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Alvespimycin is identified as a novel therapeutic agent for diabetic kidney disease by chemical screening targeting extracellular vesicles

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Extracellular vesicles (EVs) are important mediators of intercellular communication and play key roles in the regulation of pathophysiological processes. In diabetic kidney disease (DKD), it has been reported that macrophages recruited in the mesangial region may play pathogenic roles through inducing local inflammation in glomeruli. We focused on EV-mediated crosstalk between mesangial cells (MC) and macrophages as a novel therapeutic target for DKD. EVs released from MC induced inflammation in macrophages and the effect was enhanced under high-glucose conditions. For discovering novel therapeutic agents which can inhibit such EV-mediated mechanisms, drug repositioning is considered as an effective tool. We established a unique screening strategy and screened agents to aim at maximizing their specificity and potency to inhibit EV mechanisms, along with minimizing their toxicity. We succeeded in identifying alvespimycin, an HSP90 inhibitor. Treatment of diabetic rats with alvespimycin significantly suppressed mesangial expansion, inflammatory gene activation including macrophage markers, and proteinuria. The inhibitory effect on EV uptake was specific to alvespimycin compared with other known HSP90 inhibitors. MC-derived EVs are crucial for inflammation by intercellular crosstalk between MC and macrophages in DKD, and alvespimycin effectively ameliorated the progression of DKD by suppressing EV-mediated actions, suggesting that EV-targeted agents can be a novel therapeutic strategy.

Keywords Extracellular vesicle, Drug screening, Diabetic kidney disease, Intraglomerular crosstalk, Mesangial cells, Macrophages

Extracellular vesicles (EVs) are important mediators of intercellular communication and play a key role in the regulation of pathophysiological processes in various organs and cells[1]. Particular attention has been paid to intercellular crosstalk by exosomes, whose size is 30–100 nm[2]. The presence of EVs in serum and urine has attracted attention not only in the field of oncology but also in the field of kidney diseases, because EVs can influence the surrounding environment by propagating the properties of their mother cells to the neighboring cells and environment[3–5].

Diabetic kidney disease (DKD) is the most common cause of chronic kidney disease and end-stage renal disease, leading to a high risk of mortality. The pathophysiology of DKD is complicated and multifaceted, and yet to be fully elucidated. Furthermore, treatments to effectively prevent the progression of DKD are still quite limited, so that novel therapeutic strategies are strongly desired. It has been a general consensus that DKD progresses along with mesangial proliferation and matrix accumulation. Macrophages infiltrate into the mesangial areas upon mesangial activation. In particular, we already reported that endogenous ligands associated with inflammation are induced specifically in glomerular infiltrating macrophages, which may have a potential

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pathogenic role in DKD[6] and glomerulonephritis[7]. These findings suggest that, in DKD, there should be some yet undefined mechanisms that specifically activate infiltrating macrophages locally in a paracrine manner.

Recently, a potential role of intraglomerular cellular crosstalk mediated by EVs in DKD has been reported. Wu et al. reported that vascular endothelial cells exposed to hyperglycemia release EVs and activate mesangial cells, leading to renal fibrosis[8]. Liu et al. showed that macrophage-derived EVs promote activation of NLRP3 inflammasome and autophagy deficiency of mesangial cells in diabetic nephropathy[9]. However, the pathophysiological role of EVs in cell–cell interaction between mesangial cells and macrophages still remains obscure, especially those from mesangial cells toward macrophages in the glomeruli.

In the present study, we investigated the role of EVs in the intercellular crosstalk between mesangial cells and macrophages in DKD. Furthermore, in order to explore a novel therapeutic strategy to intervene the EV actions between those cells and to deter the progression of DKD, we screened chemical agents that can specifically act on the behavior or action of EVs. EVs include various kinds of proteins, messenger RNAs and microRNAs[10], and multiple EV factors are assumed to be involved in the cell–cell crosstalk, suggesting that targeting a single factor might result in only partial effects. Therefore, we focused on finding chemical compounds that have effects on the primary actions on EVs derived from mesangial cells, not pursuing particular factors present in the EV as a target. Drug repositioning is a powerful and effective tool for discovering therapeutic chemical compounds in various kinds of disease[11,12]. Herein, we performed high-throughput chemical screening using a validated compound library of existing drugs to identify novel candidate agents, and verified their efficacy in DKD.

Materials and methods

Methods of reporter analysis, cell culture, real-time quantitative RT-PCR, in vivo EV-uptake study and histological analyses are described in Supplementary Methods.

EV isolation

EVs were collected from the medium using ExoQuick-TC Exosome Precipitation Solution (System Biosciences, Mountain View, CA, USA) and MagCapture Exosome Isolation Kit PS (Wako, Osaka, Japan) in accordance with the manufacturer's instruction. Sprague–Dawley rat glomerular mesangial cells (SDMCs) were maintained in DMEM/Nutrient Mixture F-12 Ham containing 5.6 mmol/L or 25 mmol/L glucose without fetal bovine serum for 24 h before isolation of EVs. For osmotic adjustment, mannitol (Nacalai Tesque, Kyoto, Japan) was added to the 5.6 mmol/L medium (24.5 mM final concentration). For EV preparation by the ExoQuick method, the cultured medium of SDMCs was centrifuged at 3000×*g* for 10 min to remove cellular debris. One-fifth of ExoQuick-TC was added to the supernatant and incubated overnight at 4 °C. The suspension was centrifuged at 1500×*g* for 30 min. The supernatant was discarded, and the remaining pellet was subjected to another centrifugation at 1500×*g* for 5 min. The pellet was resuspended with PBS and used as EVs.

To isolate EVs by the MagCapture Exosome Isolation Kit PS, the cell culture medium was centrifuged at 300×*g* for 30 min at 4 °C to remove cells and debris. The supernatant was transferred into a new tube, and centrifuged at 1200×*g* for 20 min at 4 °C. To remove large EVs, the supernatant was transferred again into a new tube, and centrifuged at 10,000×*g* for 30 min at 4 °C. Then, the sample was concentrated by using ultrafiltration unit (Vivaspin20, Sartorius, Gottingen, Germany), transferred to a new 1.5 mL microcentrifuge tube, and suspended in Exosome Binding Enhancer at a 1:500 volume. Well mixed samples were transferred into 1.5 mL Reaction Tube containing Exosome-Capture-immobilized beads, and the mixture was rotated overnight at 4 °C. The beads were washed three times with 1 mL of washing buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.0005% Tween20, 2 mM $CaCl_2$), and the bound EVs were eluted with elution buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA)[13]. An aliquot of the EV preparation was used for EV counting by NanoSight NS300 system (Malvern Panalytical, Salisbury, UK) followed by normalization to the total cell number. EV protein content was quantified using BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

DiO labeling of EVs

To prepare labeled EVs, we conducted fluorescent staining of the supernatant of cultured SDMCs with lipophilic green fluorescent dye 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) and isolated EVs derived from SDMCs by ExoQuick-TC. We added DiO (Vybrant DiO Cell-labeling Solution; Invitrogen, Carlsbad, CA, USA) to the collected supernatant at a final concentration of 0.5 μL/mL and incubated them for 10 min at 37 °C protecting from light. Hereafter, the same procedure was performed as mentioned.

Confocal microscopy and flow-cytometry

For confocal microscopic analysis, RAW 264.7 mouse macrophages were stained with red fluorescent dye (CytoTrace Red CMTPX, AAT Bioquest Inc., Pleasanton, CA, USA) for 24 h, and culture medium is labeled with a blue water-soluble, cell-impermeant polar tracer (Cascade Blue hydrazide, Thermo Fisher Scientific). After incubation, DiO-labeled EVs (DiO-EV) were added just before microscopic observation, and the uptake of DiO-EV in macrophages was evaluated. All images were scanned with confocal microscopy, FV3000 (Olympus, Tokyo, Japan).

For flow cytometry (FCM), RAW 264.7 cells were seeded in six-well plates (5×10^5/well) and grown overnight. Prior to treatment with DiO-EV, cells were washed with PBS, and then DiO-EV in 100 μL PBS/well were added and incubated at 37 °C for 24 h. Cells stained directly with 1 μL/well DiO (1 μL/mL) served as a positive control, and unstained cells as a negative control. Pre-incubation was performed with 10 μg/mL of an endocytosis inhibitor, cytochalasin D (Cayman Chemical, MI, USA) for 30 min before adding EVs. DiO-labeled EV–treated cells were removed from plates by trypsin, centrifuged, and resuspended in 1 mL of PBS. FCM was performed by SH800S Cell Sorter (Sony Life Science, Tokyo, Japan). FCM data were analyzed with FlowJo V10 program (FlowJo LLC, Ashland, OR, USA)[7].

Screening of compounds inhibiting EV-mediated mechanisms

Compound screening was conducted using a chemical library containing 3267 compounds from Drug Discovery Initiative at the University of Tokyo. The protocol was approved prior to the initiation of this study (approved by JP23ama121053, Project No. 0202).

The screening strategy consisted of 5 steps. Briefly, in Step 1, small molecules (2 mM) in dimethyl sulfoxide (DMSO) solution from library plates were added to cultured THP-1-Dual Cells in 96 well plates, and secreted embryonic alkaline phosphatase (SEAP)–reporter activities were monitored (see Supplementary Methods). Compounds inhibiting the NF-κB activity induced by mesangial cell–derived EVs (MC-EV) over 40% were selected and proceeded to the next step. In Step 2, steroidal compounds with an apparent NF-κB inhibitory action were excluded. Step 3 was composed of two strategies: in Step 3A, compounds exhibiting concentration-dependent NF-κB inhibition were selected, in which final compound concentrations were 0.2 μM, 1 μM, and 5 μM; in Step 3B, compounds showing a high toxicity were excluded, defined as the cell survival rate < 60% determined by Cell Count Reagent SF (Nacalai Tesque). The top 160 compounds that passed both Steps 3A and 3B proceeded further. In Step 4, compounds with a higher specificity to EV-mediated inflammation compared to lipopolysaccharide (LPS)-mediated inflammation were chosen; the criteria were set as the ratio of the inhibition rate against EVs to that against LPS to be > 1.5. Finally, in Step 5, compounds that inhibited EV uptake in THP-1 cells were selected, using DiO-EV and FCM analysis.

Animal experiments

Among the compounds thus selected, we focused on one compound, alvespimycin (Alv, also known as 17-dimethylaminoethylamino-17-demethoxygeldanamycin, 17-DMAG; InvivoGen, San Diego, CA, USA) as a candidate inhibitor of EV-mediated mechanisms in vivo. Experiments were conducted using eight-week-old male Sprague–Dawley rats (purchased from Japan SLC, Shizuoka, Japan. The strain was introduced from Charles River Laboratories, Inc. USA), dividing into the following four groups: (1) wild type (WT) + vehicle, (2) WT + Alv, (3) streptozotocin (STZ) + vehicle, and 4) STZ + Alv. Rats were housed in a room maintained at constant temperature, humidity, and light cycle (12:12-h light–dark) with free access to food and water. They were maintained maximum 2 individuals in one cage. After allowing rats to adapt to their environment for 1 week, STZ group and STZ + Alv group rats were injected with STZ (50 mg/kg body weight in citrate buffer, pH 4.0; Sigma-Aldrich, St. Louis, MO, USA) from the tail vein after 16 h of fasting to develop insulin-dependent diabetes. All rats survived, and their blood glucose level increased over 280 mg/dL a week after STZ administration. Three weeks after STZ administration, Alv was administered intravenously via the tail vein twice a week at a dose of 0.5 mg/kg. After 6 weeks of treatment with vehicle (200 μL saline) or Alv, 16 h-fasted rats were anesthetized and sacrificed. Blood, urine and kidney samples were collected. Urine albumin and creatinine levels were measured by using an immunoturbidimetric method (Oriental Yeast, Shiga, Japan). Euthanasia was conducted by intraperitoneal pentobarbital (250 mg/kg). In all animal experiments, we complied with the ARRIVE guidelines.

Ethics

All animal procedures were conducted in accordance with the guidelines for care and use of laboratory animals approved by Kumamoto University (No. 29-115, 30-013).

Data deposition

The original complete datasets are openly available in repository figshare at https://figshare.com/articles/dataset/Original_raw_data_of_each_step_of_the_drug_screening_/25018745.

Statistics

Data are expressed as means ± SEM. Differences between multiple groups were assessed by ANOVA with Tukey's test using Graphpad Prism (Graphpad Software). Comparison between two groups was carried out by unpaired Student's *t* test. Statistical significance was defined as $p < 0.05$.

Role of the funding source

Funders had no input on study design, data collection, data analyses, interpretation, or writing of report.

Results

Quality assessment of mesangial cell–derived EVs and evaluation of their activity on NF-κB signaling in macrophages

It is crucial to assure the quality of extracted EVs, MC-EV, which are the target of drug screening. Hence, first, quality evaluation of the extracted EVs was performed. We compared the most widely used precipitation method using ExoQuick with that using MagCapture, which has been reported to obtain relatively purer EVs. The MagCapture method is an extraction technique based on the binding of phosphatidylserine and Tim4[14]. Although EVs by ExoQuick contained larger particles compared to those by MagCapture (Fig. 1a), particle counts gated in diameters within 30–120 nm were equivalent in the both isolation methods (Fig. 1b).

Next, the effects of MC-EV upon NF-κB signaling were evaluated by SEAP-reporter assay in THP-1 cells. EVs extracted from mesangial cells induced the NF-κB activity in a dose-dependent manner with both techniques (Fig. 1c). According to these results, we employed the ExoQuick method throughout the following all experiments.

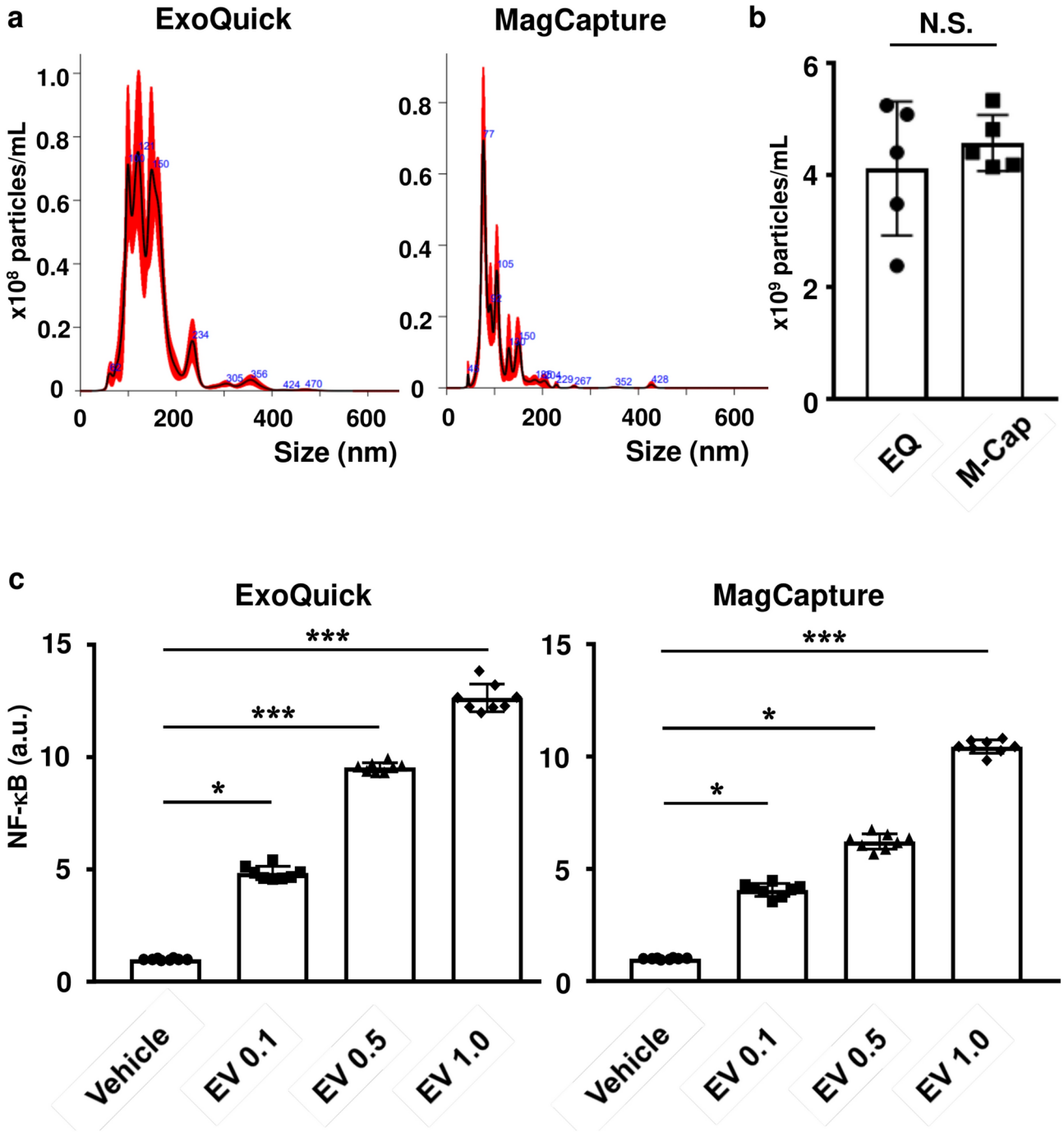

Fig. 1. Effects of mesangial cell-derived EVs upon macrophages. (**a**) Evaluation of extracted EVs from mesangial cells by nanoparticle tracking analysis. (**b**) Evaluation of extracted EVs from mesangial cells by nanoparticle counts. (particle size 30–120 nm, n = 5). (**c**) NF-κB activity in macrophages stimulated with mesangial cell-derived EVs isolated by polymer method; ExoQuick and phosphatidylserine affinity method; MagCapture (n = 8). Graph data are mean ± s.e.m. N.S., not significant; EQ, ExoQuick; M-Cap, MagCapture. $^{*}P < 0.05$, $^{***}P < 0.001$ versus control.

EVs derived from mesangial cells are endocytosed by macrophages in vitro and in vivo

In order to examine whether macrophages uptake prepared EVs, we evaluated the localization of fluorescence-labeled MC-EV stained with lipophilic green fluorescent dye DiO in tracer-labeled macrophages. Confocal microscopy findings showed that DiO-EV were colocalized with cell-impermeant polar tracer, Cascade blue in macrophages, indicating endocytosis (Fig. 2a). These uptakes were obviously suppressed by Cytochalasin D, an endocytosis inhibitor (Fig. 2b).

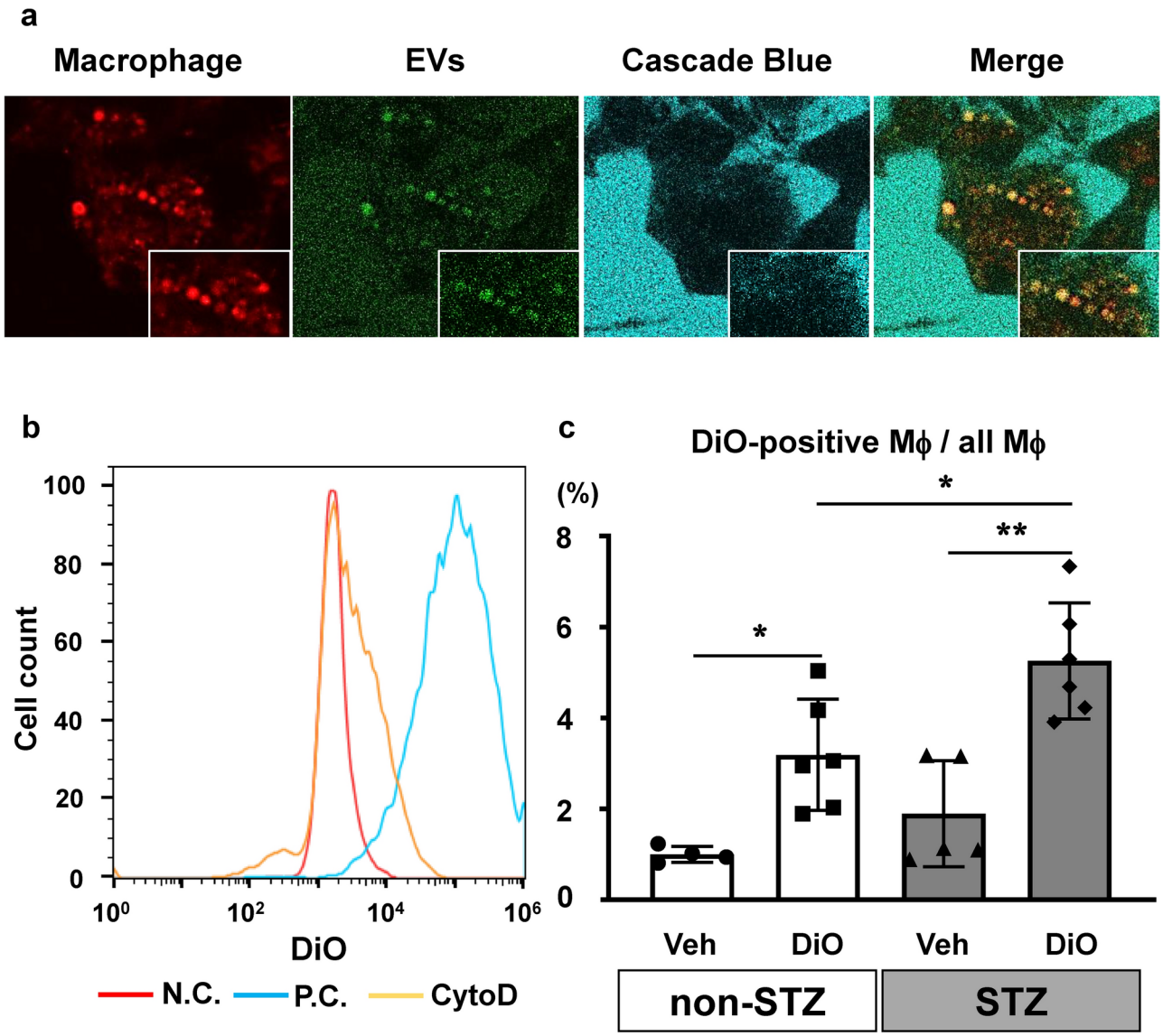

Fig. 2. EVs derived from mesangial cells are endocytosed by macrophages in vitro and in vivo. (**a**) Representative images of RAW264.7 macrophage uptaking DiO-labeled EVs. DiO-EV were extracted from DiO-labeled mesangial cells. Macrophages are stained with red fluorescent dye (CytoTrace Red CMTPX) and culture medium is labeled with blue water-soluble, cell-impermeant polar tracer (Cascade blue hydrazide). White-framed squares are magnified images of inside the cells. (**b**) Positivity of DiO-EV in RAW264.7 macrophages evaluated by FCM was reduced by cytochalasin D, an endocytosis inhibitor. N.C., untreated negative control; P.C., DiO-EV treated positive control; CytoD, DiO-EV and cytochalasin D-treated. (**c**) Percentages of DiO-positive macrophages / all macrophages in the peripheral blood of non-STZ and STZ-mice were evaluated by FCM at an hour after DiO-EV injection (n = 4–6). Graph data are mean ± s.e.m. Mϕ, macrophage; STZ, streptozotocin. $^*P < 0.05$.

We also examined the EV uptake in animal models with high-glucose condition. Non-diabetic control and STZ-mice were administered with DiO-EV by tail-vein injection and sacrificed at 1 h from injection. FCM analysis of peripheral blood was conducted and the number of DiO-positive monocytes was evaluated. The ratio of DiO-positive macrophages to all macrophages was significantly higher in STZ mice than non-diabetic mice (Fig. 2c). Thus, EVs released from mesangial cells could be uptaken through endocytosis by macrophages both in vitro and in vivo.

EVs derived from high glucose–conditioned mesangial cells augment inflammation in macrophages

Particle counts determined by NanoSight showed no significant changes in EVs prepared from mesangial cells with either high-glucose (HG-EV) or low-glucose (LG-EV) conditions (Fig. 3a). However, as to the induction of inflammatory response, corrected by particle counts, HG-EV exhibited more NF-κB activation than LG-EV

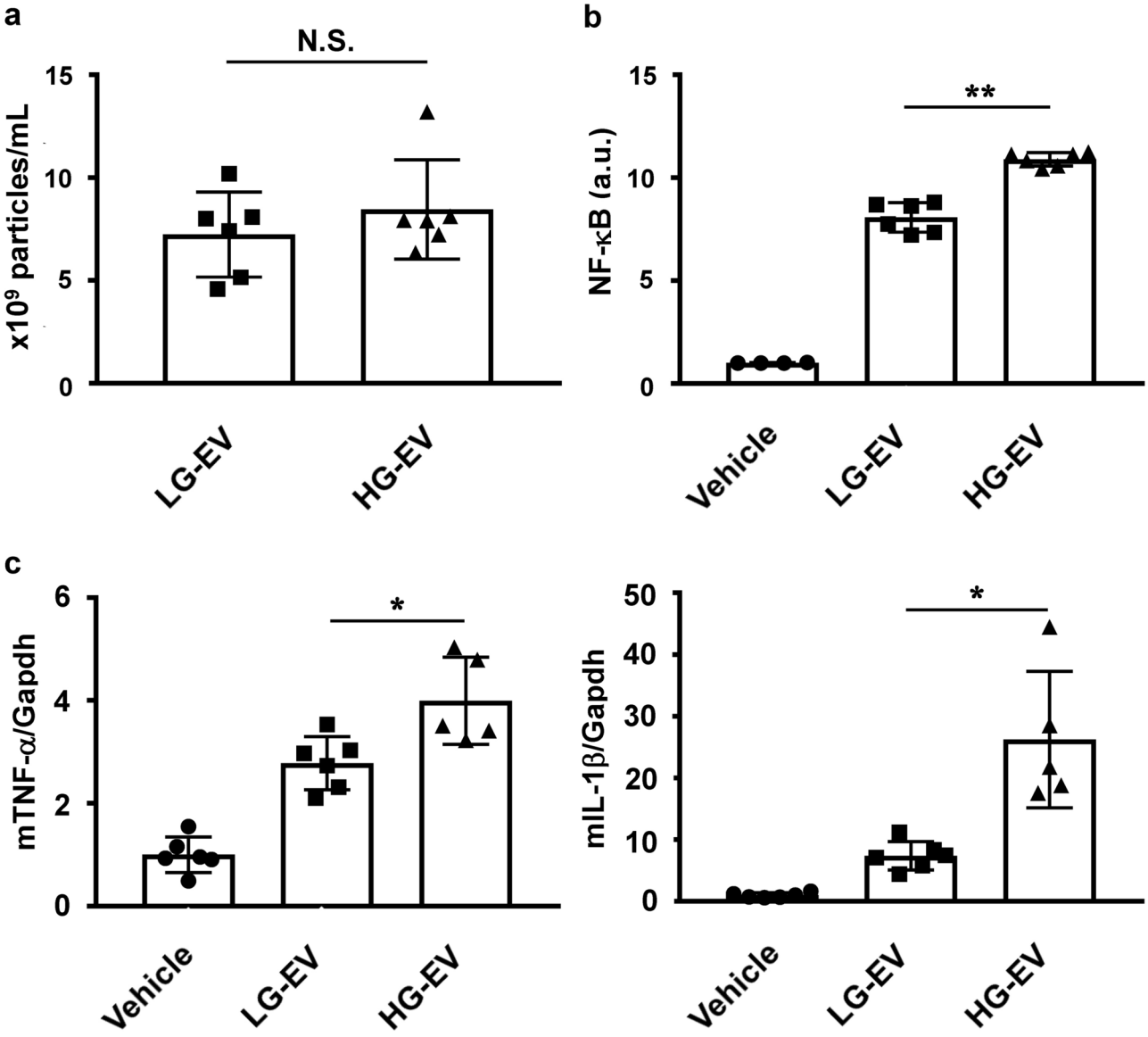

Fig. 3. Effects of EVs isolated from high-glucose and low-glucose conditioned-mesangial cells upon macrophages. (**a**) Particle counts of EVs derived from high-glucose (triangles, HG-EV) and low-glucose (squares, LG-EV) conditioned-mesangial cells (n = 6). (**b**) NF-κB activation evaluated by SEAP-reporter in macrophages stimulated with HG-EV or LG-EV (n = 4–6). (**c**) Expressions of TNF-α and IL-1β mRNA by real-time PCR in macrophages stimulated with HG-EV and LG-EV (n = 5–6). Graph data are mean ± s.e.m. N.S., not significant; low glucose: 5.6 mM, high glucose: 25 mM. $^{*}P < 0.05$, $^{**}P < 0.01$.

in macrophages, leading to the higher upregulation of TNF-α and IL-1β expressions (Fig. 3b, c). These findings suggest that the high-glucose condition affects the characteristics of the EVs rather than their quantity.

Establishment of a novel screening strategy for EV-targeting drugs

Next, we designed a high-throughput drug screening strategy using a validated compound library, in order to explore the compounds that could specifically and effectively inhibit EV-induced inflammation (Fig. 4). Before starting the screening, the study was approved by the Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS project headed by Dr. Hirotatsu Kojima, Drug Discovery Initiative, the University of Tokyo; Registration No. 03625), and the validated compound library was provided.

Step 1 was to select compounds which showed an inhibitory effect on NF-κB activation. Compounds whose inhibition rate of NF-κB pathway induction by MC-EV was over 40% were chosen (Fig. 5a). Step 2 was to exclude known anti-inflammatory compounds such as steroids. Through these steps, candidate drugs were narrowed down to 399 compounds. Step 3 was to evaluate dose-dependent effects (Fig. 5b), as well as the effects on cell viability using a WST assay (Fig. 5c). Compounds exerting cytotoxicity defined by cell viability lower than 60% were excluded (Fig. 5c). Step 4 was to evaluate the specificity to EV actions. We examined the inhibitory effect on inflammation induced by MC-EV as compared to that by LPS as a non-specific control. The compounds were

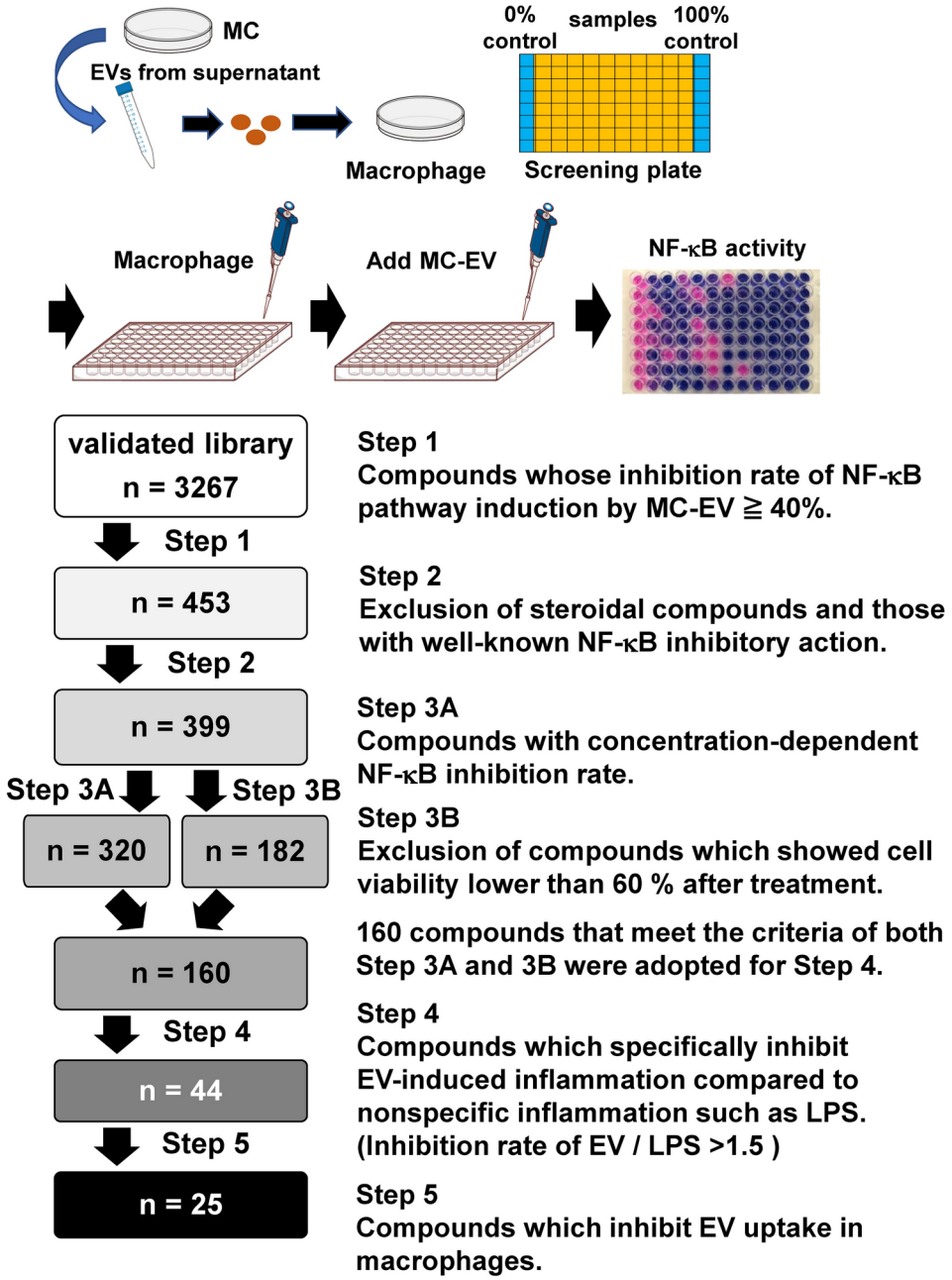

Fig. 4. EV-targeted strategy for exploring novel drug candidates for diabetic kidney disease. Schema of the part of our drug screening assay. EVs extracted from the supernatant of cultured mesangial cells were added to macrophages, and reacted with each compound. Flowchart of high-throughput drug screening strategy targeting EV is shown. From the 3267 compounds in the validated library, candidate drugs are selected through the indicated 5 steps. LPS, lipopolysaccharide.

selected to predominantly inhibit EV-induced inflammation compared to LPS (an inhibition rate of MC-EV/LPS > 1.5; Fig. 5d). For the final step, we conducted FCM analysis to evaluate an inhibitory effect on DiO-EV uptake in macrophages. Then, we integrated the results with the NF-κB inhibition rate and created a scatter plot (Fig. 5e). After completing all stages of the screening process, 25 compounds were shortlisted as final candidates, which have both NF-κB inhibitory and EV uptake inhibitory effects (Table 1). Among them, it is noteworthy that there were four compounds with a category of heat shock protein 90 (HSP90) inhibitors, of which alvespimycin (Alv) was identified to show the highest rate of inhibition of EV uptake into cells, with an NF-κB suppression rate of nearly 80% (Fig. 5e and Table 1). Therefore, we finally decided to examine the effects of Alv in vivo. The original complete datasets are openly available in repository figshare at https://figshare.com/articles/dataset/Original_raw_data_of_each_step_of_the_drug_screening_/25018745.

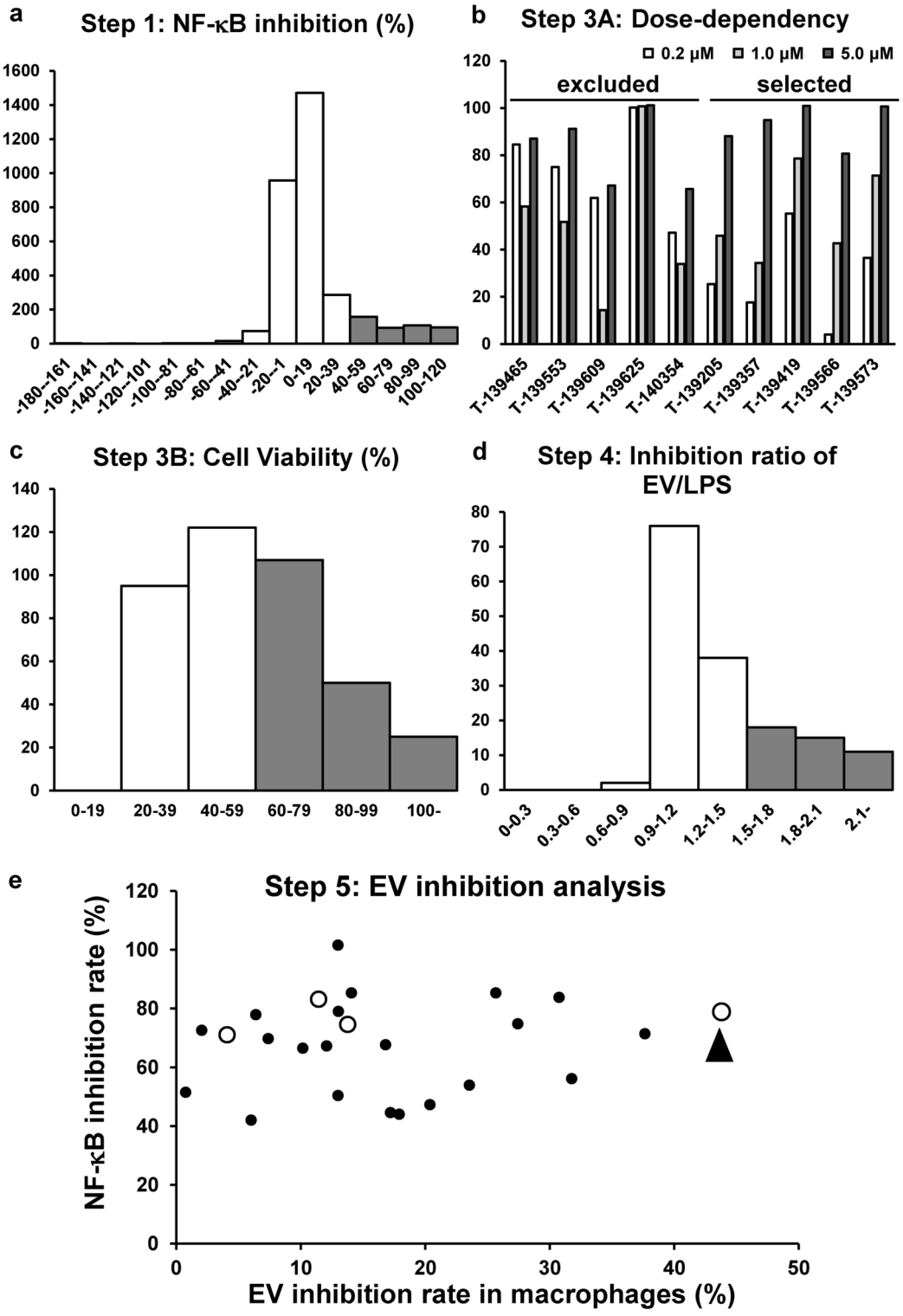

Fig. 5. Results of each step of the drug screening (Step 1–5). (**a**) Step 1. Compounds whose inhibition rate of NF-κB pathway induction by MC-EV ≧ 40% were selected. (Grey bars; 453 compounds). (**b**) Step 3A. Compounds which showed dose-dependent NF-κB inhibition rate were selected. Representative compounds are indicated. Whole data are listed in the Supplementary Table. (**c**) Step 3B. Compounds which showed cell viability higher than 60% after treatment were selected. (Grey bars; 182 compounds). (**d**) Step 4. Compounds which specifically inhibit EV-induced inflammation compared to nonspecific inflammation such as LPS. (Inhibition ratio [EV / LPS] > 1.5) Grey bars; 44 compounds. LPS, lipopolysaccharide. (**e**) Step 5. Final step was to evaluate EV inhibitory effect and integrated with the results of NF-κB inhibition rate. Open circles: HSP90-inhibitory effect-exerting compounds, arrowhead: Alvespimycin.

Compounds	Functions
T-139403	5-HT1B/1D serotonin receptor antagonist
T-139571	Peptide inhibitor of aminopeptidases
T-140671	T-type Ca2+ channel blocker
T-180698	Anti-Serum Amyloid A antibody
T-196308	Antimalarial, Heme polymerase inhibitor
T-196379	Antifungal, Inhibitor of mitochondrial electron transport
T-196595	Antibacterial, Bacterial DNA damage
T-196831	Mucolytic, Bronchitis
T-207120	Anti-cancer effects
T-207163	Inhibitor of P-glycoprotein and MRP1
T-207191	Potent inhibitor of snake venom PLA2
T-208251	p210Bcr/Abl kinase inhibitor
T-210562	XPO1 inhibitor
T-210608	Tyrosine kinase inhibitor
Alvespimycin	**HSP90 inhibitor**
T-210751	HSP90 inhibitor
T-210633	Potent Topoisomerase II inhibitor
T-196893	Antifungal, Antibacterial
T-196571	Antibacterial
T-210623	HSP90 inhibitor
T-207208	Specific inhibitor of eIF2α phosphatase
T-196778	Naturally occurring flavonoid
T-210582	HSP90 inhibitor
T-207192	ATP synthase inhibitor

Table 1. Table of final shortlisted 25 compounds and their functions.

Alvespimycin alleviates diabetic kidney disease in rats

The average area of the mesangial region of the 10 glomeruli increased in the STZ group compared to the WT group, which was significantly improved by administration of Alv (Fig. 6a, b, Supplementary Fig. 1a and b). In addition, Alv treatment significantly ameliorated the exacerbation of albuminuria in diabetic rats (Fig. 6c). It is noteworthy that expression of macrophage markers (CD68 and CD11b) was markedly suppressed by Alv administration in the glomeruli of diabetic rats together with reduction in inflammatory genes (Fig. 6d). These results suggested that Alv can mitigate diabetic renal lesions, associated with less macrophage infiltration and subsequent inflammation in glomeruli.

Alvespimycin exerts a specific inhibitory effect on EV-mediated mechanisms

We compared the effects of EV-uptake inhibition among 5 HSP90 inhibitors including Alv and pimitespib (Fig. 7), which is a recently launched HSP90 inhibitor in the clinical practice as an anti-cancer drug.[15] Alv showed an obvious inhibitory effect on EV uptake (Fig. 7a). Furthermore, the results showed that Alv was the only compound exerting a significant inhibitory effect on EV uptake among HSP90 inhibitors (Fig. 7b). These results suggested that the EV inhibitory effect of Alv may not be a class effect of HSP90 inhibitors, but rather an Alv-specific action.

Discussion

Although there have been great advances in the treatment of diabetes and its complications including sodium-glucose co-transporter 2 inhibitors, glucagon-like peptide-1 receptor agonists and mineralocorticoid receptor blockers, DKD is still a leading cause of end-stage renal disease in most countries, so that novel therapeutic strategies for DKD are urgently sought. Pathophysiology of DKD is complicated and yet to be fully elucidated; nonetheless, the pathogenic roles of macrophages, both resident and infiltrating, have been reported as the main immune cells to prepare a local inflammatory milieu in DKD[16,17]. High-glucose conditions promote expressions of several adhesion molecules and inflammatory cytokines locally, which can activate and recruit macrophages, leading to fibrosis and sclerosis in the kidney[15,16]. We previously reported that macrophages could infiltrate in the glomeruli, thereby inducing local inflammation through intraglomerular crosstalk with mesangial cells in DKD[6]. Thus, it has been postulated that such local cell–cell communication should be involved in various pathophysiology in the kidney as well as key pathogenic mechanisms in the glomeruli[6,7,16–18].

Among local mediators proposed so far, EVs have been thought to be crucial paracrine mediators of cell–cell crosstalk and assumed to be involved in several disease progression[2–5]. They contain various kinds of proteins and nucleic acids such as messenger RNAs and microRNAs[1,19], and their roles have been investigated extensively in the oncology field not only as diagnostic biomarkers but also as therapeutic targets in clinical practice[20–22]. In the field of kidney disease, it has been suggested that urinary EVs could serve as diagnostic markers of

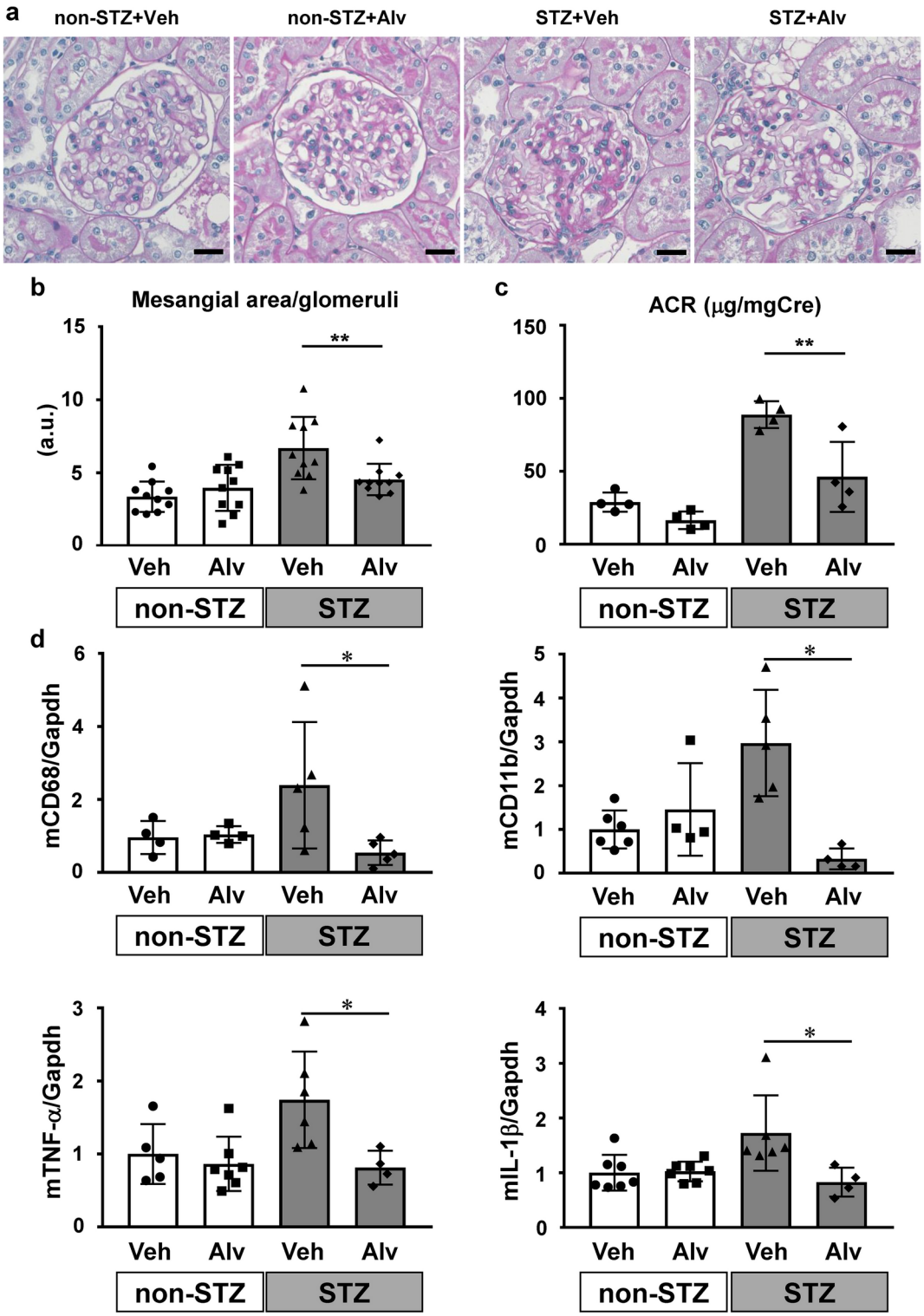

Fig. 6. Protective effects of Alvespimycin on renal pathogenesis in STZ-induced diabetic rats. (**a**) Representative images of PAS-stained kidney sections from nondiabetic control with vehicle (non-STZ + Veh), nondiabetic control treated with alvespimycin (non-STZ + Alv), STZ rats treated with vehicle (STZ + Veh) and STZ rats treated with alvespimycin (STZ + Alv) for 6 weeks. Scale bars = 20 μm. (**b**) Evaluation of the average area of the mesangial region of the randomly selected 10 glomeruli. (**c**) Evaluation of urinary protein level between the four groups. (n = 4) (**d**) mRNA expression of inflammatory genes (IL-1β, TNF-α) and macrophage markers (CD68, CD11b) in the kidney glomeruli. (n = 4–7) STZ, streptozotocin; Alv, alvespimycin; ACR, albumin creatinine ratio. $^{*}P < 0.05$, $^{**}P < 0.01$.

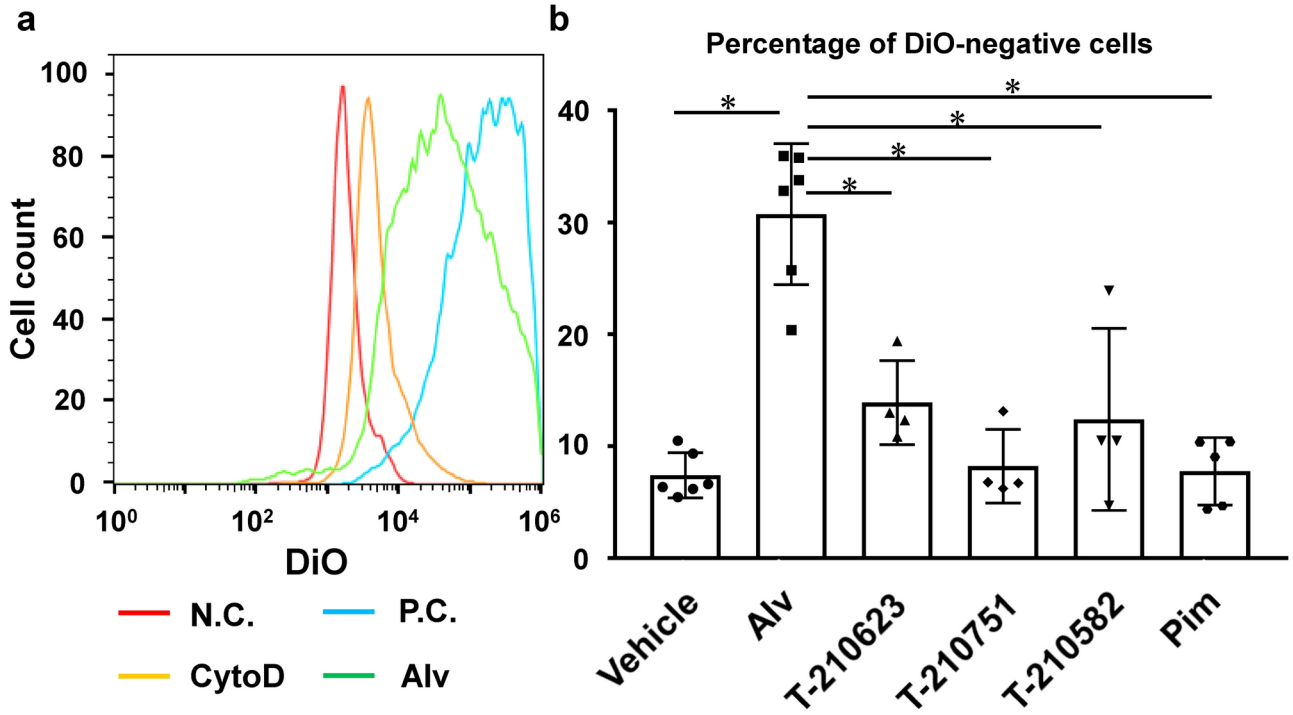

Fig. 7. Flowcytometric evaluation of the effect of alvespimycin on EV uptake inhibition. (**a**) Alvespimycin inhibits DiO-stained EV uptake in macrophages. N.C., untreated negative control; P.C., DiO-EV treated positive control; CytoD: DiO-EV and cytochalasin D-treated; Alv, DiO-EV and alvespimycin-treated. (**b**) Alvespimycin exerts significantly higher inhibitory effect of EV-uptake than other HSP90 inhibitors. T-210623, T-210751 and T-210582 are HSP90 inhibitors shortlisted in the final candidates of the screening. (n = 4–6) Alv, alvespimycin; CytoD, cytochalasinD; Pim, pimitespib. $^{*}P < 0.05$.

acute kidney injury as well as glomerular disease such as focal segmental glomerulosclerosis[23]. Moreover, EVs may modify water, electrolyte and acid–base transport in the renal tubules, thereby modulating kidney pathophysiology[24]. Despite these investigations, unveiling the role of EVs in the intraglomerular crosstalk and progression of glomerular lesions including DKD has remained unchallenged yet.

In this study, we focused on the functional role of EVs in the kidney, hypothesizing them as crucial mediators between mesangial cells and macrophages in diabetic glomeruli. The results of our study showed that MC-EV were uptaken in macrophages and induced inflammatory responses in vitro and in vivo, where the findings were more augmented under high-glucose than low-glucose conditions. These results suggested that EVs upon high-glucose conditions can affect macrophages and induce local inflammation in vivo. As shown in Supplementary Fig. 2, stimulation by supernatant of cultured mesangial cells also induced similar inflammatory response on macrophages, suggesting that several cytokines or some other factors contained in the surroundings may enhance the effects of EVs derived from mesangial cells. Based on these findings, we next tried to explore the substances that can interfere such EV-mediated mechanisms by novel screening strategy.

Drug repositioning is a powerful and effective tool for searching novel therapeutic chemical compounds. It could facilitate the discovery of novel mechanisms of action for existing drugs, thus potentially reducing clinical trial steps, the cost and time for drug development. In regard to the nephrology field, there have been a couple of promising preclinical studies so far. Hamano et al. reported the potential efficacy of diphenhydramine against cisplatin-induced kidney injury[25]. As for the treatment of autosomal dominant polycystic kidney disease, several drug candidates are listed, targeting cAMP signaling, somatostatin receptors, mTOR1 signaling, and so forth[26,27]. Such candidates and strategies should provide novel therapeutic options in clinical practice in the near future.

In this study, we screened a validated compound library of over 3,000 chemical compounds from Drug Discovery Initiative and established a unique multi-step assay to efficiently dig up substances that inhibit EV-mediated mechanisms. As a result, several candidate agents to potentially exhibit a therapeutic effect on DKD were identified. In the final candidate agents, several HSP90 inhibitors, antibacterials, P-glycoprotein inhibitors, and mitochondrial function modulators were listed. Of note, among them, four HSP90 inhibitors including Alv were shortlisted. HSP90 inhibitors have been investigated as therapeutic drugs for cancers, and pimitespib has been launched in market as the first HSP90 inhibitor for gastrointestinal stromal tumor[28]. Besides, there are some reports showing that HSP90 inhibitors can attenuate atherosclerotic vascular and renal complications, mainly by attenuating stress-induced inflammation[29–31]. Nevertheless, their effects against EV-mediated actions have never been addressed. Our study revealed for the first time that an HSP90 inhibitor Alv could inhibit the uptake of MC-EV into macrophages and relevant inflammatory responses. Administration of Alv in diabetic rats effectively suppressed local inflammation in glomeruli, leading to amelioration of diabetic glomerular lesions

(Fig. 6). As for the mechanisms, HSP90 contained within EVs or expressed on the surface of EVs may mediate membrane-deforming function and promote EV release[32]. However, our results revealed that the inhibitory effect on EV uptake of Alv was somewhat confined to Alv and not evident in other HSP90 inhibitors examined, suggesting that such inhibitory effect was not related to HSP90 inhibition, but rather an Alv-specific action. Also, Alv did not significantly affect the polarity of macrophages in animal experiments (data not shown). Further investigations are no doubt necessary to explore the precise mechanisms for this action and effect.

There are several concerns and limitations to this study. HSP90 is a chaperone protein that plays essential roles in many cellular processes including protein folding, cell cycle control and intracellular signaling pathways[33]. Thus far, HSP90 inhibitors have been investigated as potential anti-cancer therapeutic drugs. The first generation HSP90 inhibitor, geldanamycin, was abandoned for clinical usage due to its hepatotoxicity[34]. Thereafter, several derivatives including Alv have been developed to reduce their toxicity; however, we should carefully check its potential toxicity for future clinical use. Pimitespib, the first agent applied to clinical practice, is alerted to the risks of night blindness, bleeding tendency and diarrhea[15]. Actually, in our experiment, a few rats receiving Alv showed loose stool, suggesting an adverse event common to HSP90 inhibitors. Second, we showed its renoprotective effect for only 6 weeks of treatment in rodents, therefore, long-term effects are still unclear and the results could not be directly applied to humans. Also, we administered Alv intraperitoneally, but for future clinical use, orally active agents should no doubt be required. Further studies are needed to verify the effects, examine the safety and potency, and optimize drug design, route, and dose of administration in the near future. Third, our experiments lack the consistency of the cells and animal species. Our aim is to distinguish the mRNA origin by differentiating the animal species between EVs-releasing cells and EVs-uptaking cells. Preliminary experiments using murine mesangial cells and murine macrophages showed similar trends, however, the difference was smaller probably because of combined evaluation of mRNAs from both EVs and macrophages. Additionally, EVs derived from mouse podocyte did not affect macrophage inflammatory responses. And finally, all of the animal experiments were conducted using male rodents. Therefore, the results may not be able to be applied to all the genders as they are.

In conclusion, by establishing a unique multi-step screening assay, we successfully discovered a novel agent targeting EVs from existing chemical compounds. Alvespimycin, identified by our screening, may affect the action of mesangial EVs and the response of macrophages against the EVs in the intraglomerular environment of diabetic kidney. It could be a novel therapeutic strategy for DKD.

Data availability

All data are available in the main text or the Supplementary materials. Original raw data of each step of the drug screening supporting the findings of this study are openly available in repository figshare at https://figshare.com/articles/dataset/Original_raw_data_of_each_step_of_the_drug_screening_/25018745.

Received: 4 March 2024; Accepted: 15 April 2025
Published online: 25 April 2025

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Acknowledgements

This research was supported by Platform Project for Supporting Drug Discovery and Life Science Research (Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS)) from AMED under Grant Number JP23ama121053 (support number 0202). This work was also financially supported in part by research grants from JSPS KAKENHI (Grant Numbers 22K16223 to DF; 19K08729 and 22K08311 to TM; 17K09706 and 20K08611 to MM; 19K08728 and 22K08357 to T. Kuwabara), Strategic Grants from the Center for Metabolic Regulation of Healthy Aging, Kumamoto University Faculty of Life Sciences (Grant Number 09021407 to DF) and Kumamoto University Hospital Young Researcher Activation Project (Grant Number R4-6 and R5-3 to DF). We gratefully acknowledge Dr. Daisuke Nakano (independent researcher, currently working at Laboratory for Pharmacology, Asahi-Kasei Pharma Co., Ltd., Tokyo, Japan) for technical counselling about confocal exosome imaging. This study was conducted fully independent of the project of Asahi-Kasei Pharma Co., Ltd. We also appreciate Ms. Hikari Shibuta, Kazumi Saito and Naoko Hirano for technical assistance, and Ms. Noriko Nakagawa and Miki Horikiri for secretarial assistance.

Author contributions

D.F., S.U., H.K., and T. Kuwabara. designed the study. D.F., S.U., R.D. and J.Z. performed the experiments. D.F., T. Kuwabara and M.M. drafted the manuscript. D.F., S.U., T.M., T. Kanki, Y.H., Y.N., Y.K., Y.I., M.A.,H.Y., M.M. and T. Kuwabara. interpreted the results. All authors approved the final version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-98894-0.

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