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Novel carbocyclic nucleoside analogs suppress glomerular mesangial cells proliferation and matrix protein accumulation through ROS-dependent mechanism in the diabetic milieu

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ABSTRACT

The synthesis of a series of novel 3,4-cis- and 3,4-trans-substituted carbocyclic nucleoside analogs from protected uracil and thymine is described. The key reaction in the followed synthetic protocols utilized the Mitsunobu reaction to couple 3,4-substituted cyclopentanols to ^{3}N-benzoyl uracil or ^{3}N-benzoyl thymine. These molecules were evaluated with regard to their ability to treat diabetic nephropathy. Our results show that two analogs significantly reduced high-glucose induced glomerular mesangial cells proliferation and matrix protein accumulation in vitro and, more interestingly, exhibited an anti-oxidative effect suggesting that the activity may be mediated through ROS-dependent mechanism.

Diabetes is a major public health problem that affects about 250 million people worldwide. Diabetic nephropathy (DN) is a major risk factor for premature morbidity and mortality in patients with type-1 and type-2 diabetes. DN is characterized by excessive accumulation of extracellular matrix (ECM) with thickening of glomerular and tubular basement membranes and increased expression of mesangial matrix proteins, which ultimately progress to glomerulosclerosis and tubulo-interstitial fibrosis[1–5] leading to end-stage renal disease (ESRD). Recent studies have unraveled the significance of mesangial cells (MCs) proliferation in the early stages of DN.[6,7] It seems that mesangial hypercellularity precedes an increase in the extracellular matrix proteins and glomerularsclerosis. Data from animal models as well as cultured renal cells indicate that hyperglycemia and high-glucose induce proliferation of MCs, cellular hypertrophy and extracellular matrix expansion.[3,8,9] Oxidative stress has emerged as a critical pathogenic factor in the development of DN.[10–13] Diabetes is accompanied by increased generation of reactive oxygen species (ROS) in tissues including the kidney.[14–18] Treatments of diabetes and its complication remain sub-optimal. While the control of blood sugar in diabetes is essential, strict control is difficult to achieve and the blockade of the renin/angiotensin system does not result in complete protection. In addition to regulating blood glucose, identifying new drugs that can predict the onset and development of DN is of great importance and essential in developing new intervention strategies.

Carbocyclic nucleosides (carbanucleosides) displayed a wide range of biological activities up to date including antitumor, antibiotic, antimicrobial, antiviral, antimetabolite, and herbicidal activities as well as inhibition of *S*-adenosyl-L-homocysteine hydrolase and picornavirus.[19–24] One attractive feature in carbocyclic nucleosides is the replacement of the furanose ring with a cycloalkane ring that is resistant against phosphorylases that cleave the N-glycosidic bond in natural nucleosides.

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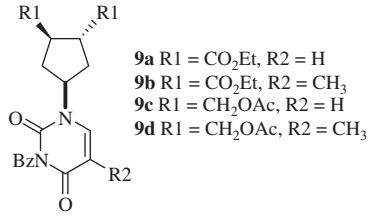
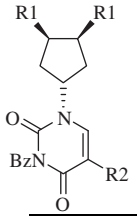


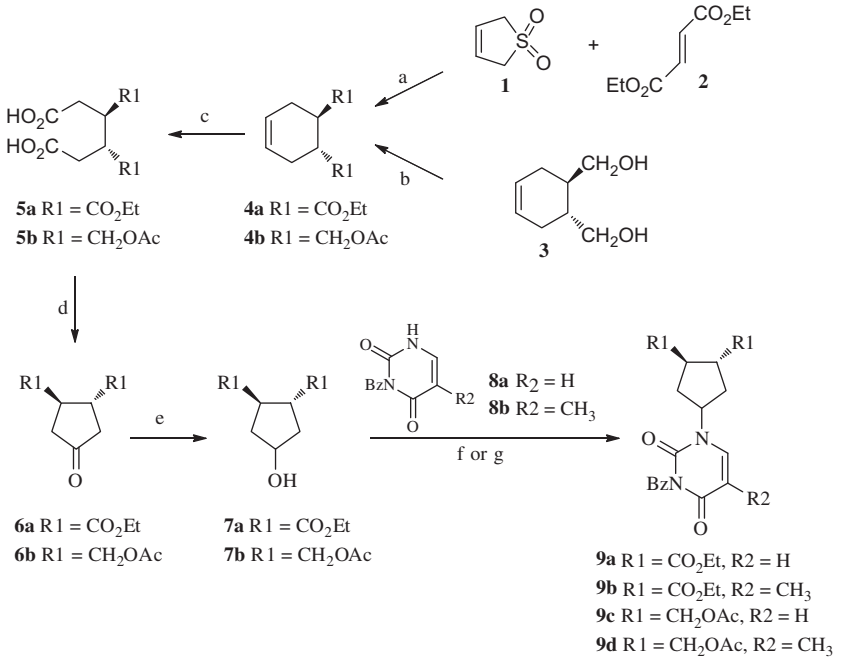
9a R1 = CO_2Et, R2 = H
9b R1 = CO_2Et, R2 = CH_3
9c R1 = CH_2OAc, R2 = H
9d R1 = CH_2OAc, R2 = CH_3



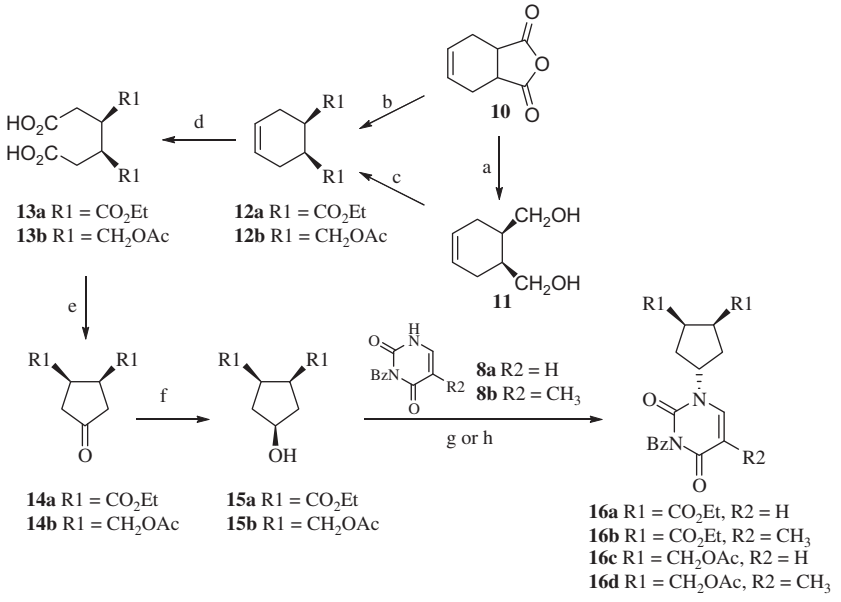
16a R1 = CO_2Et, R2 = H
16b R1 = CO_2Et, R2 = CH_3
16c R1 = CH_2OAc, R2 = H
16d R1 = CH_2OAc, R2 = CH_3

In this study, we report the synthesis of a new series of carbocyclic uridine and thymidine analogs **(±)9a–d** and **16a–d** to evaluate their effect on high-glucose (HG)-induced mesangial cells proliferation and HG-induced fibronectin expression. In parallel experiments, we evaluated whether the effect of the active molecule is mediated through ROS-dependent mechanism.

The synthesis of the *trans* and *cis* uridine and thymidine analogs **(±)9a–d** and **16a–d** was initiated with the corresponding 3,4-substituted cyclopentanones that were prepared following reported procedures (Scheme 1).[25] In short, butadiene sulfone **1** was allowed to react with diethyl fumarate **2** following the Diels–Alder reaction to yield the *rac-trans*-bis(carboxyethyl) cyclo-



Scheme 1. Reagents and conditions: (a) EtOH, 125 °C, 24 h; (b) Ac_2O, NaOAc, reflux, 30 min; (c) $KMnO_4$, H_2O, acetone, −10 °C then rt, 24 h; (d) Ac_2O, NaOAc, reflux, 3 h; (e) $NaBH_4$, MeOH, rt, 2 h; (f) DTBAD, PPh_3, dioxane, rt, 24 h; (g) DIAD, PPh_3, dioxane, rt, 24 h.



Scheme 2. Reagents and conditions: (a) LAH, THF, rt, 2 h then reflux 15 h; (b) EtOH, *p*-TsOH, reflux, 24 h; (c) Ac_2O, NaOAc, reflux, 3 h; (d) $KMnO_4$, H_2O, acetone, −10 °C then rt, 24 h; (e) Ac_2O, NaOAc, reflux, 3 h; (f) $NaBH_4$, MeOH, rt, 2 h; (g) DTBAD, PPh_3, dioxane, rt, 24 h; (h) DIAD, PPh_3, dioxane, rt, 24 h.

Table 1
Effect of the carbocyclic nucleoside on high-glucose induced fibronectin and β-actin expression[a]

Enzyme expressed	NG	HG	HG + **9a**	HG + **9b**	HG + **9c**	HG + **9d**	HG + **16a**	HG + **16b**	HG + **16c**	HG + **16d**
Fibronectin/β-actin (arbitrary units)	24 ± 2.5	65 ± 5.5[b]	67 ± 6.5	25 ± 3.0[c]	55 ± 4.5	59 ± 4.0	26 ± 2.5[c]	68 ± 6.5	64 ± 7.0	65 ± 4.5
Fibronectin/β-actin (arbitrary units)	24 ± 3.5	65 ± 6.5[b]	60 ± 5.0	20 ± 3.5[c]	52 ± 4.0	53 ± 5.5	20 ± 3.0[c]	66 ± 6.0	57 ± 5.5	40 ± 2.5[c]

[a] Quantitation of fibronectin/β-actin of Western blot results from three different experiments. Lysate were prepared from rat glomerular mesangial cells serum-deprived for 12–24 h, then treated for 24 h with high-glucose (25 mM) in the presence or absence of 1 μM (2nd row in the table) or 10 μM (3rd row in the table) in the presence or absence of the nucleoside derivative.
[b] P <0.05 versus NG.
[c] P <0.05 versus HG.

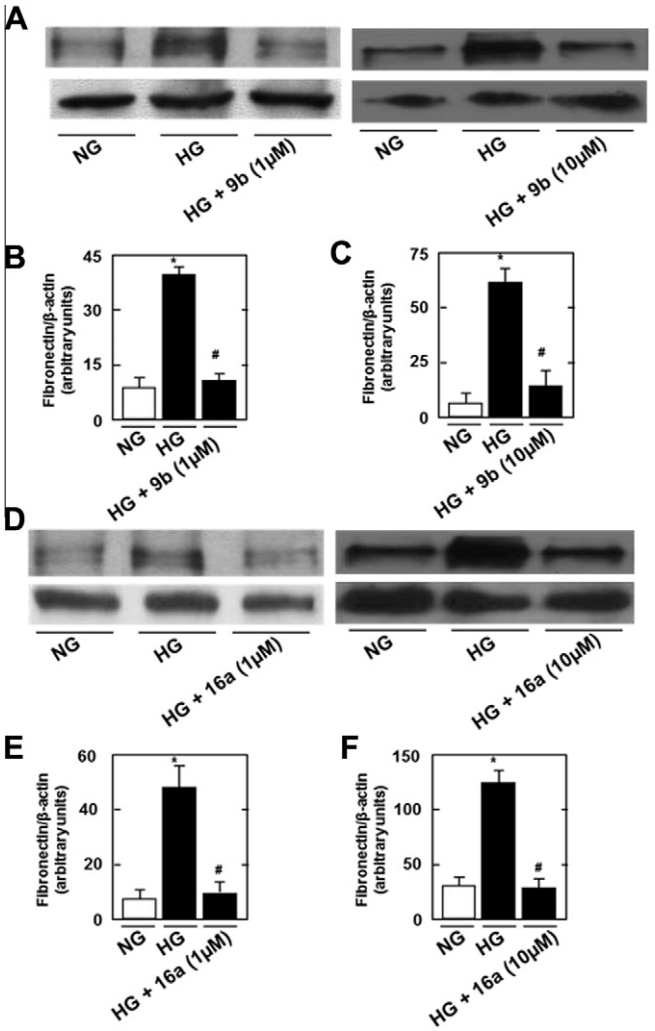


Figure 1. Effect of **9b** and **16a** on high-glucose induced fibronectin expression. (A) and (D) are representative Western blots of fibronectin (top), and β-actin (bottom) levels. (B), (C), (E) and (F) are histograms showing quantitation of Western blot results from three different experiments. *P <0.05 versus NG, #P <0.05 versus HG.

hexene **(±)4a**. Acylation of **(±)3** with acetic anhydride afforded **(±)4b** in good yields. Alkenes **(±)4a**,**b** were oxidized with potassium permanganate to form intermediates **(±)5a**,**b** that were allowed to react with acetic anhydride and sodium acetate to undergo the Dieckmann condensation and yield **(±)6a**,**b**. Reduction of the ketones with sodium borohydride afforded **(±)7a**,**b**. The Mitsunobu reaction has been effectively utilized to couple protected pyrimidine bases to secondary alcohols.[26–31] Therefore, compounds **(±)7a**,**b** were coupled to ^3N-benzoyl uracil **8a** or ^3N-benzoyl thymine **8b** via the Mitsunobu reaction with either di-*tert*-butyl-azodicarboxylate (DTBAD) or diisopropylazo dicarboxylate (DIAD) and

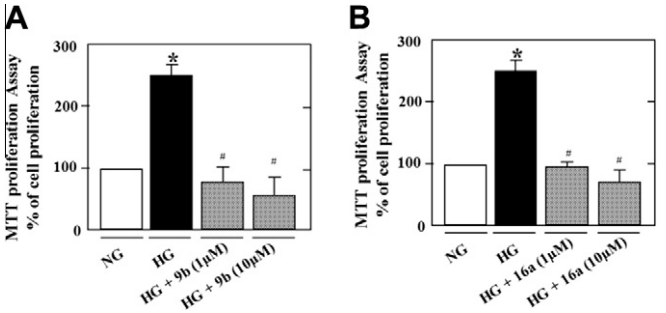


Figure 2. In vitro reduction of high-glucose induced glomerular mesangial cells proliferation.[34] *P <0.05 versus NG, #P <0.05 versus HG.

triphenyl phosphine in dry dioxane to afford compounds **(±)9a**–**d** in an overall yield of 18%, 24%, 12% and 13%, respectively.[32] The low overall yields are a result of the low isolate yields associated with the Mitsunobu reaction.

The synthesis of the *cis* 3,4-substituted cyclopentane derivatives **16a–d** was initiated with the commercially available diethyl *cis*-Δ^4-tetrahydrophthalate **10** that was either reduced with lithium aluminum hydride in dry THF to yield diol **11** or allowed to react with ethanol to form the diester **12a** (Scheme 2). Diol **11** was acylated with acetic anhydride to form diester **12b**. Compounds **12a,b** were oxidized with potassium permanganate to form intermediates **13a,b** that underwent the Dieckmann condensation to afford **14a,b**. Cyclopentanones **14a,b** were reduced and the resulting alcohols **15a,b** were coupled to ^3N-benzoyl uracil **8a** or ^3N-benzoyl thymine **8b** following the Mitsunobu reaction to yield compounds **16a–d** in an overall yield of 11%, 11%, 17% and 26%, respectively.[33]

The effect of these derivatives on fibronectin expression was investigated in cultured rat mesangial cells incubated for 24 h with high-glucose (HG: 25 mM) in the presence or absence of 1 or 10 μM, of the tested carbocyclic nucleoside (Table 1).

The trypan blue exclusion test was used to monitor the viability of cells. Cells were preincubated with the drug of interest for one hour before high-glucose exposure. Even though compounds **9a,c,d** and **16b,c,d** displayed a slight reduction in fibronectin expression, this effect is not significant enough to evaluate them further as potent inhibitors of renal cellular injury in the diabetic milieu. In contrast, the *trans*-thymine derivative **9b** (Fig. 1A–C) and the *cis*-uracil derivative **16a** (Fig. 1D–F) displayed a significant decrease in HG-induced fibronectin expression at the different tested concentrations.

Kidney cells proliferation is another marker for cellular injury in diabetic nephropathy. Several studies have recently unraveled the significance of mesangial cells proliferation in the early stages of diabetic nephropathy.[6,7] It seems that mesangial hypercellularity precedes an increase in the extracellular matrix proteins and glomerular sclerosis, hallmarks of diabetic nephropathy. Hence, we assessed the effect of **9b** and **16a** on HG-induced mesangial cells injury (1 and 10 μM). Cultured rat mesangial cells were incubated

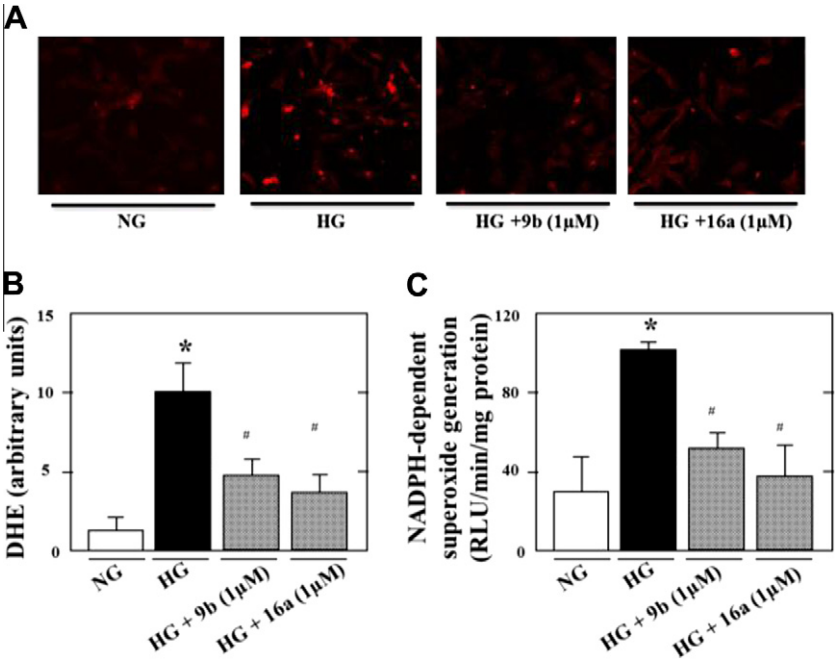


Figure 3. Reduction of high-glucose induced Reactive Oxygen Species (ROS) production.[37] (A) DHE staining of rat glomerular mesangial cells in the presence or absence of **9b** or **16a**. (B) Mean fluorescence intensity of the digitized images. (C) NADPH-dependent superoxide generation (NADPH oxidase activity).[35–37]

with NG or HG in the presence or absence of different concentrations of **9b** or **16a** and cell proliferation was determined after 24 h. Our data clearly show that **9b** and **16a** significantly reduced HG-induced mesangial cells proliferation (Fig. 2).

It has been shown that ROS are produced under diabetic conditions.[10] In this study, ROS production increased in the presence of HG, however, this effect was clearly attenuated in the presence of **9b** or **16a** (Fig. 3). More importantly, these two derivatives inhibited HG-induced NADPH oxidase activity (Fig. 3), known to be a major source of ROS production in diabetic nephropathy.[35,36] Our results suggest that these two derivatives may possess antioxidant activity.

We have designed and synthesized novel carbocyclic nucleosides that suppress mesangial cells proliferation and matrix protein accumulation in vitro. We envisage the synthesis of analogs with other nucleic bases and different substituents at the 3- and 4-position of the cyclopentane ring to confirm and refine our initial results, and therefore, to carry out a thorough quantitative structure–activity relationships (QSAR) study.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.10.122.

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34. The cell proliferation was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Rat glomerular mesangial cells were serum-deprived for 12 h and treated for 24 h with high-glucose (25 mM) in the presence or absence of **9b** (A) or **16a** (B). After treatment, mesangial cells

were incubated with MTT (0.5 mg/ml). Results are presented as percentage of cell proliferation ($n = 3$).
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37. Rat glomerular mesangial cells were serum-deprived for 12 h and treated for 24 h with high-glucose (25 mM) in the presence or absence of **9b** or **16a**. (A) Superoxide production was assessed by DHE staining. (B) Mean fluorescence intensity of the digitized image was measured with ImageJ software (version 1.35, National Institutes of Health, Bethesda, MD) for quantification ($n = 3$). (C) NADPH dependent superoxide generation (NADPH oxidase activity). Superoxide production was expressed as relative light units per milligrams of protein. Protein content was measured using a Bio-Rad protein assay reagent.