaling, Massachusetts, USA), CTGF (Proteintech), Beclin 1 (Cell Signaling), p62 (MBL, Woburn, USA), LC3 (Cell Signaling), NLRP3 (Proteintech), ASC (AdipoGene, San Diego, USA), caspase 1 (Proteintech), and GAPDH (Proteintech) overnight at 4°C. After washing three times with TBS-T at room temperature, the membranes were incubated with secondary antibodies for 1 h at room temperature and washed with TBS-T three times. Immunoreactive bands were visualized by a chemiluminescence detection system (Thermo Fisher Scientific, Waltham, MA, USA).

2.9. Statistical analysis

The two-sample t test or one-way analysis of variance with Dunnett's multiple comparison test was used to estimate the differences between groups of all experimental data, and the plot of each data set was expressed as the means ± standard deviations (SD). p values < 0.05 in all statistical tests were considered statistically significant.

3. Results

3.1. RNA sequencing with NGS analysis in phosphoramidon-treated HK-2 cells

The precise pathways and potential targets induced by an ECE inhibitor (phosphoramidon) were evaluated by NGS in human kidney proximal tubular epithelial cells. Total RNA was extracted from HK-2 cells after treatment with or without phosphoramidon to create a stranded RNA-seq library (Fig. 1A). To determine the gene expression under phosphoramidon treatment, transcriptomics datasets were measured by NGS analysis and then normalized to identify significant targets in response to phosphoramidon. The normalized NGS data showed that the top-ranking candidate genes were found to have a fold change (FC) ≥ 1.5 ($\log_2$ FC of 0.58) as

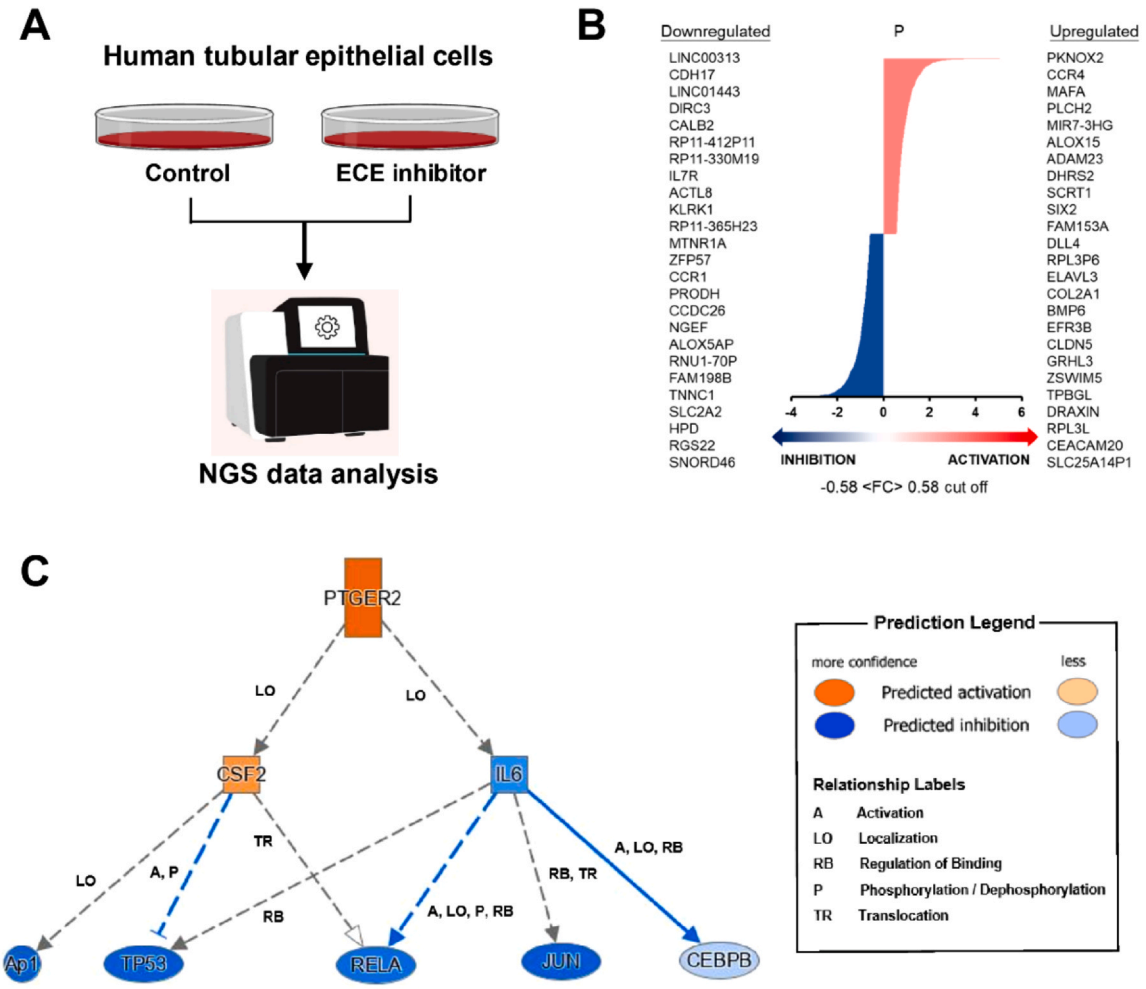

Fig. 1. Several genes and pathways were affected by the ECE inhibitor (phosphoramidon) in HK-2 cells. (A) The procedure of NGS data obtained from human kidney cells after treatment with or without phosphoramidon. Figures were created with BioRender.com. (B) The significantly up- and downregulated genes at a threshold of 1.5-fold change were highlighted for the mRNA levels after treatment with phosphoramidon. (C) The prediction of the PTGER2 network was generated from a comparison between the NGS data and the IPA database with a 1.5-fold change cutoff from HK-2 cells. The activating or inhibiting are colored orange or blue after phosphoramidon treatment, respectively.

Table 1
The top-ranking gene candidates by using the IPA database from phosphoramidon treatment compared with untreated HK-2 cells.

Gene name	Activation z-score	p-value
PTGER2	4.282	1.82E-09
KDM1A	3.289	3.14E-04
TBX2	3.207	4.47E-03
IKZF1	3.167	2.05E-04
FOXM1	2.861	3.63E-07
PPARGC1A	2.613	8.46E-03
MYC	2.541	1.96E-02
ZNF217	2.530	4.47E-03
DDIT3	2.514	1.21E-01
S100A6	2.514	2.94E-02

| FC | > 0.58 cut off

activation and FC ≤ 1.5 as inhibition in phosphoramidon relative to the control (Fig. 1B). The normalized NGS data were further analyzed using IPA to define specific molecular targets induced by phosphoramidon exposure in HK-2 cells. The data revealed that PTGER2 was the top suggested candidate to be activated in response to phosphoramidon exposure, with a z score of 4.282 and a p-value of 1.82E-09 (Table 1). The prediction of the PTGER2 network was derived from the common signature by comparison of the IPA database with NGS data from HK-2 cells with a 1.5-fold-change cutoff (Fig. 1C). The intensity of the color indicated the degree of activating (orange) or inhibiting (blue) regulation after phosphoramidon treatment. The predicted relationships of the PTGER2 network indicated that phosphoramidon may activate the expression of PTGER2 in HK-2 cells. In addition, prostaglandin E2 was predicted to be activated and further regulate its downstream genes, including AP1, TP53, CEBPB, RELA, and JUN, through upregulation of CSF2 or downregulation of IL6 after treatment with phosphoramidon.

3.2. Homology modeling of the EP2 protein by a homologous template 3D structure

Before constructing a three-dimensional structure for the EP2 protein, its amino acid sequence was used to identify conserved or similar sequences with a related homologous protein. For the first step of homology modeling, the sequence of EP2 was regarded as a target, while the EP4 protein with a tertiary structure was used as a template structure. The sequence alignments between EP2 and EP4 are displayed in Fig. 2A. The pairwise sequence alignments revealed that the sequence identity and similarities were 30.0% and 47.2%, respectively. Therefore, EP4 can be considered a candidate template structure for EP2 protein model generation. The homology model of the EP2 protein structure was built by the I-TASSER web server (Fig. 2B). For EP2 protein model validation, the Ramachandran plot indicated that 86.250% of residues were located in the favored region, 8.125% of residues were located in the semi-allowed region,

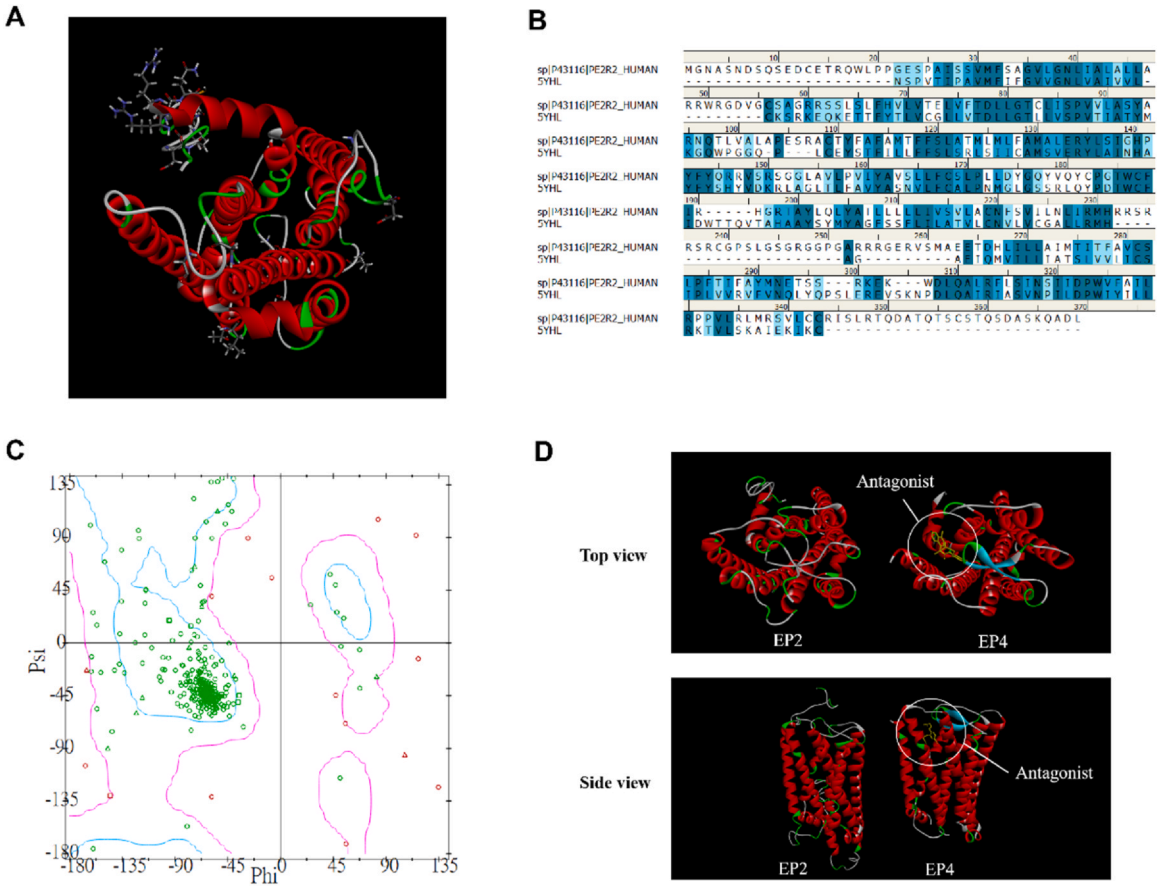

Fig. 2. Homology modeling of the EP2 protein structure according to the 3D conformation of the EP4 crystal structure via the I-TASSER web server. (A) The most reasonable model of the EP2 protein structure. (B) Ramachandran plot of each amino acid of the EP2 modeling protein. (C) Sequence comparison between the target protein (EP2) and template structure (EP4). The identity and similarity scores are 30.0% and 47.2%, respectively. (D) The binding site of the EP2 structure is defined by the site of the antagonist on the EP4 crystal structure.

and 5.625% were located in the disallowed regions (Fig. 2C). For binding site definition, the druggable site of the EP2 receptor for database virtual screening was determined according to the location of the antagonist in the crystal structure of EP4 (Fig. 2D).

3.3. Database virtual screening to identify potential candidates to fit the EP2 receptor

The docking results were ranked based on the LibDock score and are tabulated in Table 2. Among the top 10 compounds, we selected four purchasable candidates, ritodrine, dofetilide, dobutamine, and citalopram, for further analysis. The 2D diagram of the docking poses of the above four candidates in the EP2 binding site is displayed in Fig. 3. The docking pose of dobutamine generated four carbon H-bonds with SER24 and SER305, while the two residues VAL89 and MET31 contained phenyl groups and had pi-alkyl interactions with dobutamine. Dofetilide forms two carbon H-bonds with THR82 and ARG302, and three pi-alkyl interactions were found to interact with residues ILE27, MET31, and VAL89. In addition, it was found that ARG302 generates one pi-cation interaction with dofetilide. The 2D diagram of citalopram has no H-bond interaction with any residues but revealed pi-interactions such as pi-cation, pi-sulfur, and pi-alkyl with residues ARG302, MET31, and VAL89, respectively. For the docking pose of ritodrine in the EP2 binding site, the docked ligand generated two H-bonds with residues ARG302 and SER305. A pi-alkyl interaction was found between ritodrine and three residues, including MET31, ILE85, and ILE27.

Table 2
The top ten candidates were selected from the virtual screening of the drug bank database that ranked by Libdock score.

Ranking	DRUGBANK_ID	LibDock Score	Name	CAS No.
1	DB00640	99.9810	NA	NA
2	DB01102	99.9800	Arbutamine	128470–16–6
3	DB00867	99.9785	Ritodrine	23239–51–2
4	DB00204	99.9721	Dofetilide	115256–11–6
5	DB00841	99.9642	Dobutamine	49745–95–1
6	DB00179	99.9630	Masoprocol	500–38–9
7	DB01187	99.9509	NA	NA
8	DB00215	99.9503	Citalopram	59729–32–7
9	DB00131	99.9479	NA	NA
10	DB01145	99.9476	Sodium sulfoxone	144–75–2

3.4. Cell viability analysis of drug-treated HK-2 cells

We evaluated the toxic effects of the four candidate drugs in HK-2 cells before western blot analysis (Fig. 4). The viability of HK-2 cells treated with dobutamine was notably decreased at concentrations of 40 μM and 80 μM after 24 h of incubation. In contrast, the least toxic were dofetilide, citalopram, and ritodrine, with no inhibitory effects under 80 μM. For all candidate compounds, the cell viability of HK-2-treated cells did not significantly decrease at a concentration of 40 μM. Therefore, the concentration of 40 μM can be considered in the following experimental design for the test compounds.

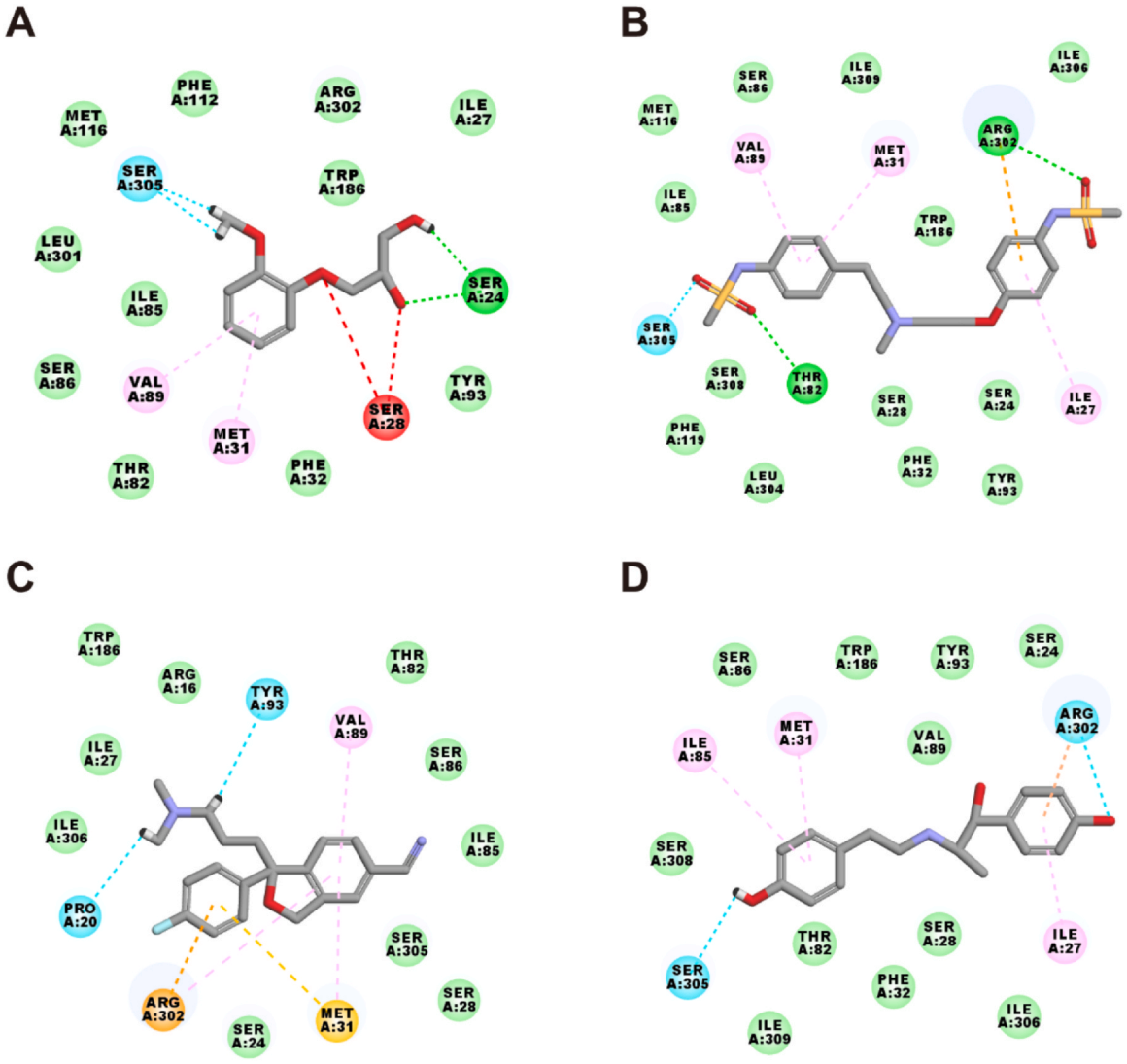

Fig. 3. Docking poses of selected candidates on the binding site of the EP2 protein structure: (A) dobutamine, (B) dofetilide, (C) citalopram, and (D) ritodrine. Chemical interactions such as conventional H-bonds, carbon H-bonds, pi-donor H-bonds, pi-cation, pi-alkyl, pi-sulfur, and van der Waals interactions are colored green, cyan, purple, orange, pink, and yellow, respectively.

3.5. The candidate drugs increase the autophagy-related proteins in HK-2 cells

To investigate whether the aforementioned candidate drugs affect the autophagy signaling pathway, HK-2 cells were exposed to dobutamine, dofetilide, citalopram, and ritodrine at specific concentrations of 0, 10, 20, and 40 μM for 24 h. Subsequently, we examined whether phosphoramidon induced autophagy. We analyzed autophagy-related proteins via Western blotting to understand whether the four candidate drugs induced autophagy (Fig. 5). Our results showed that HK-2 cells treated with all tested drugs exhibited increased levels of Beclin 1, p62, and LC3-II in a concentration-dependent manner. Moreover, we analyzed the protein expression ratio for all autophagic markers by comparing it with the internal control of GAPDH from Western blot experiments. Specifically, cells treated with 40 μM dobutamine had significantly higher Beclin 1 expression than the other two autophagic markers. Dofetilide-treated cells increased the protein expression ratio of Beclin 1 and LC3-II at specific concentrations of 20 μM and 40 μM. For citalopram-treated cells, the levels of LC3-II were significantly increased at both concentrations of 20 μM and 40 μM compared to other autophagic markers. HK-2 cells treated with ritodrine exhibited significantly increased Beclin 1 expression at specific concentrations of 20 μM and 40 μM. In addition, the protein expression ratio of LC3-II was increased in HK-2 cells after treatment with 40 μM ritodrine. These results suggest that dobutamine, dofetilide, citalopram, and ritodrine at 40 μM could induce autophagy markers in HK-2 cells.

3.6. The candidate drugs suppress LPS+ATP-induced NLRP3 inflammasome activity in HK-2 cells

We further studied the anti-NLRP3 inflammasome activity in LPS +ATP-treated HK-2 cells after treatment with dobutamine, dofetilide, citalopram, and ritodrine at different concentrations of 0, 10, 20, and 40 μM for 24 h. The expression of NLRP3 and cleaved caspase-1 was significantly increased after LPS+ATP-induced activation in HK-2 cells. The study of anti-NLRP3 inflammasome activation revealed that the expression of NLRP3 and cleaved caspase-1 was concentration-dependently decreased after treatment with the four candidate drugs in LPS+ATP-treated HK-2 cells compared to the control group (Fig. 6). Dobutamine, dofetilide, and ritodrine suppressed the expression of NLRP3 at a concentration of 20 μM, which was higher than that of LPS+ATP-treated HK-2 cells without drug treatment. In addition, the protein expression of cleaved caspase-1

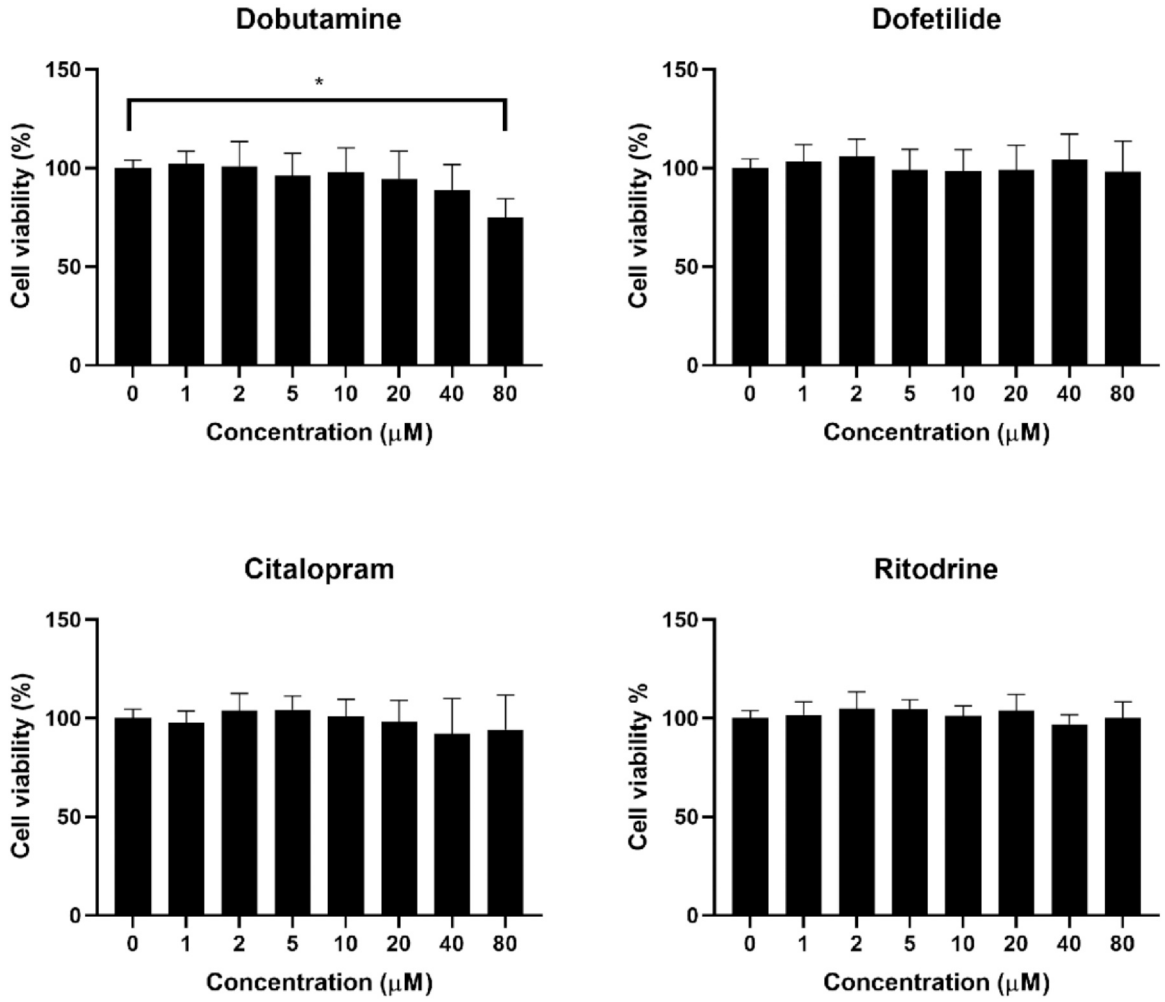

Fig. 4. Cell viability of HK-2 cells after treatment with four drug candidates for 1 d. The cell viability data were obtained using the SRB assay. Each cell viability data point was normalized to the control group at a concentration of 0 μM and represented as the percentage of cell viability (%). Data are shown as the means ± standard deviations of three individual experiments, n = 3, * $p < 0.05$ between the indicated groups.

displayed a significant decrease at a concentration of 40 μM after treatment with the four candidate drugs. These results suggested that dobutamine, dofetilide, citalopram, and ritodrine suppressed LPS+ATP-induced activation of the NLRP3 inflammasome in the HK-2 cell injury model.

3.7. The candidate drugs trigger anti-fibrosis in HK-2 cells

We measured the levels of fibrosis factors (including collagen type 1, CTGF, and PAI-1). Western blot analysis of anti-fibrosis revealed that the expression of fibrosis-related proteins, including collagen type 1, CTGF, and PAI-1, was significantly upregulated after treatment with transforming growth factor β (TGF-β) in HK-2 cells (Fig. 7). The data showed that HK-2 cells treated with TGF-β and four candidate drugs had antifibrotic effects. All candidate drugs significantly suppressed the expression levels of fibrosis-related proteins at concentrations of 10, 20, and 40 μM compared to TGF-β-induced cells without treatment after 24 h of incubation. These results indicated that dofetilide, citalopram, and ritodrine at 40 μM could reduce fibrosis effects in response to injury-related signals in HK-2 cells.

4. Discussion

The G-protein-coupled E-prostanoid including EP1, EP2, EP3, and EP4 are important mediators of PGE2 actions in the renal microvasculature [34]. The activation of EP2 and EP4 contribute to anti-inflammatory effects through PGE2 interaction, while EP1 and EP3 mediate potentiate inflammation [35]. Previous reports have illustrated that PGE2 attenuates the endothelin-induced vasoconstriction responses through EP2 leading to vasorelaxation in the kidney [36–38]. In addition, PGE2 also has potent anti-fibrogenic potential to ameliorate intestinal fibrosis in rat liver macrophages through the receptors EP2 and EP4, which inhibit the release of the fibrogenic-associated factors TNF-α, ET-1, and IL-lα while enhancing the anti-fibrogenic mediators IL-6 and IL-10 [39]. According to a previous report, EP2 knockout transgenic mice contribute to a reduction in the proinflammatory response after injury [40]. In other cases, PGE2-EP2/EP4 signaling mediates the expression of anti-inflammatory cytokines through anti-inflammatory cytokine IL-10 signaling and function [41]. The inhibition of cytokine TGF-β-induced cell proliferation in vivo is directly inhibited by PGE2 through EP2 and EP4 [42], which might be involved in the PKC–Raf1–MEK1/2–ERK1/2, PKA/AKT, and MAPK/ERK signaling pathways in the prevention of the progression of CKD [43]. In our present study, the normalized NGS gene expression data successfully identified a significant PTGER2 gene, the EP2 protein, that is activated in response to human

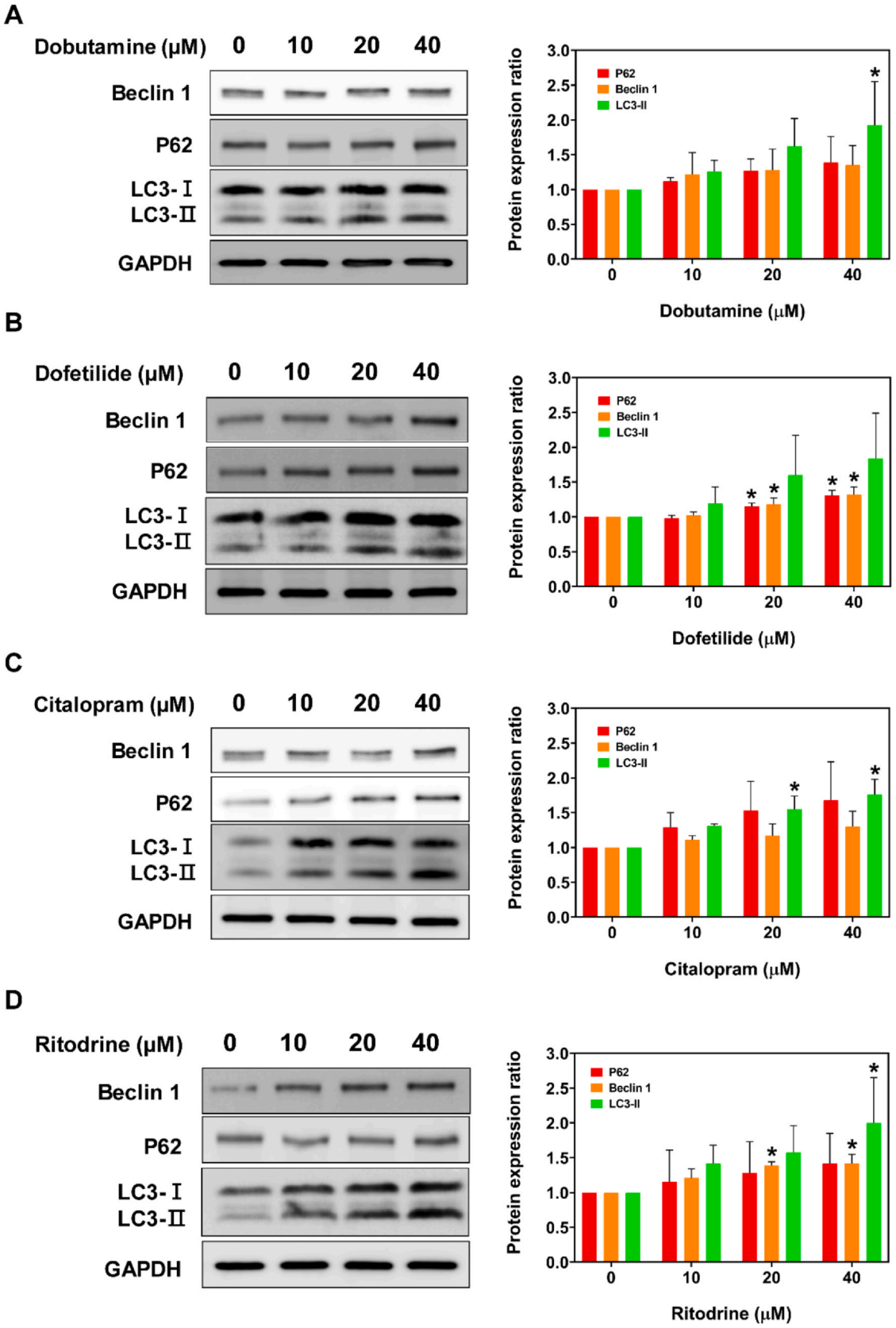

Fig. 5. The autophagy-related protein levels of LC3-I, LC3-II, Beclin 1, and p62 were measured in drug-treated HK-2 cells at concentrations of 0, 10, 20, and 40 μM for 24 h: (A) dobutamine, (B) dofetilide, (C) citalopram, and (D) ritodrine. GAPDH was regarded as the internal control. Data are shown as the means ± standard deviations of three individual experiments, n = 3. $^{*}p < 0.05$ compared with the control.

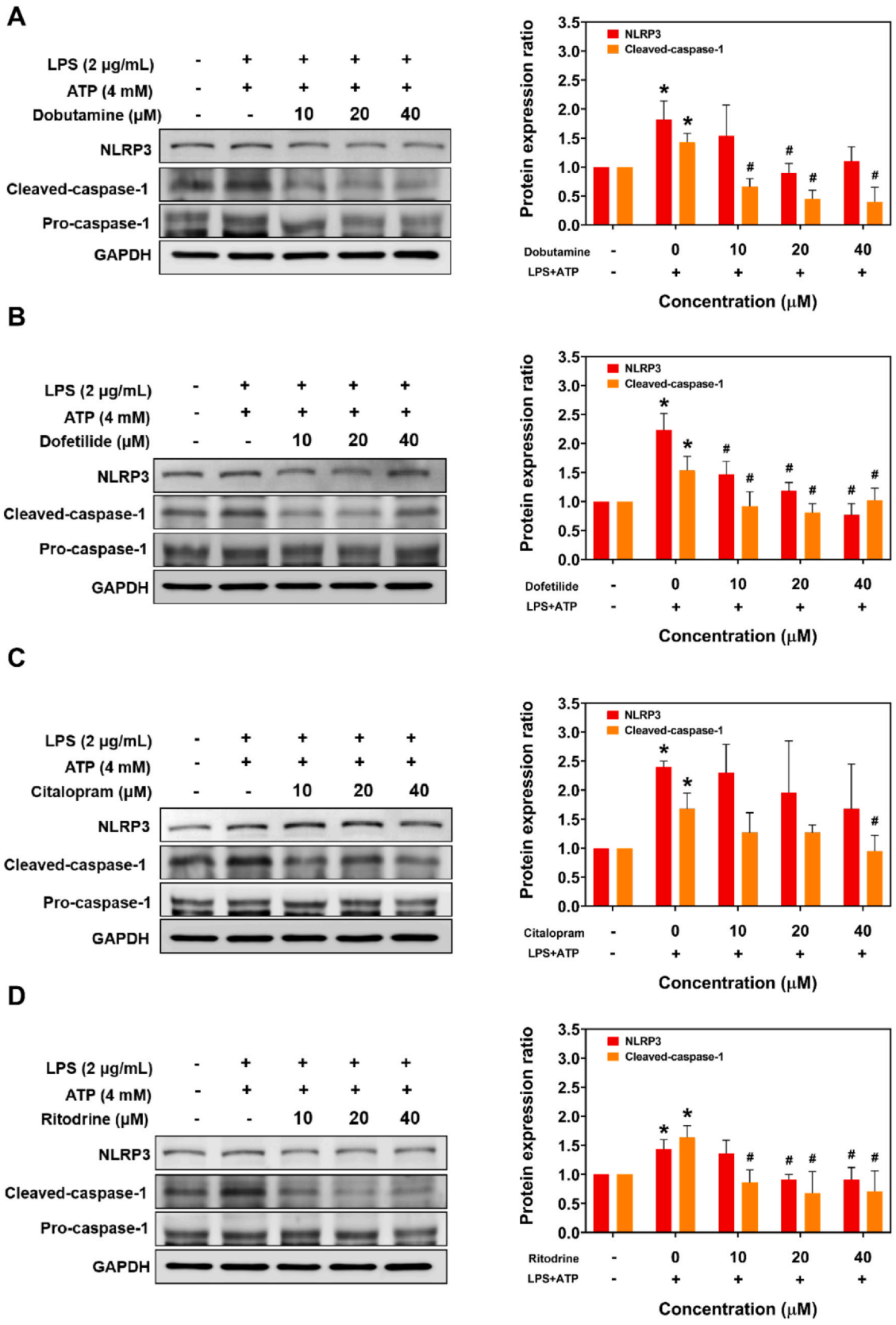

Fig. 6. Western blot analysis of NLRP3 inflammasome-related protein expression in drug-treated HK-2 cells at concentrations of 0, 10, 20, and 40 μM: (A) dobutamine, (B) dofetilide, (C) citalopram, and (D) ritodrine. GAPDH was used as the internal control. The LPS+ATP-induced NLRP3 inflammasome in HK-2 cells was incubated with the test compounds and LPS (2 μg/mL) for 24 h and subsequently treated with ATP (4 mM) for 2 h. # $p < 0.05$ compared with the LPS+ATP group. * $p < 0.05$ compared with the control group. Data are shown as the means ± standard deviations of three individual experiments, n = 3.

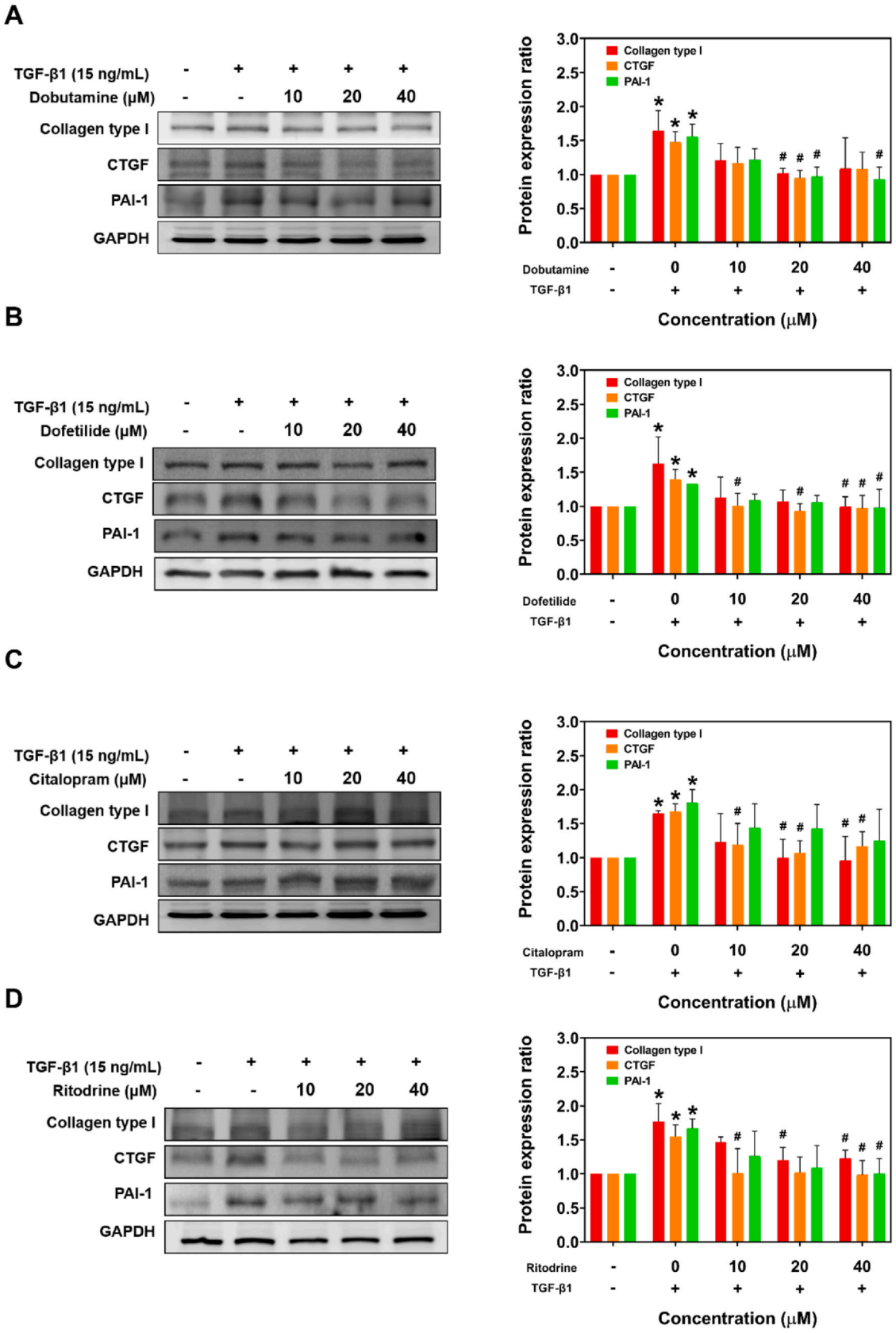

Fig. 7. Expression levels of fibrosis-related proteins in HK-2 cells via western blot analysis. Cells were treated with (A) dobutamine, (B) dofetilide, (C) citalopram, and (D) ritodrine at concentrations of 0, 10, 20, and 40 μM for 24 h. GAPDH was regarded as the internal control. The fibrosis in drug-treated HK-2 cells was treated with TGF-β1 (15 ng/mL) and subsequently incubated for 24 h. # $p < 0.05$ compared with the TGF-β1 group. * $p < 0.05$ compared with the control group. Data are shown as the means ± standard deviations of three individual experiments, n = 3.

kidney cells after treatment with an ECE inhibitor by using IPA database analysis. According to our predictive signaling network, the activation of PTGER2-upregulated expression of CSF2 has high confidence in the downregulation of AP1 and RELA genes via activation, localization, and regulation of binding. In addition, PTGER2 increased CSF2, contributing to decreased phosphorylation and activation of the TP53 protein. The IL6 gene was downregulated by activated PTGER2 in the predicted network, which downregulated TP53, RELA, and JUN with high confidence but inhibited CEBPB with less confidence. Importantly, previous reports have demonstrated that the downregulation of AP1 [44], TP53 [45], RELA [46], IL6 [47], and CEBPB [48] are critical elements of the inflammatory and fibrosis responses in sepsis or fibrosis models. Therefore, this evidence illustrated that the EP2 is the putative candidate to be activated in response to phosphoramidon, which is the top ranking of candidate genes and displayed > 1.5-fold differential expression in ECE inhibitor relative to control from the results.

An early study demonstrated that inhibition of the PGE2/EP2R and coupled cAMP–PKA–CREB signaling pathways ameliorated diabetes-associated inflammation, which was associated with acute activation of the NLRP3 inflammasome [49]. In addition, another previous study also illustrated that the activation of PGE2 signaling could attenuate the activation of macrophages induced by LPS stimulation and reduce TNF-α expression levels [50]. PGE2 and its receptor EP2 are effective at attenuating NLRP3 inflammasome activation against the LPS-induced classical activation of macrophages. Kidney infiltration by macrophages is common in human CKD. So far, many previous studies have used tubular cells to uncover the mechanism of NLRP3 inflammasome after injury [51]. Besides, the tubular cells can also be used to study the nephroprotective effects of active compounds in Chinese herbs through the pathway of NLRP3 inflammasome [52,53]. Moreover, our present study emphasized that the EP2 receptor is strongly expressed in response to phosphoramidon after exposure in human kidney cells from the results of IPA database analysis. Therefore, targeting EP2 is a promising therapeutic strategy that reduces both inflammatory and fibrotic responses produced by macrophage activation [54]. Therefore, the magnitude of EP2 activation correlates with the anti-inflammatory and antifibrotic effects, suggesting an effector function in CKD treatment.

In clinical data, TGF-β plays a key role in pulmonary fibrosis and can be detected in the lung tissue of patients with chronic, progressive, fibrotic interstitial lung disease [55]. However, several studies have shown that EP2 receptor activation can attenuate renal fibrogenesis [56]. Stimulation of the EP2 receptor effectively mitigates kidney fibrosis in several models of renal injury [57]. A selective EP2 antagonist contributes to more severe cystic disease and renal fibrogenesis [58]. Unfortunately, the underlying antifibrotic effect of human kidney disease is not fully understood. To date, there is growing research showing that autophagy attenuates tubulointerstitial fibrosis by suppressing NLRP3 inflammasome signaling and TGF-β/Smad signaling in renal injuries. Moreover, our previous report found that the ECE inhibitor phosphoramidon induces autophagy-associated proteins without any injury or stress in kidney cells. Thus, the activation of autophagy might have renoprotective functions via inhibition of the NLRP3 inflammasome and suppression of renal fibrosis. Due to autophagy being a dynamic process, the protection of kidney injury needs to depend on the time and the molecular mechanism of autophagy and the intensity of induction [59].

In our current study, the in vitro pharmacology of the four candidate drugs, including ritodrine, dofetilide, dobutamine, and citalopram, displayed significant effects on the induction of autophagy, attenuation of NLRP3 inflammasome activation, and inhibition of fibrosis-related proteins. These findings were consistent with our previous report that the ECE inhibitor phosphoramidon triggered autophagy to suppress NLRP3 inflammasome signaling and attenuate tubulointerstitial fibrosis in human kidney cells. Thus, our experimental data confirmed that the EP2 receptor suggested by NGS data is a potential target for the development of a renoprotective drug to attenuate the progression of CKD. The abovementioned candidate drugs filtered from EP2 protein structure can induce autophagy-related proteins, which also ameliorate NLRP3 inflammasome and renal fibrosis expression. In this report, we demonstrate that these suggested candidate drugs can be regarded as potential new treatments for chronic inflammatory disease in the human kidney.

5. Conclusions

The present findings showed that ritodrine, dofetilide, dobutamine, and citalopram are high-affinity protein-binding drugs for EP2 receptors. We found that these abovementioned candidates induced autophagy in HK-2 cells, which inhibited NLRP3 inflammasomes and reduced fibrosis-related proteins. Collectively, combining computational modeling and cell-based method provides potent clinically used drugs as an alternative therapeutic effect for new renoprotective treatments to delay the progression of kidney injury in CKD patients.

CRediT authorship contribution statement

Hung-Jin Huang: Investigation, Methodology, Software, Formal analysis, Writing - original draft, Writing - review & editing. **Yu-Hsuan Lee:** Conceptualization, Funding acquisition. **Li-Chin Sung:** Conceptualization, Funding acquisition. **Yi-Jie Chen:** Formal analysis, Methodology. **Yu-Jhe Chiu:** Formal analysis, Methodology, Data curation, Software. **Hui-Wen Chiu:** Conceptualization, Funding acquisition, Writing - original draft, Writing - review & editing. **Cai-Mei Zheng:** Conceptualization, Funding acquisition, Supervision, Project administration.

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.

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