22-Oxacalcitriol prevents progression of endothelial dysfunction through antioxidative effects in rats with type 2 diabetes and early-stage nephropathy

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ABSTRACT

Background. Vitamin D deficiency is associated with endothelial dysfunction in type 2 diabetes patients, but the effectiveness of vitamin D supplementation remains controversial. We assessed whether 22-oxacalcitriol (OCT) could prevent endothelial dysfunction in type 2 diabetes mellitus (DM) rats.

Methods. DM rats with early-stage nephropathy were treated for 10 weeks with OCT (0.2 μg/kg) three times per week or by an implanted insulin pellet. Endothelial dysfunction was assessed by femoral flow-mediated dilation (FMD).

Results. Insulin significantly improved FMD as blood glucose levels normalized. OCT also improved FMD without hypercalcemia or hyperphosphatemia and without affecting blood glucose or blood pressure. In femoral arteries, OCT significantly suppressed the elevated expression of p22phox, a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit, and improved the endothelial nitric oxide synthase (eNOS) dimer-to-monomer ratio. In cultured endothelial cells, OCT significantly suppressed high-glucose (HG)-induced reactive oxygen species (ROS) production. Simultaneously, OCT significantly suppressed HG-induced p22phox expression and improved eNOS uncoupling as was observed in the in vivo study.

Conclusion. In DM rats, OCT improved endothelial dysfunction, at least in part, by suppressing ROS generation through hypercalcemia or hyperphosphatemia and without affecting blood glucose or blood pressure.
p22phox expression, which might contribute to improving eNOS uncoupling.

INTRODUCTION

Chronic kidney disease (CKD) is a major risk factor for cardiovascular disease [1, 2]. Diabetes mellitus (DM) is associated with CKD progression [3], and diabetes itself is a critical risk factor for development of cardiovascular disease [4, 5].

Endothelial dysfunction, another critical risk factor for cardiovascular disease [6], can be noninvasively evaluated by measuring post-ischemic flow-mediated dilation (FMD) of conduit arteries [7]. Endothelial dysfunction is observed in the early phase of atherosclerosis, and FMD decreases in type 2 diabetic patients [5]. Moreover, left ventricular diastolic function and FMD are impaired in type 2 diabetic patients with microalbuminuria [8]. Progression of endothelial dysfunction in diabetes involves the following general mechanism: high glucose (HG) levels increase reactive oxygen species (ROS) generation in vascular tissues [9]; this leads to endothelial nitric oxide synthase (eNOS) uncoupling, followed by reduced NO production and induction of ROS production [10, 11] which quenches NO. Thus, hyperglycemia decreases endothelium-derived NO, leading to endothelial dysfunction.

In type 2 diabetic patients, serum 25-hydroxyvitamin D (25OHD) concentration is inversely correlated with prevalence of cardiovascular disease [12, 13]. In addition, the serum 25OHD status is significantly associated with brachial artery FMD in patients with type 2 diabetes [14]. Therefore, 25OHD and active vitamin D are expected to benefit type 2 diabetic patients, but the clinical evidence remains unclear.

1,25-Dihydroxyvitamin D3, calcitriol, is an active form of vitamin D3, which functions via binding to vitamin D receptors (VDRs). Calcitriol and its analogs such as 22-oxacalcitriol (OCT) and paricalcitol are selective VDR activators (VDRAs) commonly used to manage hyperparathyroidism associated with CKD [15] and may play a role in modulating cardiovascular function [16]. Non-diabetic animal studies, such as the study of calcitriol in hypertensive rats [17], do in fact indicate that VDRAs may favorably affect endothelial function. Because it is expected that the risk of vascular calcification is lower with OCT than with calcitriol [18], these observations offer further support for the possibility that endothelial function is more favorably affected by OCT than calcitriol. Therefore, it would be intriguing to determine whether VDRAs can exert protective effects towards endothelial dysfunction in type 2 diabetes.

The spontaneously diabetic Torii (SDT) rat is a type 2 diabetes model without hyperinsulinemia, hypertension or obesity [19], which allows these influences on progression of vascular dysfunction to be excluded. In addition, kidney disease in SDT rats progresses by oxidative and nitrosative stress, as it does in other type 2 diabetes models such as Zucker diabetic fatty rats [20], and the deterioration is rescued by insulin treatment [21].

We used SDT rats to assess whether OCT could prevent diabetes-induced endothelial dysfunction, and we used cultured human coronary artery endothelial cells (HCAECs) to clarify the underlying mechanisms.

MATERIALS AND METHODS

Animal model

Male SDT rats and age-matched male Sprague Dawley (SD) rats (normal control group; n = 6) were used (CLEA Japan, Tokyo, Japan). All rats were fed ordinary laboratory chow and allowed free access to water under a constant 12-h light/dark cycle. At 20 weeks of age, SDT rats with blood glucose levels >250 mg/dL were selected and put into metabolic cages to collect 24-h urine samples for measuring urinary total protein (uTP). Rats were then divided into three groups: SDT diabetic control rats (DM, n = 6); SDT rats receiving insulin (DM + INS, n = 6) and SDT rats receiving OCT (Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) (DM + OCT, n = 6). OCT was dissolved in phosphate-buffered saline (pH 8.0) containing 0.2% ethanol and 0.01% Tween 20. Rats in the DM + INS group were subcutaneously implanted every 2 weeks with an insulin pellet (LinShin Canada, Scarborough, ON, Canada) that releases a controlled amount of insulin (2 U/day). Rats in the DM + OCT group were intraperitoneally administered OCT at a dose of 0.2 µg/kg body weight three times per week from 20 weeks of age for 10 weeks. At Week 10, blood pressure was measured with a tail cuff and the rats were put into metabolic cages to collect 24-h urine samples before FMD assessment. All animal procedures were approved by the Institutional Animal Care and Use Committee at Chugai Pharmaceutical Co., Ltd, and all experimental protocols were approved by the Animal Care Committee of the institution and conform to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Blood, plasma, serum and urine measurements

Blood from the abdominal aorta and urine were collected before treatment (Week 0) and every 2 weeks until Week 10. HbA1c was measured by autoanalyzer (TBA-120FR; Toshiba Medical Systems, Tochigi, Japan) at Weeks 0, 6 and 10 by dissolving 3 µL of blood in 300 µL of hemolyzing reagent (Auto-Wako HbA1c; Wako, Osaka, Japan). Plasma and urine samples were stored at −30°C until analysis. Plasma calcium (Ca), plasma phosphate (Pj), urine calcium (uCa), urine phosphate (upj) and uTP were measured by an autoanalyzer (Hitachi 7170; Hitachi, Tokyo, Japan). Blood glucose was measured with a digital glucose meter and test strips (NIPRO FreeStyle; NIPRO, Osaka, Japan) immediately after sampling. Plasma parathyroid hormone (PTH) was measured by Rat Intact PTH ELISA kit (Immutopics, San Clemente, CA). Serum 25OHD was measured by 25(OH)-vitamin D direct ELISA kit (Immundiagnostik AG, Bensheim, Germany).

FMD measurements

Rats were anesthetized with thiobutabarbital, and the rectal temperature was constantly monitored. FMD in rats was measured as previously described [22]. Briefly, a high-resolution ultrasound system with a 30- or 40-MHz transducer...
H2DCF-DA for 45 min at 37°C, and then (1 × 10^5 cells/2 mL/dish) and cultured in monolayers under ROS production, the cells were seeded onto plastic dishes. 5 were cultured in endothelial basal medium-2 containing 5.6 mM glucose and 5% fetal bovine serum (Lonza). To measure ROS production, the cells were seeded onto plastic dishes (1 × 10^5 cells/2 mL/dish) and cultured in monolayers under 5% CO₂ in a humidified incubator at 37°C. After overnight incubation, HCAECs were cultured in the medium described above with additional added glucose (total 35.6 mM) for 24 h. OCT (10 nM) was added and incubated over the same period.

**Cell culture**

Normal HCAECs (Lonza, Walkersville, MD) of passages 2–5 were cultured in endothelial basal medium-2 containing 5.6 mM glucose and 5% fetal bovine serum (Lonza). To measure ROS production, the cells were seeded onto plastic dishes (1 × 10^5 cells/2 mL/dish) and cultured in monolayers under 5% CO₂ in a humidified incubator at 37°C. After overnight incubation, HCAECs were cultured in the medium described above with additional added glucose (total 35.6 mM) for 24 h. OCT (10 nM) was added and incubated over the same period.

**ROS production measurements**

The ROS level was monitored with the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA; Invitrogen). Briefly, the cultured cells were incubated with 10 μM H₂DCF-DA for 45 min at 37°C, and then fluorescent images were captured with a confocal microscope (Zeiss Axiovert 200) and quantified with the Image J program (version 1.240; rsb.info.nih.gov). At least 20 randomly selected cells were scanned in each dish, using three dishes for each experimental condition.

**Western blot analysis**

Harvested femoral arteries or cultured cell lysates were frozen in liquid nitrogen immediately after isolation and stored at −80°C until the measurement of proteins by western blotting. Each of the femoral arteries or cultured cell lysates was homogenized in a lysis buffer composed of 25 mM Tris–HCl (pH 7.4), 1 mM dithiothreitol (Invitrogen, Carlsbad, CA), 25 mM sodium fluoride (Alexis Biochemicals, Lausen, Switzerland), 1 mM sodium orthovanadate (Calbiochem, San Diego, CA), protease inhibitor cocktail tablet (Roche Diagnostics, Basel, Switzerland), phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and 1% Triton X-100. The homogenates were centrifuged at 14 000 g for 20 min at 4°C. The supernatants were collected, and the protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). Equal amounts of protein extracts were separated on 10% Mini-Protean TGX precast gel and transferred onto polyvinylidene difluoride (PVDF) membranes using the Trans-Blot Turbo Transfer Pack Mini format (Bio-Rad Laboratories, Hercules, CA). Samples were heated at 95°C for 5 min for immunoblot analysis of total eNOS. For immunoblot analysis of dimeric and monomeric forms of eNOS, the samples were not heated and sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed on ice. The membranes were blocked with a PVDF blocking reagent (Toyobo, Osaka, Japan), and incubated with either anti-eNOS antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-p22phox antibodies (Santa Cruz Biotechnology). After washing, the membranes were incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Immunoreactive bands were visualized with a SuperSignal West Dura Extended Duration substrate (Thermo Fisher Scientific) and detected using a ChemiDoc XRS system (Bio-Rad Laboratories). Each protein signal was normalized to GAPDH expression (Cell Signaling Technology, Danvers, MA) from the same sample.

**Statistical analysis**

All data are expressed as mean ± SEM. An n value refers to the number of individual animals on which the experiments were performed. For longitudinal data, the statistical significance of differences between the normal group and the DM group, and between the DM group and the DM + INS or DM + OCT group, was determined using a two-way ANOVA followed by the Bonferroni post hoc test. For the other biological data and the western blot analysis data, statistical significance of differences was determined using Tukey’s multiple comparison test. A P value of <0.05 was considered significant. Statistical analysis was performed using SAS version 8.2 software (SAS Institute, Cary, NC).
increased blood glucose and HbA1c levels, and glucose was completely controlled to normal levels (Figure 1). With respect to changes in plasma Ca and plasma Pi, the levels of these parameters were significantly lower in DM rats than in Control rats, but there was no significant difference between DM rats and OCT-treated DM rats throughout the treatment period (Figure 2A and B). Urinary Ca and Pi excretion were significantly higher in DM rats than in Control rats. On the other hand, OCT further elevated urinary Ca excretion, but did not affect urinary Pi excretion. Insulin ameliorated the levels of these parameters to the levels in Control rats (Figure 2C and D). Plasma PTH levels in DM rats were slightly (but not significantly) lower than those in Control rats, and OCT significantly inhibited PTH levels (Table 1). There was no significant lowering of serum 25OHD levels in DM rats, but OCT significantly suppressed the levels in DM rats (Table 1).

**FMD and NTG-induced vasodilation in OCT-treated DM rats**

To evaluate the effect of OCT on preventing endothelial function deterioration, we measured FMD in rat femoral arteries after 10 weeks’ treatment with OCT (Figure 3). In DM rats, changes in FMD (percentage increase in arterial diameter after reperfusion compared to pre-ischemic baseline arterial diameter) were significantly lower than those in control rats, and insulin completely restored the impaired FMD in DM rats (Figure 3A). OCT also significantly improved the reduced FMD similarly to insulin treatment (Figure 3A). NTG-induced vasodilation, which is endothelium-independent vasodilation, was similar in control and DM rats (Figure 3B). These results demonstrated that OCT ameliorated endothelial dysfunction without affecting vascular smooth muscle function in DM rats.

**Evaluation of femoral NADPH oxidases and eNOS uncoupling in OCT-treated DM rats**

To provide insight into the mechanisms underlying the observed protective effects of OCT treatment on endothelial function, we investigated the effect of OCT on the expression of nicotinamide adenine dinucleotide phosphate oxidase (NADPH) oxidases and eNOS in femoral arteries. Expression of p22phox protein was significantly elevated in the femoral arteries of DM rats, and OCT treatment significantly inhibited this increase (Figure 4). There was no significant difference in total eNOS expression between control, DM and OCT-treated DM rats (Figure 5A). On the other hand, the dimer-to-monomer ratio of eNOS protein expression was significantly

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**Table 1. Effect of OCT on several parameters at the end of 10 weeks’ treatment**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DM</th>
<th>DM + OCT</th>
</tr>
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<tbody>
<tr>
<td>BW, g</td>
<td>816.1 ± 27.5</td>
<td>467.3 ± 3.3</td>
<td>466.6 ± 7.1</td>
</tr>
<tr>
<td>uTP, mg/day</td>
<td>11.8 ± 1.3</td>
<td>181.9 ± 25.1</td>
<td>119.4 ± 20.8</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>137.9 ± 7.1</td>
<td>153.6 ± 5.0</td>
<td>152.8 ± 4.5</td>
</tr>
<tr>
<td>PTH, pg/mL</td>
<td>553.3 ± 123.5</td>
<td>254.0 ± 72.9</td>
<td>109.8 ± 22.5</td>
</tr>
<tr>
<td>25(OH)D, nmol/L.</td>
<td>21.6 ± 5.3</td>
<td>33.2 ± 4.7</td>
<td>11.4 ± 2.3</td>
</tr>
</tbody>
</table>

BW, body weight; uTP, urinary total protein; SBP, systolic blood pressure; PTH, parathyroid hormone; data are expressed as means ± SEM, n = 6, *P < 0.05 versus control, #P < 0.05 versus DM
lower in the femoral arteries of DM rats, and OCT treatment significantly increased the ratio (Figure 5B).

**Effect of OCT on high-glucose (HG)-induced ROS generation in HCAECs**

To evaluate the effect of OCT on oxidative stress, we measured ROS production in HCAECs exposed to HG. We confirmed that VDR protein was expressed in HCAECs (data not shown). Generation of ROS was significantly increased in HCAECs exposed to HG (Figure 6) and significantly inhibited by OCT; however, OCT did not influence ROS generation at normal glucose levels. OCT significantly suppressed the increased expression of p22phox protein induced by HG, but did not influence the expression of p22phox protein at normal glucose levels (Figure 7A). The dimer-to-monomer ratio of eNOS protein expression tended to be lowered by HG levels \((P = 0.056)\). OCT significantly improved the decreased dimer-to-monomer ratio of eNOS protein expression induced by HG, but did not influence the eNOS dimer-to-monomer ratio at normal glucose levels (Figure 7B).

**DISCUSSION**

We investigated here whether OCT could prevent endothelial dysfunction in rats with early-stage type 2 diabetic nephropathy, and we used HCAECs to explore the mechanisms underlying the effect of OCT. We used SDT rats, a model of early-stage type 2 diabetic nephropathy [21]. After 20 weeks of age, 100% of male SDT rats spontaneously developed impaired insulin secretion and hyperglycemia without hypertension or obesity that persists until 40 weeks of age [19]. Moreover, SDT rats can survive for a long time even without insulin treatment. In addition to these characteristics, the DM rats in our study did not show an insufficiency of serum 25OHD levels. Although the DM rats did not develop a vitamin D deficiency, impaired FMD due to continuous hyperglycemia was observed in these rats as is observed in type 1 diabetes rats [22]. Insulin therapy completely reversed the decline in FMD by controlling blood glucose to normal levels.

Although a clinical study has reported that vitamin D3 has no effect against endothelial dysfunction in type 2 diabetic patients with vitamin D deficiency [23], the clinical benefit of supplementation with other forms of vitamin D to these patients is unclear. Here, we confirmed for the first time that long-term repeated dosing with OCT significantly halted the decline in FMD in type 2 diabetes rats without affecting glycemic control. In addition, this effect was exerted at the early stage of diabetic nephropathy even in animals without vitamin D deficiency.

Endothelial dysfunction in diabetes is attributable to endothelial ROS production [24]. A clinical study has shown that among the many enzymatic systems capable of producing superoxide, NADPH oxidase and uncoupled eNOS are the main sources of superoxide in the vascular wall in diabetic patients [25]. HG stimulates the generation of endothelial superoxide [9], which reacts rapidly with NO resulting in the elevation of peroxynitrite anions [26]. Although we could not clarify the in vivo effect of OCT on ROS generation by measuring oxidative stress markers, such as 8-hydroxydeoxyguanosine or malondialdehyde levels, either in serum or in urine (data not shown), our in vitro study clearly showed that OCT...
acted to reduce HG-induced ROS production (Figure 6). In addition, OCT significantly suppressed the elevated expression of p22phox protein induced by HG in our in vitro study (Figure 7A) and in the DM rats in our in vivo study (Figure 4). Therefore, these findings suggest that OCT could alleviate the generation of superoxide in the local blood vessels in diabetes, and the inhibition of p22phox might partially contribute to this alleviation. The fact that there were no changes in the other NADPH oxidase subunits, such as p47phox or gp91phox (data not shown), suggests the critical role of p22phox in ROS production.

**FIGURE 3:** OCT prevents the impairment of FMD but not NTG-induced vasodilation in DM rats. Time course of changes in (A) FMD after reperfusion and (B) NTG-induced vasodilation in DM rats at the end of the 10 weeks' treatment. Normal control rats (open circles), DM rats (filled circles), insulin-treated DM rats (triangles) and OCT-treated DM rats (squares). The values are expressed as means ± SEM. Two-way ANOVA followed by the Bonferroni post hoc test was used to compare groups. *P < 0.05 versus normal control; *P < 0.05 versus DM (n = 6).

**FIGURE 4:** OCT prevents NADPH oxidase subunit p22phox protein expression in femoral arteries of DM rats. Top: representative western blots for p22phox protein in rat femoral arteries. DM + OCT rats were treated with OCT for 10 weeks. Bottom: results are expressed as a ratio of control intensity (control = 1). The values are expressed as means ± SEM. Tukey's multiple comparison test was used to compare groups. *P < 0.05 versus control; *P < 0.05 versus DM (n = 5–6).

**FIGURE 5:** OCT improves eNOS dimer-to-monomer ratio but not total eNOS in femoral arteries of DM rats. (A) Total eNOS protein expression and (B) eNOS dimer-to-monomer ratio in rat femoral arteries. Top: representative western blots for total eNOS and eNOS dimer and monomer in control, DM and OCT-treated DM rats. Bottom: results are expressed as a ratio of control intensity (control = 1). The values are expressed as means ± SEM. Tukey’s multiple comparison test was used to compare groups. *P < 0.05 versus control; *P < 0.05 versus DM (n = 5–6).
Peroxynitrite formation is a nitrosative stress causing kidney disease progression in SDT rats [27]; therefore, we evaluated nitrotyrosine protein expression induced by peroxynitrite in the femoral arteries. We found that OCT tended to suppress nitrotyrosine expression, but not significantly (data not shown). Increased peroxynitrite leads to eNOS uncoupling due to the reduction of the tetrahydrobiopterin (BH4)/dihydrobiopterin (BH2) ratio [28, 29]. Uncoupled eNOS acts as an oxidative stressor by reducing NO and generating ROS, thereby reducing NO bioavailability which decreases endothelium-dependent vasodilation [29–31]. Treatment with superoxide dismutase [28] or BH4 [31] reportedly protects against endothelial dysfunction in diabetes through correcting NO bioavailability. In this study, we observed a significant decrease in the eNOS dimer-to-monomer ratio in the femoral arteries of DM rats (Figure 5B). We also examined whether OCT could improve the eNOS dimer-to-monomer ratio in HCAECs and found that OCT improved HG-induced eNOS uncoupling. Taken together, the mechanism underlying the improvement of endothelial function shown by OCT could possibly increase the bioavailability of NO due to OCT inhibiting ROS generation via the reduction of p22phox expression and improving uncoupled eNOS.

Vitamin D can also improve endothelial function in type 2 diabetes by suppressing PTH [32], lowering blood pressure [33], inhibiting the production of advanced glycation end products [34], and via its anti-inflammatory effects [35, 36]. Because the PTH levels in DM rats were not elevated compared with normal rats (Table 1), FMD deterioration would be not related to PTH levels, an observation that may support a

![Figure 6](image_url)

**Figure 6:** OCT treatment decreases high-glucose (HG)-induced ROS production in HCAECs. HCAECs were exposed to both high levels of glucose and OCT for 24 h for ROS measurement. Top: representative images show DCF fluorescence in control cells and in cells treated with HG, HG + OCT and OCT alone. Bottom: quantification of ROS levels. At least 20 randomly selected cells per dish from three dishes were scanned. Results are expressed as a ratio of control intensity (control = 1). The values are expressed as means ± SEM. Tukey’s multiple comparison test was used to compare groups. #P < 0.05 versus control; *P < 0.05 versus HG.

![Figure 7](image_url)

**Figure 7:** OCT improves NADPH oxidase subunit p22phox protein expression and eNOS dimer-to-monomer ratio in HCAECs. Western blotting for (A) p22phox protein and (B) eNOS dimer-to-monomer ratio in OCT-treated HCAECs. The total eNOS and dimer-to-monomer eNOS ratio is expressed as a ratio of control intensity (Control = 1). The values are expressed as means ± SEM. Tukey’s multiple comparison test was used to compare groups. #P < 0.05 versus control; *P < 0.05 versus HG.
previous report stating that vitamin D derivatives improved FMD without changing PTH levels in type 2 diabetes patients [37]. In addition, in our study, OCT did not affect systolic blood pressure (Table 1) or glucose levels (Figure 1A). Therefore, OCT showed a significant beneficial effect on the vascular tone without affecting either blood pressure or blood glucose.

Plasma PTH levels in OCT-treated rats were significantly lower than those in control rats (Table 1). To evaluate whether the PTH levels suppressed by OCT encourage the bad trend observed by DM, we checked femoral bone quality. The bone mineral density, max load and stiffness were significantly lower in DM rats than in control rats (Supplementary Table). In contrast, these parameters were not changed by OCT treatment. Thus, OCT treatment did not affect the bone quality decreased in DM rats despite OCT showing excessive suppression of serum PTH levels.

OCT significantly inhibited the progression of uTP (Supplementary Figure S1). However, the peak values of FMD were not significantly correlated with the levels of uTP (Supplementary Figure S2), which is in agreement with observations in a clinical setting [38]. Therefore, we believe that the improvement of FMD by OCT may not be due to inhibition of the progression of proteinuria.

In conclusion, OCT exerted a protective effect on endothelial function in type 2 diabetes rats without inducing hypercalcemia or hyperphosphatemia. The efficacy was exerted without affecting blood pressure or glycemic control. The underlying mechanism might be, in part, attributable to the reduction of oxidative stress through suppression of p22phox expression and to the improvement of eNOS coupling.

### SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.

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### CONFLICT OF INTEREST STATEMENT

M.F. received research funding from CHUGAI Pharmaceutical Co., Ltd. The other authors are employees of CHUGAI Pharmaceutical Co., Ltd.

### REFERENCES


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