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Research Paper

# Hypercholesterolaemia induces early renal lesions characterized by upregulation of MMP-9 and iNOS and $ET_AR$: alleviated by a dual endothelin receptor antagonist CPU0213 and simvastatin

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## Abstract

**Objectives** We aimed to investigate hypercholesterolaemia-induced early renal lesions which result in abnormal expression of endothelin A receptor ($ET_AR$), induced nitric oxide synthase (iNOS) and matrix metalloproteinase 9 (MMP-9). We hypothesized that this is due to an upregulated endothelin (ET) pathway consequent to hypercholesterolaemia and that CPU0213, a dual ET antagonist, could mitigate these changes.

**Methods** Rats were randomly divided into four groups: (1), control; (2), high-fat diet for 60 days (HFD); HFD rats medicated in the last 15 days with either (3) CPU0213 (30 mg/kg daily, s.c.) or (4) simvastatin (4 mg/kg daily, p.o.).

**Key findings** Body weight, serum triglycerides, total cholesterol and low-density-lipoprotein cholesterol were significantly increased, whereas high-density lipoprotein cholesterol decreased in the HFD group, relative to normal. Meanwhile, these changes were associated with upregulation of mRNA and protein of $ET_AR$, iNOS and MMP-9 in the kidney. The lipid-lowering effect of simvastatin was predominant, lessening abnormal expression of these molecules in the kidney dramatically. Interestingly, CPU0213 significantly normalized expression of mRNA and protein of $ET_AR$, iNOS and MMP-9, comparable with simvastatin, leaving no changes in hyperlipidaemia.

**Conclusions** CPU0213 relieves renal lesions by blunting hypercholesterolaemia caused by the upregulated ET system, iNOS and MMP-9 in the kidney. This indicates that CPU0213 is promising in treating patients with end stage renal disease.

**Keywords** endothelin receptor antagonist; ET-1; hypercholesterolaemia; iNOS; MMP-9; simvastatin

## Introduction

Hypercholesterolaemia is actively involved in renal disease[1] and is predictive in the progression of chronic renal failure.[2] Hypercholesterolaemia may increase renal deposition of extracellular matrix, inflammatory cell infiltration, cellular proliferation and glomerulosclerosis.[3] Inflammatory mediators and cytokines that are produced in response to hypercholesterolaemia may facilitate renal remodelling and dysfunction associated with glomerulosclerosis and tubulointerstitial sclerosis.[4] In addition, hypercholesterolaemia accompanying chronic renal failure likely exacerbates renal lesions attributed to accumulating inflammatory factors.[5] Activated renal induced nitric oxide synthase (iNOS) as a source of reactive oxygen species (ROS) is implicated in the pathology of renal disease.[6,7]

In the presence of an increased genesis of ROS, excessive matrix metalloproteinase 9 (MMP-9) is released from mesangial cells, participating in remodelling of the glomerular basement membrane, the glomerular apparatus and tubules leading to sclerotic changes in the kidney.[8] Increasing evidence suggests that chronic kidney disease is strongly linked with abnormal MMP-9 associating with proinflammatory cytokines, including endothelin-1 (ET-1), iNOS, interleukin-1 (IL-1), tumour necrosis factor-$\alpha$ (TNF$\alpha$), transforming growth factor-$\beta$ (TGF$\beta$), etc. Low-density lipoprotein cholesterol stimulates mesangial cells to generate TNF$\alpha$[9] and chronic renal failure is always associated with hypercholesterolaemia, which produces more ET-1[10] activating multi-signal transduction pathways to elicit more pro-inflammatory factors in the kidney. Thus, the ET pathway is likely to be

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central to the recruitment of ROS, iNOS and abnormal activity of MMP-9, and probably leucocytes[11] in diseased kidney.

CPU0213 is a low-selective endothelin receptor antagonist (ETRA) that has shown promise in attenuating cardiac[12] and vascular lesions[13] and renal dysfunction.[14] Blockade of the ET receptors brings about benefits in renal dysfunction and efficacy of ETRAs in chronic renal failure is being investigated in ongoing clinical trials.[15,16]

Simvastatin, a classic lipid-lowering agent, inhibits 3-hydroxy-3-methylglutaryl coenzyme A reductase and it, hopefully, eradicates changes caused by hypercholesterolaemia in chronic renal failure; unfortunately, it does not prolong the lifespan of patients. Thus, it is necessary to find an alternative way to mitigate the further renal sufferings from hypercholesterolaemia in patients with chronic renal disease. Herein, we hypothesize that hypercholesterolaemia may activate upregulation of renal endothelin A receptor ($ET_AR$), iNOS and MMP-9, causing further harm to the affected kidney, which is likely to be critically mediated by an abnormal ET pathway. Our aim was to verify whether a high-fat diet could induce abnormal expression of iNOS, $ET_AR$ and MMP-9 in the kidney and, secondly, whether a dual endothelin receptor antagonist, CPU0213, is sufficient to relieve renal changes resulting from hypercholesterolaemia, and to compare its effects with those of simvastatin.

## Materials and Methods

### Drugs and reagents

Simvastatin was obtained from Hangzhou MSD Pharmaceutical Company Ltd. (Lot No. W1049; Hangzhou, China). Kits for biochemical measurements (triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C)) were provided by Jiancheng Bio-engineering Company (Nanjing, China). The reverse transcription–polymerase chain reaction (RT-PCR) reagents (oligo d(T)18, dNTP, Trizol, RNase inhibitor, avian myeloblastosis virus (AMV) reverse transcriptase and Taq DNA polymerase) were obtained from Promega (Madison, USA). CPU0213 was supplied by the Medicinal Chemistry Department of China Pharmaceutical University (CPU), Nanjing, China.

### Animals and diets

Male Sprague–Dawley rats (qualified No. SCXK (SU) 2002-0018), 40 in total, weighing 200–220 g, were obtained from the Animal Center of Qinglongshan in Nanjing. All procedures were approved by the University Ethic Committee, in accordance with the Guidelines for the Care and Use of Laboratory Animals in Jiangsu Province, China.

Rats were kept in a temperature-controlled (25°C) room under natural lighting and had free access to water. High-fat diet was purchased from the Animal Center of the University, containing, as percentage of energy: 10% lard, 10% egg yolk powder, 1% cholesterol, 0.2% bile salt and 78.8% basal chow.

### Experimental procedure

Rats were randomly divided into four groups as follows: (1) normal rats with food supply restricted to 15 g per rat on average (control), reported previously;[17] (2) high-fat diet with no food restriction for 60 days (HFD); rats on the high-fat diet were administered, on the last 15 days, with either (3) CPU0213 (HFD + CPU0213) (30 mg/kg daily, s.c.) or (4) simvastatin (HFD + SIM) (4 mg/kg daily, p.o.). The rats were allowed free access to water. Body weight was monitored weekly. Serum total cholesterol was monitored at the end of the first and second month, confirming sustained hypercholesterolaemia. Drugs were suspended in 0.5% carboxymethylcellulose sodium (CMC-Na) before medication.

Rats, fasted overnight, were anaesthetized with urethane (1.5 g/kg, i.p.), and blood samples were taken by a catheter inserted into the right common carotid. Then serum was separated and collected by centrifugation and stored at −20°C before biochemical assays. Rats were sacrificed and kidneys were quickly harvested and placed under −80°C in liquid nitrogen before analysis.

### Biochemical assays

Serum levels of TC, TG, LDL-C and HDL-C were measured according to instructions from the kit manufacturer.

### RT-PCR

Renal tissue samples were processed as previously reported.[17,18] After the supernatant was discarded, to the remainder was added cold 75% ethanol; this was then centrifuged, dried and extraction of RNA was conducted. RT-PCR of iNOS, $ET_AR$ and MMP-9 was performed in several steps and the amplified products were measured by imaging analysis.

### Western blot

Renal tissue, 100 mg in 400 $\mu$l, was homogenized and protein was extracted and stored at −20°C for further analysis, and Western blot was performed according to previous reports.[17,18] Briefly, samples were separated on a 10% SDS polyacrylamide gel, transferred to nitrocellulose membrane, blocked in 5% TBS–Tween and then probed with the first antibodies against $ET_AR$, iNOS and MMP-9, separately (from Santa Cruz Biotechnology, Santa Cruz, CA, USA), and a secondary anti-goat antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) for 1 h each at room temperature. After extensive washes, bands were visualized using reagents for enhanced chemiluminescence.

### Statistical analysis

Results were expressed as mean ± SD. Analysis of variance was used and the difference between two groups was calculated by the Student–Newman–Keuls test for the statistical meanings; a level of $P < 0.05$ was considered as significant.

## Results

### Body weight and blood lipids

The body weight of HFD rats was significantly increased, relative to normal ($P < 0.05$), and was significantly reduced by simvastatin, but was not affected by CPU0213. Serum levels of TC, TG and LDL-C in the high-fat diet group were significantly elevated ($P < 0.01$), while HDL-C was reduced ($P < 0.01$), relative to normal, suggesting that hypercholesterolaemia had been well established. Simvastatin reversed the changes in serum lipids completely, relative to the untreated HFD rats ($P < 0.05$). In contrast, rats medicated with CPU0213 did not exhibit a blood lipid-lowering effect, with serum lipids being no different from the HFD group (Table 1).

### Endothelin A receptor and induced nitric oxide synthase

In the presence of hypercholesterolaemia, increased inflammatory factors such as iNOS and $ET_AR$ were found in the kidney. iNOS mRNA and protein were upregulated significantly following 60 days of HFD, relative to control (Figure 1a, b). We evaluated $ET_AR$ mRNA and protein expression in the HFD group, and these were upregulated significantly relative to the normal group (Figure 2a, b). CPU0213 exerted a beneficial effect on these changes, comparable with that of simvastatin ($P < 0.05$), by reducing the upregulation of $ET_AR$ and iNOS.

### MMP-9

As we found above, there was increase in iNOS and $ET_AR$ in renal tissue of HFD rats, which may increase MMP-9 in the renal tissue. As predicted, an increase in expression of mRNA and protein of MMP-9 was found in the kidney in response to hypercholesterolaemia, which accounted for renal remodelling and fibrosis, resulting in further damage to the renal cells in HFD rats, relative to control ($P < 0.01$) (Figure 3a, b). Both simvastatin and CPU0213 were effective in attenuating these changes.

## Discussion

Renal disease may present with dyslipidaemia, manifesting as an increase in serum triglycerides in the early stages, then a reduction in HDL followed by elevated LDL associated with the advanced stage of renal disease.[5] These changes

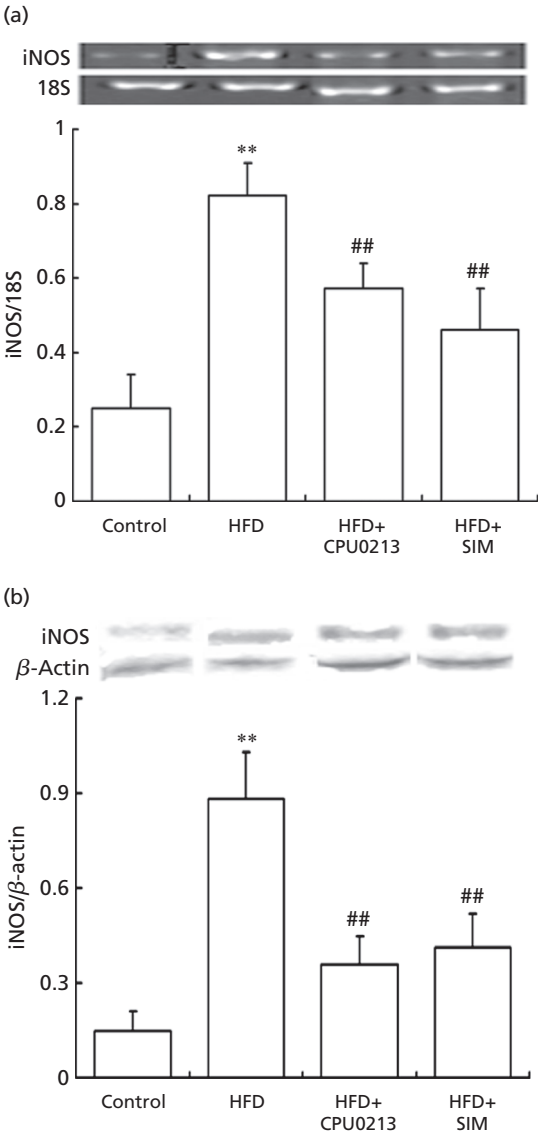


**Figure 1** mRNA and protein expression of induced nitric oxide synthase in the kidney of rats fed with a high-fat diet. Upregulation of mRNA (a) and protein expression (b) of induced nitric oxide synthase (iNOS) was caused by hypercholesterolaemia in the kidney of rats fed with a high-fat diet (HFD). These changes were suppressed by either simvastatin (HFD + SIM) or CPU0213 (HFD + CPU0213). Data are mean ± SD, $n = 6$. $^{**}P < 0.01$ vs control; $^{\#\#}P < 0.01$, vs HFD.

**Table 1** Changes in body weight, total cholesterol, triglyceride, high-density lipoprotein and low-density lipoprotein in rats fed with high-fat diet

| | BW (g) | TC (mmol/l) | TG (mmol/l) | HDL (mmol/l) | LDL (mmol/l) |
|---|---|---|---|---|---|
| Control | 338 ± 45 | 1.15 ± 0.27 | 1.25 ± 0.32 | 0.83 ± 0.12 | 0.31 ± 0.07 |
| HFD | 437 ± 35* | 2.27 ± 0.15** | 2.31 ± 0.32** | 0.55 ± 0.15** | 0.82 ± 0.15** |
| HFD + CPU0213 | 395 ± 42 | 1.85 ± 0.29 | 1.95 ± 0.23 | 0.59 ± 0.12 | 0.75 ± 0.14 |
| HFD + SIM | 376 ± 57$^{\#}$ | 1.66 ± 0.36$^{\#}$ | 1.75 ± 0.31$^{\#}$ | 0.71 ± 0.13$^{\#}$ | 0.58 ± 0.10$^{\#}$ |

BW, body weight; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein. The increases in BW, TC, LDL-C and TG and decrease in HDL-C found in the high-fat diet (HFD) rats were reversed by simvastatin (HFD + SIM), but no blood-lipid lowering effect was brought about by CPU0213 (HFD + CPU0213), a dual endothelin receptor antagonist. Data are mean ± SD, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control; $^{\#}P < 0.05$ vs HFD.

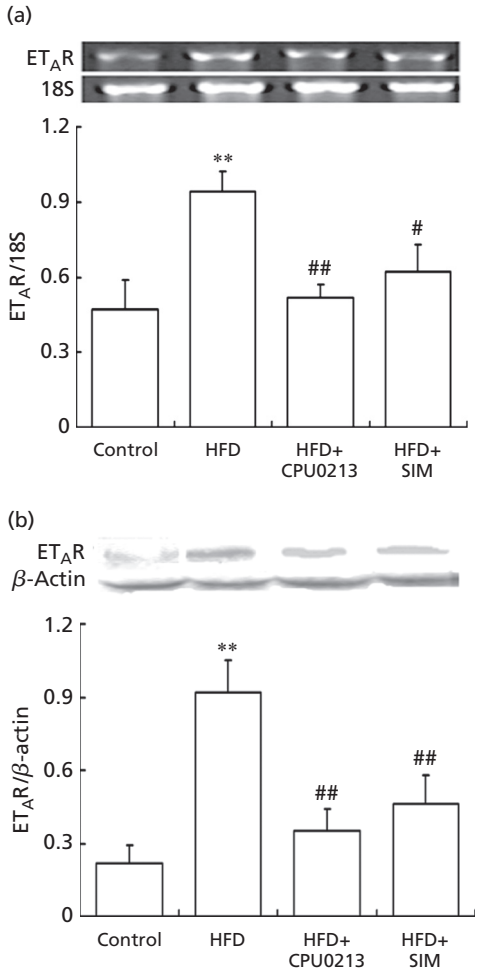


**Figure 2** mRNA and protein expression of endothelin A receptor in the kidney of rats with hypercholesterolaemia induced by a high-fat diet. Upregulation of mRNA (a) and protein (b) of endothelin A receptor ($ET_AR$) was found in the kidney in response to hypercholesterolaemia in the rats fed on a high-fat diet (HFD). These changes were suppressed by either simvastatin (HFD + SIM) or CPU0213 (HFD + CPU0213). Data are mean ± SD, $n = 6$. $^{**}P < 0.01$ vs control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs HFD.

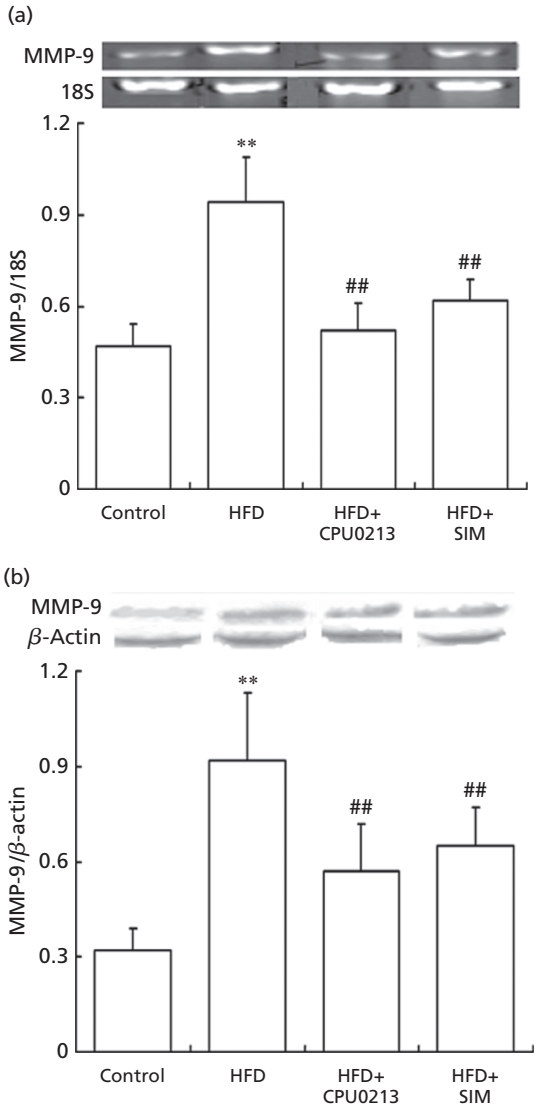


**Figure 3** mRNA and protein expression of matrix metalloproteinase 9 in the kidney of rats with hypercholesterolaemia induced by a high-fat diet. Upregulation of mRNA (a) and protein expression (b) of matrix metalloproteinase 9 (MMP-9) was found in the kidney in response to hypercholesterolaemia in the rats fed on a high-fat diet (HFD). These changes were suppressed by either simvastatin (HFD + SIM) or CPU0213 (HFD + CPU0213). Data are mean ± SD, $n = 6$. $^{**}P < 0.01$ vs control; $^{\#\#}P < 0.01$ vs HFD.

may serve as a causal factor in renal deterioration into uraemia.[19] Thus, abnormal blood lipids, which are the consequence of disturbance of lipid metabolism in chronic kidney disease, act inversely as an aetiological factor, not only eliciting atherosclerosis and coronary disease[20] but also damaging renal tissue leading to exacerbation of renal dysfunction and remodelling.

Atherosclerosis is initiated by hypercholesterolaemia and is recognized as a chronic inflammatory disease. Hypercholesterolaemia elicits an inflammatory reaction, which harms the vascular endothelium, and its role in the cardiovascular system has been fully recognized.[20] These are considered as 'partners in crime' in the pathology of atherosclerosis.[21] It is of interest that the glomerular apparatus and renal tissue respond to elevated LDL-C, presenting lesions that share similarity with those in the intima and vasculature of the renal vascular system. A high-fat diet, in this study, elevated levels of TC, TG, LDL-C and reduced HDL-C in the serum of rats, causing abnormal expression of iNOS, ET-1 and MMP-9 in the renal tissue. These are considered as markers of early renal lesions at the molecular level caused by hypercholesterolaemia. As expected, benefits to the kidney were shown by the lipid-lowering effect of simvastatin, leading to complete blunting of the molecular events in response to hypercholesterolaemia. These findings were in line with those reported for statins in the literature.[22,23]

Endothelin-1, a powerful vasoconstrictor and proliferator, is synthesized and secreted in mesangial, endothelial, epithelial and tubular cells in the kidney.[24] An excess of ET-1, as found in this study, is revealed by significant upregulation of its protein and mRNA; thus, an activated ET-1

pathway in the kidney is a consequence of hypercholesterolaemia. Expression of ET-1 mRNA and protein is low in the normal kidney, as well as in the normal aorta or in early atherosclerotic lesions, but this expression can be dramatically increased in aortic neo-intima and adventitia when a status of atherosclerosis has been established,[10] coincident with the present findings in the kidney. In the kidney, ET-1 acts in a paracrine/autocrine manner regulating renal and intrarenal vasoconstriction, mesangial cell contraction and proliferation of renal cells, modulating the amount of MMP released and regulating water–sodium retention.[24] It has been established that an abnormality of the ET system serves as a participatory factor in glomerular and tubular fibrosis in diabetic nephropathy relating to renal oxidase stress by activation of NADPH oxidase.[14] Therefore, ET receptor antagonists have become a major focus in treating renal disease[15,25,26] and hypertension[27] in certain situations. However, few studies have been performed to investigate changes in biomolecular events in the kidney in hypercholesterolaemia relating to an activated renal ET pathway.

It is of interest to address whether a blockade of ET receptors by CPU0213 blunts changes in iNOS and MMP-9 compared with the lipid-lowering effect of simvastatin. Benefits from this lipid-lowering effect of simvastatin are profound in ameliorating the progression of chronic renal disease. Statins decrease the odds of developing renal dysfunction by 13% ($P < 0.01$) and the benefits of statins to patients may be independent of decreasing cholesterol and are promising in dealing with chronic renal disease.[28] The blood lipids and mortality of cardiovascular events in chronic renal disease are greatly reduced in statin users but the overall death rate does not decline, thus leading to the concept that the final place for the reno-protective effect of statins in treating chronic renal disease has not yet been established.[29] The benefits of statins in alleviating renal disease may stem from their antioxidative effect,[30] and this idea is supported by our study in that simvastatin suppresses iNOS mRNA and protein expression contributing, at least partly, to the mitigation of the increase in MMP-9 in hypercholesterolaemia. An antioxidant activity is also delivered by the dual endothelin receptor antagonist CPU0213; an intimate interaction of ET-1 with ROS has been well established.[31] A vicious cycle connects activation of the ET pathway with an excess of ROS and an exacerbated ET pathway in the presence of hypercholesterolaemia is mediated by ROS.[32] ROS mediate an activation of the ERK1/2 pathway by ET-1,[33] thus blunting of iNOS and MMP-9 upregulation caused by abnormal blood lipids is achieved by CPU0213 medication. In addition CPU0213 is effective in suppressing NAPDH oxidase in the vasculature,[14] resulting in an improved vasodilative response and attenuating the oxidative response by isoproterenol in the myocardium.[34]

MMP-9, which degrades extracellular matrix (ECM), closely participates in a variety of renal diseases.[35] Statins suppress MMP-9 in the affected vasculature.[36] Tubular epithelial cells can be converted into stromal cells, causing renal fibrosis via an epithelial–mesenchymal transformation mechanism, which is attributed to an activation of MMP-9 by the combined effects of epithelial growth factor (EGF) and transforming growth factor (TGF)-$\beta$1.[37] Thus, activation of MMP-9 serves as a key step to deliver signals in renal remodelling, thereafter followed by renal dysfunction. In this study we proved that both endothelin receptor antagonist CPU0213 and simvastatin diminished the over-expression of MMP-9 in the renal lesions caused by a high-fat diet, suggesting that an activation of $ET_AR$ is critically involved in renal disorders and that an abrogation of the insult of hypercholesterolaemia by simvastatin is attributed to suppression of renal $ET_AR$. Thus, ET-1 exerts an important action on the epithelial–mesenchymal transformation mechanism through activating MMP-9.

The limitations of our study include the fact that we did not demonstrate the functional and morphological derangement caused by hypercholesterolaemia and that these changes can be reversed by either CPU0213 or simvastatin. CPU0213, as a dual endothelin receptor antagonist, may be favourable for dealing with renal disease. In this aspect, we did not demonstrate an upregulation of $ET_BR$. However, this has been shown in cardiomyopathy and heart failure in several previous reports,[17,18] and also in renal failure[6] and diabetic nephropathy.[32]

## Conclusions

In this study, we demonstrated that a dual endothelin receptor antagonist, CPU0213, suppressed the responses consequent to hypercholesterolaemia by normalizing expression of $ET_AR$, iNOS and MMP-9 in renal tissue. The favourable outcome with CPU0213 was comparable with the lipid-lowering effect of simvastatin. An elevation in cholesterol may be beneficial for better survival of patients with renal failure[38] and a higher incidence of adverse reactions to statins is not acceptable in patients with end stage renal disease.[39] Thus, the beneficial effect from the use of statins in treating chronic renal failure patients is controversial. As compared with simvastatin, a dual endothelin receptor antagonist is promising in relieving renal lesions at the molecular level in hypercholesterolaemia. Because CPU0213 relieves renal damage without affecting the elevated cholesterol and triglycerides, the pattern of drug effect of endothelin receptor antagonists may be suitable in treating patients with end stage renal disease. Endothelin receptor antagonists could be an alternative to statin therapy in the clinical setting.

## Declarations

### Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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