

C66 ameliorates diabetic nephropathy in mice by both upregulating NRF2 function via increase in miR-200a and inhibiting miR-21

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Abstract

Aims/hypothesis Diabetic nephropathy is the leading cause of end-stage renal disease. Previously we reported that C66, a novel analogue of curcumin with a very high bioavailability, ameliorated diabetic nephropathy in mice, with little known about the mechanism. The present study aimed to define the mechanism by which C66 ameliorates diabetic nephropathy. **Methods** Our aim was to discover whether C66 acts through the activation of nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or NRF2), which governs the antioxidant response.

Streptozotocin-induced *Nrf2* (also known as *Nfe2l2*)-knock-out and wild-type (WT) diabetic mice were treated with C66. To determine whether the actions of C66 on NRF2 are mediated by microRNA (miR)-200a, WT diabetic mice were treated with C66 in the presence or absence of an in vivo miR-200a inhibitor (locked nucleic acid-modified anti-miR-200a [LNA-200a]) for 6 months. To determine whether miR-21 downregulation provided an NRF2-independent basis for C66 protection, *Nrf2*-knockout diabetic mice were treated with either C66 or an inhibitor of miR-21 (locked nucleic acid-modified anti-miR-21 [LNA-21]).

Results Deletion of *Nrf2* partially abolished diabetic nephropathy protection by C66, confirming the requirement of NRF2 for this protection. Diabetic mice, but not C66-treated diabetic mice, developed significant albuminuria, renal oxidative damage and fibrosis. C66 upregulated renal miR-200a, inhibited kelch-like ECH-associated protein 1 and induced NRF2 function, effects that were prevented by LNA-200a. However, LNA-200a only partially reduced the protection afforded by C66, suggesting the existence of miR-200a/NRF2-independent mechanisms for C66 protection. C66 was also found to inhibit diabetes induction of miR-21. Both C66 and LNA-21 produced similar reductions in miR-21, albuminuria and renal fibrosis.

Conclusions/interpretation The present study indicates that in addition to upregulating NRF2 by increasing miR-200a, C66 also protects against diabetic nephropathy by inhibiting miR-21.

Keywords Albuminuria · Curcumin · Diabetic nephropathy · Fibrosis · KEAP1 · miR-200 · miR-21 · NRF2

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Abbreviations

3-NT	3-Nitrotyrosine
4-HNE	4-Hydroxynonenal
CA	Cinnamic aldehyde

COL4	Collagen 4
DTT	Dithiothreitol
FN	Fibronectin
JNK	c-Jun N-terminal kinase
KEAP1	Kelch-like ECH-associated protein 1
LNA-200a	Locked nucleic acid-modified anti-miR-200a
LNA-21 miR	Locked nucleic acid-modified anti-miR-21 microRNA
NFE2L2, NRF2	Nuclear factor (erythroid-derived 2)-like 2
PAS	Periodic acid–Schiff
PDCD4	Programmed cell death protein 4
pri-miR-21	Primary-miR-21
SFN	Sulforaphane
STZ	Streptozotocin
UACR	Urinary albumin-to-creatinine ratio
WT	Wild-type

Introduction

Diabetic nephropathy is the leading cause of end-stage renal disease [1], resulting in high mortality. As a long-term complication of diabetes, diabetic nephropathy still cannot be effectively treated and so there is an urgent need to develop more effective medicines to prevent or slow down its progression [2].

Oxidative stress contributes to the pathogenesis of diabetic nephropathy [3]. Nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or NRF2) controls cellular defence mechanisms against oxidative stress [4] by turning on transcription of antioxidant genes, such as *Ho1* and *Nqo1* [5]. NRF2 has been demonstrated to play a key role in protection from diabetic nephropathy [2, 3]. Curcumin is a natural compound that induces NRF2 [6] and prevents diabetes-induced renal injury in animal models [7, 8]. Although curcumin has beneficial effects in animal models of many diseases, poor bioavailability limits its clinical application. The doses of curcumin used in animal models of diabetic nephropathy range from 50 mg/kg to 200 mg/kg daily [7, 9–11]. C66 is a novel curcumin analogue with a much lower effective dose of 5 mg/kg administered every other day, and has been shown to establish protection from diabetic nephropathy and diabetic cardiomyopathy [12, 13]. We have also demonstrated that C66 activated NRF2 in diabetic aorta [14]. However, it is not certain that activation of NRF2 is necessary for C66 to provide protection against diabetic nephropathy. Therefore our first question to address is whether renal protection by C66 in diabetes is mediated by NRF2.

Kelch-like ECH-associated protein 1 (KEAP1) sequesters NRF2 in the cytoplasm, promoting its proteasomal degradation [3]. Thus, one strategy for increasing the activity of NRF2 is to reduce cytoplasmic KEAP1. MicroRNAs (miRs) are short non-coding RNAs that modulate gene expressions [15]. miR-200a ameliorates diabetic nephropathy and promotes degradation of

Keap1 mRNA [16]. In diabetic mice, suppression of renal miR-200a increases KEAP1, decreases NRF2 and worsens renal fibrosis and albuminuria [17]. Interestingly, a difluorinated analogue of curcumin upregulates expression of the miR-200 family [18, 19]. Curcumin analogue C66 may also upregulate miR-200a which could mediate the ability of C66 to upregulate NRF2 and prevent diabetic nephropathy. miR-21 also has a key role in the pathogenesis of diabetic nephropathy [20–22] but unlike miR-200a, it exacerbates diabetic nephropathy. Curcumin downregulates miR-21 [18, 23] and this likely contributes to the beneficial effect of curcumin against diabetic nephropathy. C66 has not been tested for its ability to modulate renal levels of miR-200a or miR-21; this could be an essential step in the pathway by which C66 provides protection from diabetic nephropathy.

The present study was performed to determine the mechanism(s) by which C66 provides protection against diabetic nephropathy by answering the following questions: (1) does prevention of diabetic nephropathy by C66 require NRF2?; (2) does C66 upregulate miR-200a or downregulate miR-21?; (3) is C66 upregulation of NRF2 mediated by miR-200a? and (4) is C66 modulation of either miR-21 or miR-200a sufficient to protect against diabetic nephropathy? To these ends, *Nrf2* (also known as *Nfe2l2*)-knockout (*Nrf2*-null) mice and their wild-type (WT) counterparts were used with and without C66 treatment, and in combination with specific in vivo inhibitors of miR-200a or miR-21.

Methods

Animal treatment *Nrf2*-null (*Nrf2*^{-/-}) male mice with C57BL/6J background (WT) were obtained through breeding of homozygote (*Nrf2*^{-/-}) with heterozygote (*Nrf2*^{+/-}) following the mating system suggested by Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6J male mice (*Nrf2*^{+/+}) were also purchased from the Jackson Laboratory. All mice were housed in University of Louisville Research Resources Center at 22°C, on a 12 h light–dark cycle, with free access to standard rodent feed and tap water. The Institutional Animal Care and Use Committee at University of Louisville approved all experimental procedures for these animals, and all procedures complied with the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health (2011, eighth edition). To test the role of NRF2 in C66 protection against diabetic nephropathy, 8-week-old male WT mice were randomised into the following four groups (*n*=7): control (Ctrl), control treated with C66 (Ctrl/C66), diabetes (DM) and diabetes treated with C66 (DM/C66). To further explore whether miR-200a mediates NRF2 activation by C66, 8-week-old male WT mice were randomised into six groups (*n*=7): Ctrl, Ctrl/C66, DM, DM/C66, C66/locked nucleic acid-modified anti-miR-200a (LNA-200a) negative control co-treated DM (DM/C66/LNA-

200a-NC) and C66/LNA-200a co-treated DM (DM/C66/LNA-200a). To explore whether the inhibition of miR-21 by C66 provides additional protection against diabetic nephropathy in the absence of NRF2, 8-week-old male *Nrf2*-null mice were randomised into six groups ($n=7$): Ctrl, Ctrl/C66, DM, DM/C66, locked nucleic acid-modified anti-miR-21 (LNA-21) negative control treated DM (DM/LNA-21-NC) and LNA-21 treated DM (DM/LNA-21). Mice received an intraperitoneal injection of either sodium citrate or streptozotocin (STZ) (Sigma-Aldrich, St Louis, MO, USA; 50 mg/kg, dissolved in 0.1 mol/l sodium citrate, pH 4.5) on five consecutive days. One week after the last injection of STZ, fasting glucose levels (4 h fast) were measured and mice with a fasting glucose level above 13.89 mmol/l were considered diabetic. Diabetic mice and age-matched controls were then treated by gavage with C66 (5 mg/kg, dissolved in 1% carboxymethyl cellulose-Na solution) or vehicle every other day for 24 weeks. LNA-200a, LNA-21 and their respective mismatched controls (Exiqon, Woburn, MA, USA; 2 mg/kg, dissolved in normal saline [154 mmol/l NaCl]) were delivered subcutaneously at the same time as C66, twice weekly for 24 weeks. Blood glucose levels were recorded on days 28, 56, 84, 112, 140 and 168 after diabetes onset. Mice were then killed and their kidneys taken

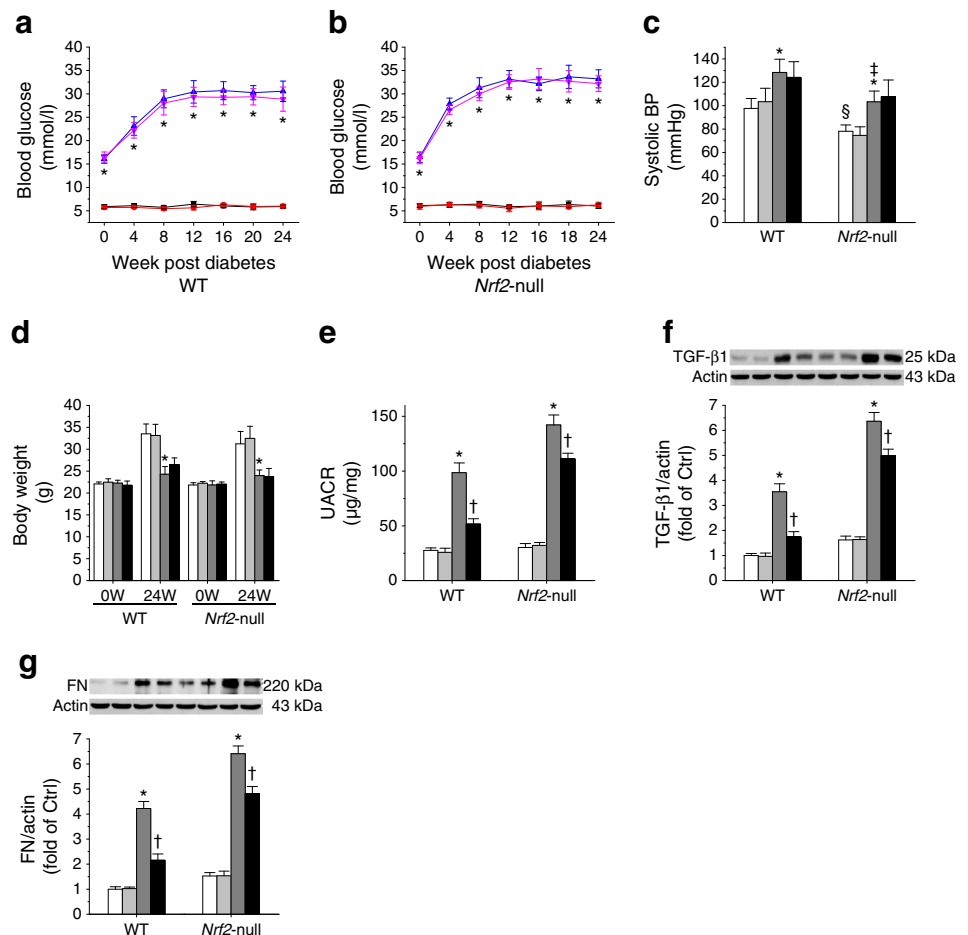
for analysis. Experimenters of this study were blind to group assignment and outcome assessment.

Analysis of kidney function Urine albumin and creatinine were measured on a spot urine sample by a mouse albumin ELISA kit (Bethyl Laboratories, Montgomery, TX, USA) and a QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA, USA). The urinary albumin-to-creatinine ratio (UACR) was calculated.

Measurement of blood pressure Systolic BP was recorded on day 168 after the onset of diabetes with a non-invasive tail cuff Plethysmograph system (CODA6TM; Kent Scientific, Torrington, CT, USA) as previously described [24].

Western blot analysis Western blot was performed using kidney cortex as described in our previous study [25]. The primary antibodies were anti-KEAP1 (Santa Cruz Biotechnology, Dallas, TX, USA; 1:1,000), anti-NRF2 (Santa Cruz Biotechnology; 1:1,000), anti-Histone H3 (Santa Cruz Biotechnology; 1:500), anti-4-HNE (Alpha Diagnostic, San Antonio, TX, USA; 1:3,000), anti-3-NT (Millipore, Temecula, CA, USA; 1:1,000), anti-TGF- β 1 (Cell Signaling, Beverly, MA, USA; 1:500),

Fig. 1 Deletion of the *Nrf2* gene partially abolished the renal protection afforded by C66 against diabetes-induced albuminuria. **(a, b)** Blood glucose levels in C57 WT mice **(a)** and *Nrf2*-null mice **(b)** were monitored at 0, 4, 8, 12, 16, 20 and 24 weeks after diabetes onset. **(c)** Systolic BP was recorded at 24 weeks. **(d)** Body weight was measured at 0 and 24 weeks (W). **(e)** UACR in C57 WT and *Nrf2*-null mice at 24 weeks. **(f, g)** Protein levels of TGF- β 1 **(f)** and FN **(g)** were evaluated by western blot. Ctrl, control. Black squares and white bars, Ctrl; red circles and light grey bars, Ctrl treated with C66; blue triangles and dark grey bars, diabetes; pink inverted triangles and black bars, diabetes treated with C66. Data were normalised by Ctrl and presented as means \pm SD ($n=7$). * $p<0.05$ vs control; † $p<0.05$ vs diabetes; ‡ $p<0.05$ vs WT diabetes; § $p<0.05$ vs WT control



anti-COL4 (Abcam, Cambridge, MA, USA, 1:500), anti-FN (Santa Cruz Biotechnology; 1:500), anti-Smad7 (Santa Cruz Biotechnology; 1:1,000), anti-Smad3 (Santa Cruz Biotechnology; 1:1,000), anti-p-Smad3 (Cell Signaling; 1:500), anti-p-JNK (Cell Signaling; 1:500), anti-PDCD4 (Santa Cruz Biotechnology; 1:1,000), anti-t-JNK (Cell Signaling; 1:1,000), anti-Actin (Santa Cruz Biotechnology; 1:2,000) and anti-GAPDH (Santa Cruz Biotechnology; 1:3,000). These antibodies were routinely validated when they arrived from suppliers with previous positive tissues that had been defined either based on the knockout or overexpression of the target protein.

Quantitative reverse transcription PCR

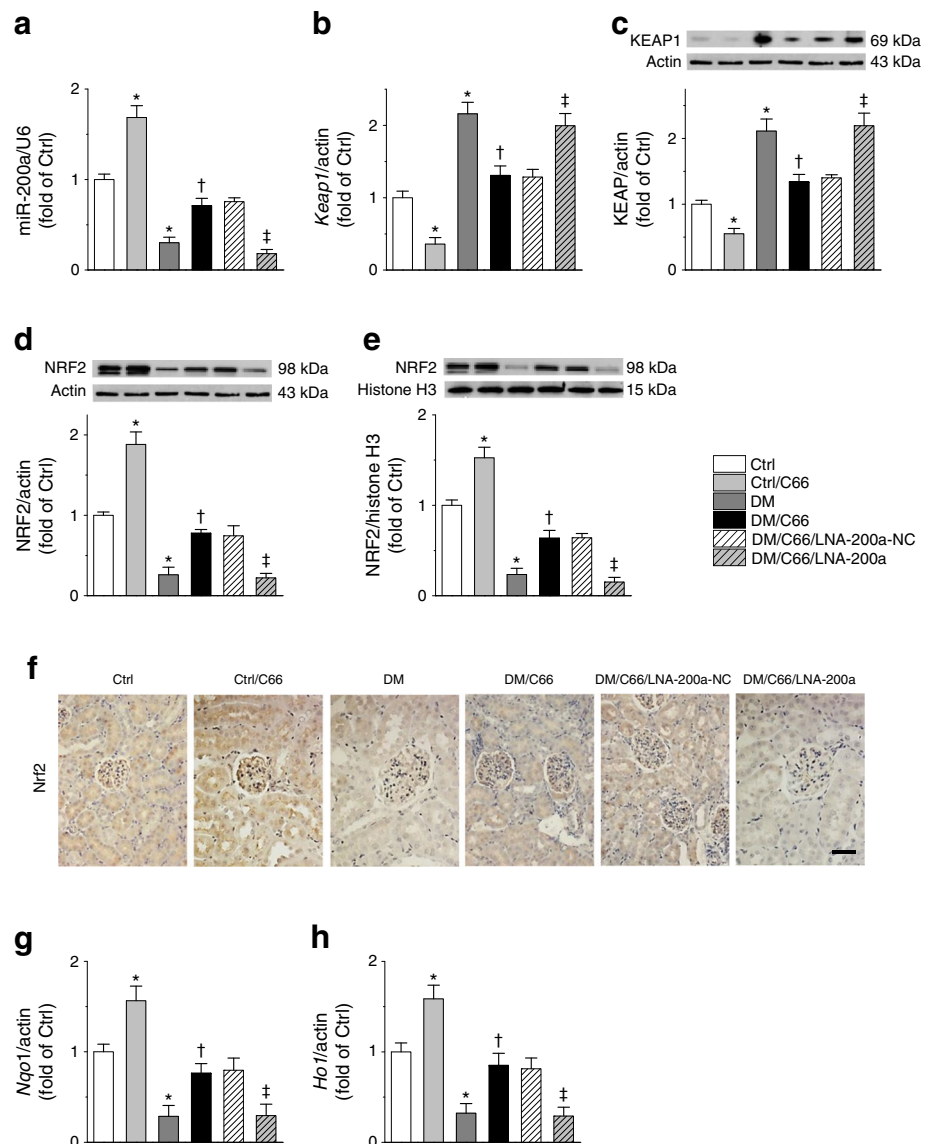
Quantitative RT-PCR (qRT-PCR) was performed as described in our previous studies [26, 27]. qRT-PCR for miRNAs was

performed with the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, Grand Island, NY, USA). Primers for *Keap1*, NAD(P)H quinone oxidoreductase (*Nqo1*), haem oxygenase-1 (*Ho1*), primary-miR-21 (pri-miR-21), miR-21, *Smad7*, programmed cell death protein 4 (*Pdcd4*), actin and U6 were all purchased from Life Technologies.

Histological and immunohistochemical staining Kidney tissues were fixed immediately in 10% buffered formalin solution after harvesting and were embedded in paraffin and sectioned into 5 μm thick sections onto glass slides. The sections were processed for periodic acid–Schiff (PAS), Masson's trichrome and immunohistochemical staining using antibody against NRF2 (Santa Cruz Biotechnology; 1:100 dilution).

Isolation of nuclei The renal nuclei were isolated using a nuclei isolation kit (Sigma-Aldrich). Kidney tissue (30 mg)

Fig. 2 C66 upregulation of renal NRF2 requires an increase in miR-200a. (**a–e**) The effects of C66, diabetes, LNA-200a and their combinations were compared on RNA levels of miR-200a (**a**) and *Keap1* (**b**) and on protein levels of KEAP1 (**c**), total NRF2 (**d**) and nuclear NRF2 (**e**). (**f**) Immunohistochemical staining for NRF2 (Ctrl, control; DM, diabetes). Scale bar, 50 μm . (**g, h**) RNA levels of *Nqo1* (**g**) and *Ho1* (**h**). Data were normalised by Ctrl and presented as means \pm S D ($n=7$). * $p<0.05$ vs control; † $p<0.05$ vs diabetes; ‡ $p<0.05$ vs DM/C66/LNA-200a-NC



from each mouse was homogenised for 45 s in 150 μ l of cold lysis buffer containing 0.5 μ l of dithiothreitol (DTT) and 0.1% Triton X-100. After that, 300 μ l of cold 1.8 mol/l cushion solution (Sucrose cushion solution:sucrose cushion buffer:DTT 900:100:1) was added to the lysis solution. The mixture was transferred to a new tube preloaded with 150 μ l of 1.8 mol/l sucrose cushion solution followed by centrifugation at 30,000 g for 45 min. The supernatant fraction containing cytosolic components was aspirated and the nuclei were visible as a thin pellet at the bottom of the tube.

Morphometric analyses Morphometric analyses were performed using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). Areas to be photographed were selected by people blind to the identity of the samples, as was scoring.

Statistical analysis Seven mice per group were studied. The measurements for each group were summarised as means \pm SD. Image Quant 5.2 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) was used to analyse western blots. One-way ANOVA was performed for comparisons among different groups, followed by post hoc pairwise comparisons using Tukey's test with Origin 8.6 data analysis and graphing software Lab (OriginLab, Northampton, MA, USA). In addition, we constructed a t test to compare the effect of C66 on WT diabetic mice and *Nrf2*-null diabetic mice (Fig. 1). Differences were significant if $p < 0.05$.

Results

C66 retains partial renal protection against diabetes-induced albuminuria despite deletion of the *Nrf2* gene

Diabetic WT and *Nrf2*-null mice had higher blood glucose levels compared with their respective controls (Fig. 1a, b). Blood glucose levels were not altered by C66 (Fig. 1a, b). *Nrf2*-null mice had lower systolic BP compared with WT mice (Fig. 1c), which was in line with findings reported in a previous publication [28]. Diabetes mildly increased BP (Fig. 1c) and decreased body weight (Fig. 1d) in both strains 24 weeks post diabetes. C66 had no impact on either BP or body weight (Fig. 1c, d). Diabetes increased UACR 2.57-fold in WT mice and 3.70-fold in *Nrf2*-null mice (Fig. 1e), suggesting the importance of NRF2 in preventing diabetic nephropathy. Protein levels of TGF- β 1 (Fig. 1f) and FN (Fig. 1g) were increased by diabetes in both strains but these elevated levels were decreased by C66. C66 decreased UACR, TGF- β 1 and FN protein by 44.72%, 50.80% and 48.94% in WT diabetic mice and by 21.77%, 21.68% and 24.83% in *Nrf2*-null diabetic mice; these effects were all significantly lower in *Nrf2*-null diabetic mice than in WT diabetic mice. This confirmed the requirement of NRF2 in C66 protection. On the other hand, the fact that C66 still provided protection (Fig. 1e–g) suggested the existence of

an NRF2-independent protective effect of C66 against diabetic nephropathy.

C66 upregulated renal NRF2 expression via miR-200a targeting *Keap1*

The next study was to address whether C66 upregulates miR-200a and, if so, whether this is involved in upregulation of NRF2 function by C66. Diabetes significantly decreased renal miR-200a, while C66 increased miR-200a in both control and diabetic groups (Fig. 2a). C66 decreased levels of *Keap1* mRNA (Fig. 2b) and KEAP1 protein (Fig. 2c) in the control group and also decreased diabetes-induced high levels of *Keap1* mRNA and KEAP1 protein (Fig. 2b, c). Total and nuclear NRF2 protein levels were decreased in diabetic mice and increased in C66-treated control and diabetic mice (Fig. 2d, e). Immunohistochemical staining of NRF2 showed its expression to be predominantly located in the glomeruli (Fig. 2f). The C66-induced expression of nuclear NRF2 protein (Fig. 2e) was accompanied by elevated mRNA levels of *Nqo1* (Fig. 2g) and *Ho1* (Fig. 2h). These

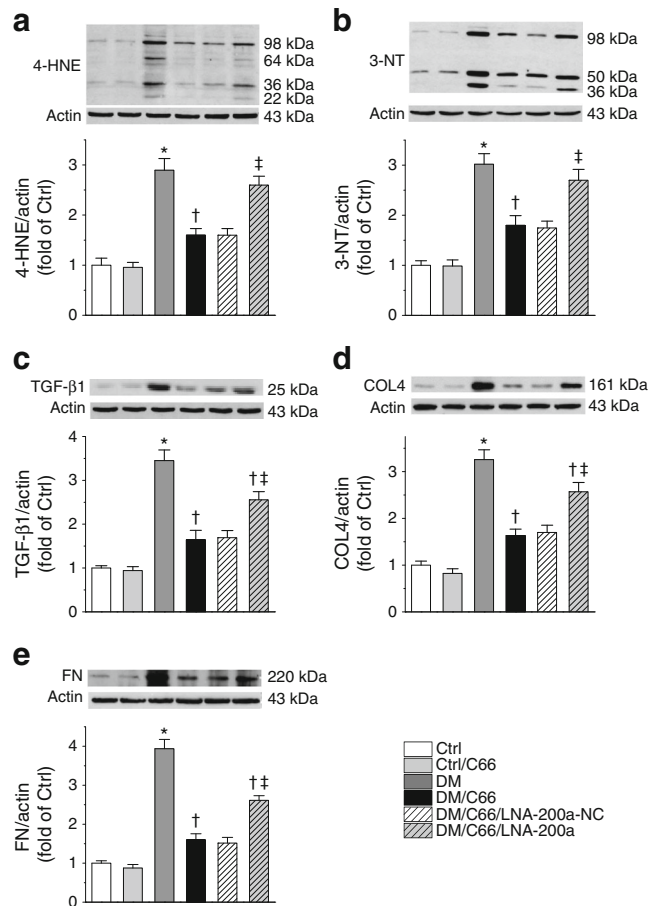


Fig. 3 LNA-200a blocks renal C66 protection against diabetic oxidative damage more effectively than C66 protection against diabetic renal fibrosis. Protein levels of 4-HNE (a), 3-NT (b), TGF- β 1 (c), COL4 (d) and FN (e) were evaluated by western blotting. Data were normalised by control (Ctrl) and presented as means \pm SD ($n = 7$). * $p < 0.05$ vs control; † $p < 0.05$ vs diabetes; ‡ $p < 0.05$ vs DM/C66/LNA-200a-NC

results suggest that the induction of NRF2 expression and function by C66 may be due to stimulation of miR-200a expression. Supporting this notion, administration of LNA-200a completely prevented the actions of C66, including induction of miR-200a (Fig. 2a), suppression of diabetes induction of *Keap1* mRNA and KEAP1 protein (Fig. 2b, c), prevention of diabetes reduction of total and nuclear NRF2 protein (Fig. 2d–f) and suppression of diabetes-induced downregulation of *Nqo1* and *Ho1* mRNA (Fig. 2g, h)

LNA-200a blocks renal C66 protection against diabetic oxidative damage more effectively than C66 protection against diabetic renal fibrosis, remodelling or albuminuria

In the following studies, we defined which variables of diabetic nephropathy were most dependent on C66 stimulation of miR-200a. As shown in Fig. 3a, b, the ability of C66 to reduce diabetes-induced high levels of 4-hydroxynonenal (4-HNE) and 3-nitrotyrosine (3-NT), indices of oxidative and nitrosative protein damage, was largely eliminated by co-administration of LNA-200a. C66 effectively reduced diabetes-increased protein levels of TGF- β , collagen 4 (COL4) and FN (Fig. 3c–e). This

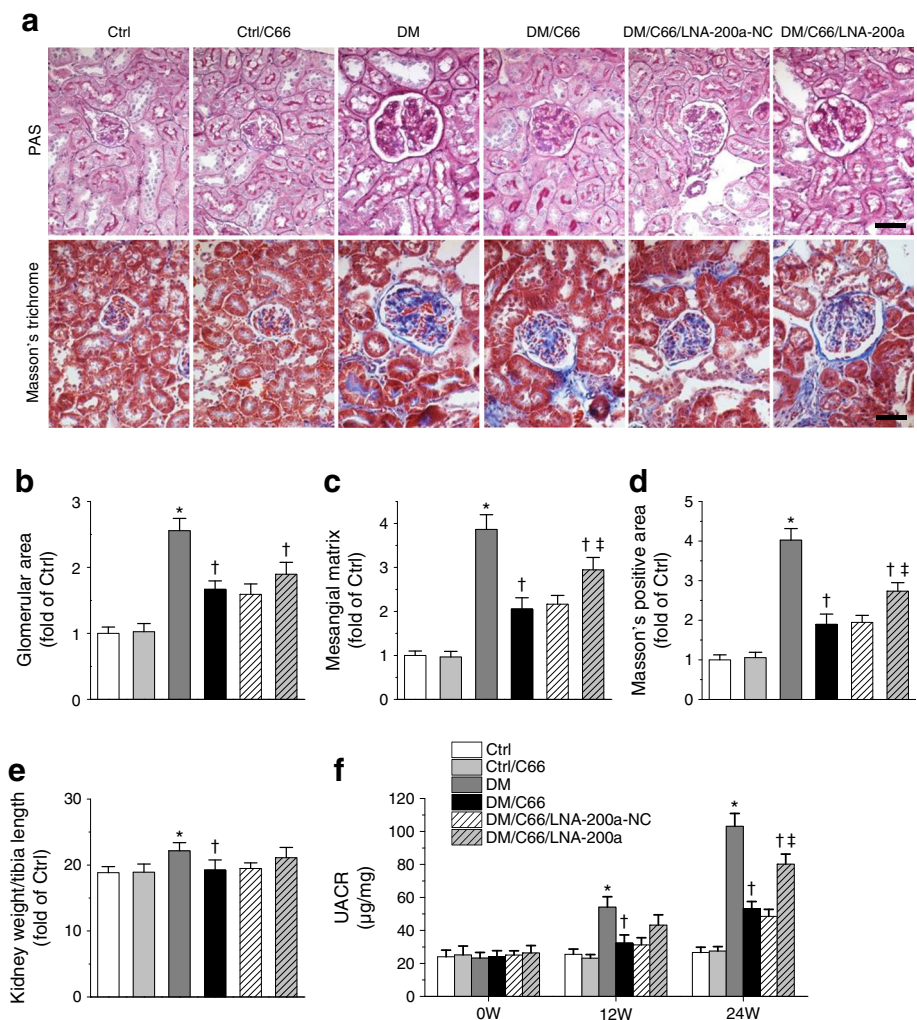
reduction was partially abolished by LNA-200a despite its much stronger effects on 4-HNE and 3-NT.

Significantly enlarged glomerular area (Fig. 4b), mesangial matrix expansion (Fig. 4c) and trichrome-positive area (Fig. 4d) were observed in diabetic kidney, effects that were all attenuated by C66 treatment. LNA-200a partially reduced the efficacy of C66 for all three variables.

Diabetes-induced renal structural remodelling was accompanied by a slight increase in kidney size, shown by increased ratio of kidney weight to tibia length (Fig. 4e). Diabetic renal dysfunction was reflected by increased UACR at 12 and 24 weeks post diabetes and this effect was significantly reduced by C66 treatment (Fig. 4f). The protective effect of C66 on UACR was partially abolished by LNA-200a after 24 weeks of treatment (Fig. 4f).

In summary, LNA-200a completely abolished the effect of C66 on KEAP1–NRF2 signalling (Fig. 2) and oxidative damage (Fig 3a, b) but only partially abolished the protective effect of C66 against renal fibrosis (Fig. 3c–e), remodelling (Fig. 4a–d) and albuminuria (Fig. 4f). These results suggest that the C66-stimulated miR-200a–KEAP1–NRF2 pathway is

Fig. 4 C66 can still provide partial protection against diabetes-induced renal pathological changes and albuminuria despite antagonism by LNA-200a. (a) PAS and Masson’s trichrome staining. Scale bar, 50 μ m. Control, Ctrl; DM, diabetes. (b, c) Glomerular area (b) and mesangial matrix expansion (c) quantified from PAS staining. (d) Fibrosis accumulation quantified from Masson’s trichrome staining. (e) Kidney weight normalised by tibia length. (f) UACR at 0, 12 and 24 weeks (W). Data were normalised by Ctrl and presented as means \pm SD ($n = 7$). * $p < 0.05$ vs control; † $p < 0.05$ vs diabetes; ‡ $p < 0.05$ vs DM/C66/LNA-200a-NC



not the full explanation for C66 protection against diabetic nephropathy and this conclusion is consistent with the finding that C66 retains partial efficacy against albuminuria in diabetic *Nrf2*-null mice (Fig. 1e).

C66 decreased miR-21 in normal and diabetic WT mice accompanied by downregulation of p-Smad3 In diabetes, increased miR-21 downregulates its direct target, Smad7, which thereby releases Smad7 inhibition of Smad3 phosphorylation [29]. Increased phosphorylated Smad3 (p-Smad3) promotes renal fibrosis in diabetic nephropathy [22, 30]. Since the beneficial actions of curcumin are related to downregulation of miR-21 [18, 23], we evaluated this pathway in C66 protection. As shown in Fig. 5a, b, diabetes induced pri-miR-21 and mature miR-21, and this was reversed by C66. *Smad7* mRNA and Smad7 protein levels were decreased by diabetes (Fig. 5c, d) and these actions were also reversed by C66. p-Smad3 protein was markedly reduced by C66 under normal and diabetic conditions (Fig. 5e). These results suggest that C66 can reverse diabetes-increased p-Smad3 levels by initially downregulating miR-21.

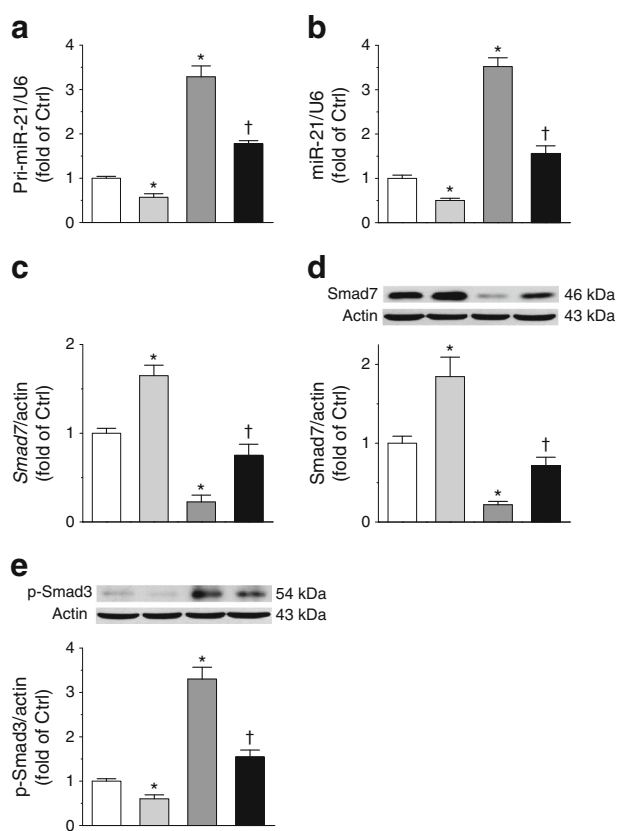


Fig. 5 C66 decreases miR-21 leading to downregulation of p-Smad3. RNA levels for pri-miR-21 (a) miR-21 (b) and *Smad7* (c) were determined by quantitative RT-PCR and protein levels of Smad7 (d) and p-Smad3 (e) by western blot. Ctrl, control. White bars, Ctrl; light grey bars, Ctrl treated with C66; dark grey bars, diabetes; black bars, diabetes treated with C66. Data were normalised by Ctrl and presented as means \pm SD ($n = 7$). * $p < 0.05$ vs control; † $p < 0.05$ vs diabetes

Both C66 and LNA-21 downregulate miR-21 in *Nrf2*-null diabetic mice, resulting in partial amelioration of diabetes-induced renal fibrosis To firmly establish that miR-21 inhibition is an NRF2-independent mechanism for C66 protection against diabetic nephropathy, it is necessary to test this pathway in *Nrf2*-null diabetic mice. To this end *Nrf2*-null diabetic mice were treated with C66 or LNA-21. Both C66 and LNA-21 partially, but significantly, downregulated elevated miR-21 levels in *Nrf2*-null diabetic mice (Fig. 6a), leading to reversal of low diabetic expression of *Smad7* mRNA (Fig. 6b) and Smad7 protein (Fig. 6c). Furthermore, diabetes-increased p-Smad3 protein in *Nrf2*-null diabetic mice was partially reduced by both C66 and LNA-21 (Fig. 6d), along with a decrease in TGF- β 1 and FN levels (Fig. 6e, f). To further test whether c-Jun N-terminal kinase (JNK) phosphorylation is affected by C66 or LNA-21, mRNA and protein levels of *Pdcd4*, a recognised miR-21 target [31] as well as a JNK inhibitor [32], were determined. C66 and LNA-21 similarly increased the diabetes-reduced levels of *Pdcd4* mRNA and PDCD4 protein (Fig. 6g, h), and inhibited the diabetes-increased phosphorylation of JNK (Fig. 6i).

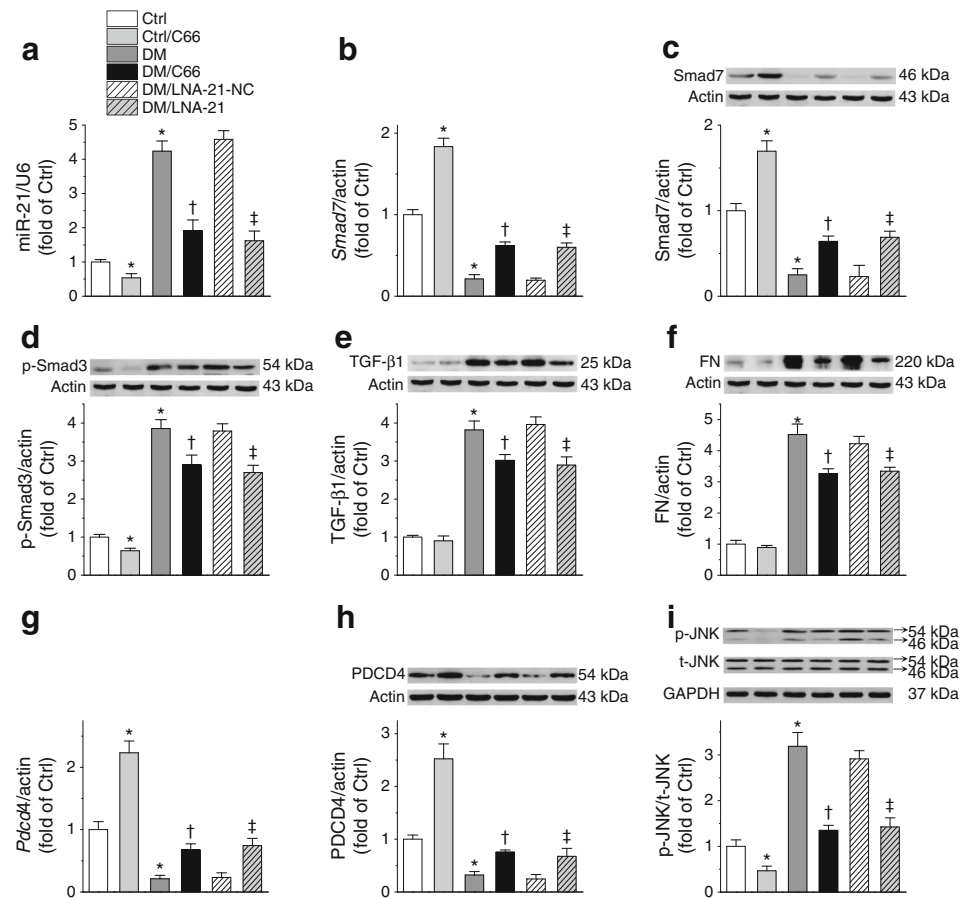
Morphological and pathological changes were evaluated by PAS and Masson's trichrome staining (Fig. 7a). Diabetes increased glomerular area (Fig. 7b), mesangial matrix expansion (Fig. 7c) and accumulation of fibrosis (Fig. 7d) in *Nrf2*-null mice. Notably, these diabetes-induced changes were attenuated to a similar extent by C66 and LNA-21 (Fig. 7a–d). Furthermore, UACR increased time dependently in diabetic mice and C66 and LNA-21 afforded similar, partial, attenuation of diabetes-increased UACR at 12 and 24 weeks post diabetes (Fig. 7e). These results demonstrated that in *Nrf2*-null diabetic mice blocking miR-21 with LNA-21 produced almost the same benefits as C66. This may indicate that all NRF2-independent benefits of C66 are mediated by miR-21 inhibition.

Discussion

The present study addressed whether NRF2 induction mediates, and is required for, C66 protection against diabetic nephropathy. In studies with diabetic *Nrf2*-null mice, we demonstrate that NRF2 plays an important role in the protection afforded by C66. By applying the miR-200a blocker, LNA-200a, we show that the miR-200a-KEAP1-NRF2 pathway enables C66 to up-regulate NRF2 function. miR-21 is shown to be the basis of NRF2-independent C66 protection. In *Nrf2*-null mice diabetes induces miR-21 and this is suppressed by treatment with LNA-21 or C66. Both C66 and LNA-21 produce similar partial renal protection in diabetic *Nrf2*-null mice. Therefore, downregulation of renal miR-21 by C66 in diabetic mice may be responsible for the NRF2-independent component of C66 protection against diabetic nephropathy (Fig. 8).

Oxidative stress contributes to the pathogenesis of diabetic nephropathy [33] and the KEAP1-NRF2 system even governs

Fig. 6 Both C66 and LNA-21 downregulate miR-21 in *Nrf2*-null mice, resulting in reduced levels of stimulators for diabetic renal fibrosis. **(a, b)** In *Nrf2*-null mice, expression levels of renal miR-21 **(a)** and *Smad7* **(b)** RNA were evaluated by RT-PCR. **(c–f)** Protein levels of Smad7 **(c)**, p-Smad3 **(d)**, TGF- β 1 **(e)** and FN **(f)** were evaluated by western blot. **(g–i)** Levels of *Pdcd4* mRNA **(g)**, PDCD4 protein **(h)** and JNK protein **(i)** were further determined. Data were normalised by Ctrl and presented as means \pm SD ($n = 7$). * $p < 0.05$ vs Ctrl; † $p < 0.05$ vs DM; ‡ $p < 0.05$ vs DM/LNA-21-NC



antioxidant redox signalling to prevent the initiation of diabetes mellitus [34, 35]. We and others have demonstrated the beneficial role of NRF2 induction in the prevention of diabetic nephropathy [2, 36]. High BP accelerates diabetic nephropathy. In the present study, the systolic BP of *Nrf2*-null mice was lower than that of WT mice. This result is in accordance with the finding by Erkens et al [28], which showed that elevated endothelial nitric-oxide synthase in the heart and aorta of *Nrf2*-null mice was the reason for the low BP. Given that high BP accelerates diabetic nephropathy whereas the *Nrf2*-null mice had lower BP along with severer diabetic renal injury, we may further conclude that NRF2 does play an important role in the prevention of diabetic nephropathy.

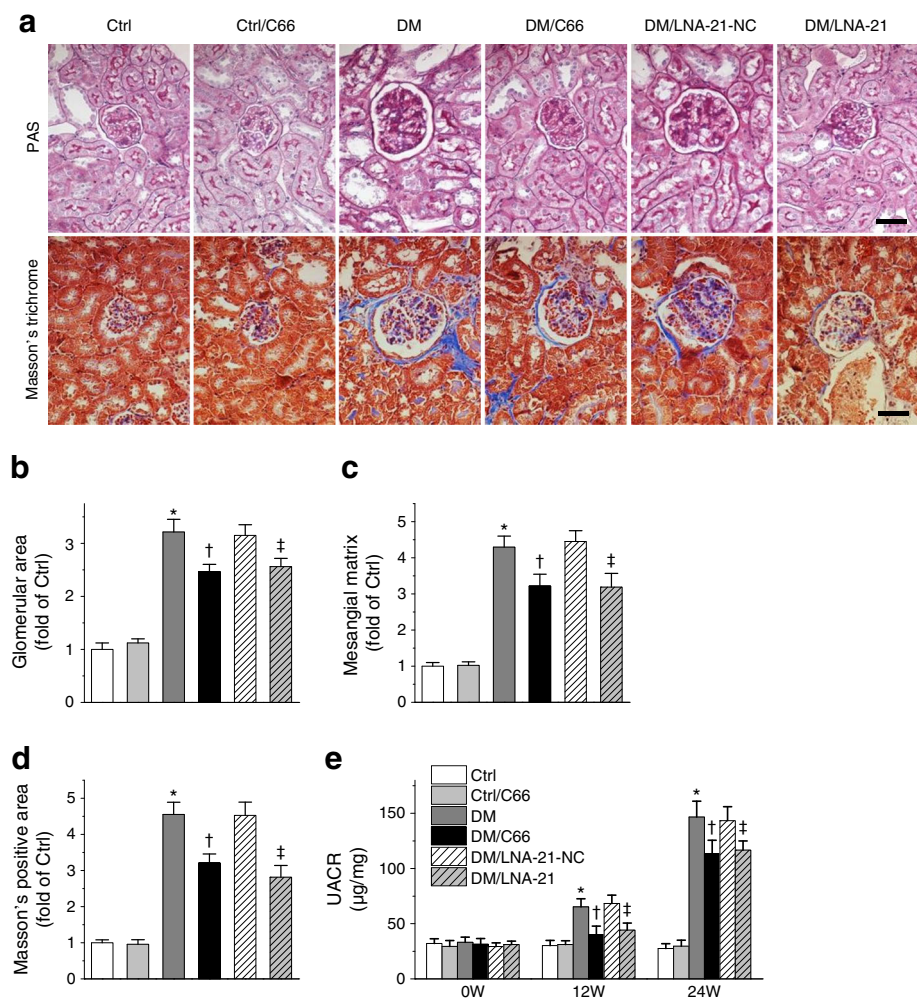
Curcumin is reported to be an activator of NRF2 [6, 37], although little is known about how this occurs. Curcumin disrupts the binding between KEAP1 and NRF2, leading to the release of NRF2 [38]. However, the fact that curcumin downregulates KEAP1 expression [8] challenges the notion that curcumin only modifies the KEAP1–NRF2 complex. Our results also demonstrate that the curcumin analogue C66 decreases the expression of *Keap1* mRNA and KEAP1 protein. Therefore, in addition to modification of the KEAP1–NRF2 complex, curcumin-decreased expression of *Keap1* may also account for NRF2 induction. The present study identified miR-200a as the

mediator between C66 and *Keap1* mRNA. miR-200a degrades *Keap1* mRNA [16]. Moreover, LNA-200a exacerbates diabetic cortical and glomerular fibrogenesis and increases urinary albumin excretion [17]. Using LNA-200a, we provide the first evidence that C66 stimulates miR-200a to downregulate *Keap1* expression, resulting in the activation of NRF2.

C66, sulforaphane (SFN) and cinnamic aldehyde (CA) all activate NRF2. The protection from diabetic nephropathy afforded by SFN and CA is abolished in *Nrf2*-null diabetic mice [2]. The present study showed that C66 still provided significant protection in the absence of NRF2 (Fig. 1e–g). The difference between C66 and the other two NRF2 activators must be due to additional actions of C66. SFN and CA exclusively provide protection through NRF2 and, accordingly, no preventive effect on diabetic nephropathy is observed in *Nrf2*-null diabetic mice [2]. Unlike SFN and CA, we show that C66 has additional function [39] other than NRF2 upregulation [6, 37].

Curcumin is a regulator of epigenetic events, including miRNAs [40], among which miR-21 has been demonstrated to play a key role in the pathogenesis of diabetic nephropathy [20–22]. Recent findings shed light on the involvement of miR-21 as a key component of TGF- β –Smad and PDCD4–JNK signalling. miR-21 degrades *Smad7* or *Pdcd4* mRNA to lower their protein translation [22], leading to impaired

Fig. 7 Both C66 and LNA-21 attenuated diabetes-induced pathological changes and albuminuria in the absence of NRF2. (**a–d**) In *Nrf2*-null mice, PAS and Masson's trichrome staining (**a**) were performed with glomerular area (**b**) and mesangial matrix expansion indexes (**c**) quantified from PAS staining; fibrosis accumulation was reflected by Masson's-positive area (**d**) and was quantified from Masson's trichrome staining. Scale bar, 50 μ m. (**e**) UACRs at weeks (W) 0, 12 and 24 after diabetes initiation. Data were normalised by Ctrl and presented as means \pm SD ($n = 7$). * $p < 0.05$ vs Ctrl; † $p < 0.05$ vs DM; ‡ $p < 0.05$ vs DM/LNA-21-NC



function of Smad7 or PDCD4 in the inhibition of Smad3 or JNK phosphorylation [32, 41]. The transcription factors p-Smad3 or p-c-Jun turn on the expression of a cohort of renal fibrotic and inflammatory genes [41–45]. Therefore, inhibition of miR-21 may exert an effect different from activation of NRF2. This notion is supported by our results that knock-down of NRF2 by LNA-200a in WT mice completely abolished C66 protection against renal oxidative damage (Fig. 3a, b) whereas C66 still protected against renal fibrosis and kidney function (Figs 3c–e and 4d, f). Furthermore, inhibition of miR-21 by either C66 or LNA-21 negatively altered TGF- β –Smad3 signalling, PDCD4–JNK signalling and albuminuria even in the absence of NRF2 (Figs 6 and 7), confirming that inhibition of miR-21 offered an additional role for C66 distinct from that of inducing NRF2 function.

Inhibition of diabetic nephropathy-inducing miRNAs has attracted a great interest in recent years. miRNA inhibitors or mimics have even been studied in clinical trials [46]. The advantage of C66 compared with LNA-21 is that C66 also upregulates NRF2 in addition to knockdown of miR-21. NRF2 activation has already been applied in clinical trials.

Initial human studies of bardoxolone methyl were promising but its use was terminated in a phase III study due to heart complications [47]. However, NRF2 remains a viable drug target as demonstrated by the approval of BG-12 (dimethyl fumarate) for use in multiple sclerosis [48]. In addition to initiating treatment at an earlier stage of diabetic nephropathy and diminishing drug–drug interactions, greater attention should have been paid to the dosage of bardoxolone [49]. More recently, Tan et al showed that dh404, a derivative of bardoxolone methyl, prevented diabetic nephropathy [50], although inflammation was triggered at higher doses. These studies indicate a sweet spot for NRF2 activation [47]. Therefore the dosage of NRF2 activators should be carefully considered. The dose-effectiveness of C66 is very much better than native curcumin (20- to 80-fold), and this confers a unique advantage in future clinical trials.

In summary, the present study demonstrates for the first time that C66 ameliorates diabetic nephropathy by both inhibiting miR-21 and activating NRF2 through the stimulation of miR-200a. Thus, this novel curcumin analogue with dual effects and high bioavailability urgently needs to be

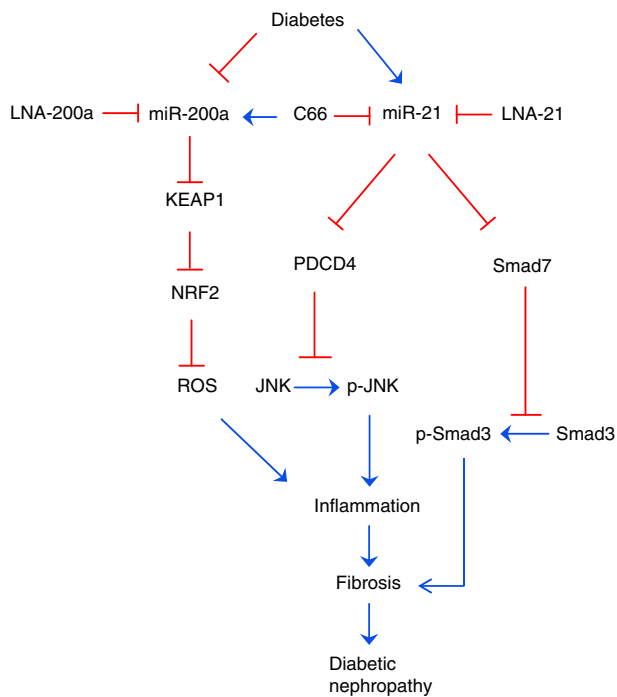


Fig. 8 Possible mechanisms for the prevention of diabetic nephropathy by C66. On one hand, C66 upregulates miR-200a to enhance NRF2 function by targeting *Keap1*, leading to alleviation of renal oxidative damage. On the other hand, C66 inhibits miR-21-induced activation of JNK and Smad3. These protective effects of C66 attenuate renal fibrosis and finally lead to the amelioration of diabetic nephropathy. Blue, activation; red, inhibition; ROS, reactive oxygen species

further tested in various diabetic nephropathy models and small clinical trials.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement HW and LK researched the data. YT, JZ, JG, PNE and XC were involved in the experiments and acquisition of data. MK did essential statistical analysis. HW, LM, LC and GL contributed to the initial discussion and design of the project. GL provided compound C66 used in the study. LC, LM, GL, PNE and XC provided funding for this work. HW, PNE and LC wrote the manuscript. LK, YT, PNE, JZ, JG, GL, MK, XC, LM and LC reviewed and revised the manuscript. YT, PNE and XC were involved in discussion of the manuscript. All the authors approve the version to be published. LC and LM are guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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