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RESEARCH ARTICLE

Correlation study between A_3 adenosine receptor binding affinity and anti-renal interstitial fibrosis activity of truncated adenosine derivatives

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Abstract Truncated 4′-thionucleosides **1**–**4** and 4′-oxonucleosides **5**–**8** as potent and selective A_3AR antagonists were synthesized from D-mannose and D-erythronic acid γ-lactone, respectively. These nucleosides were evaluated for their anti-fibrotic renoprotective activity in TGF-β1-treated murine proximal tubular (mProx) cells. Their antagonistic activities for A_3AR were proportional to their inhibitory activities against TGF-β1-induced collagen I upregulation in mProx cells. This result suggests that the binding affinity of A_3AR antagonists is closely correlated with their anti-fibrotic activity. Thus, A_3AR antagonists might be novel therapeutic candidates for treating chronic kidney disease.

Keywords A_3 adenosine receptor · Antagonist · Binding affinity · Truncated adenosine · Renal fibrosis

Introduction

Adenosine is an endogenous signaling molecule that modulates many physiological processes through binding to four subtypes of adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3ARs) (Ralevic and Burnstock 1998). These four subtypes are classified based on their ability to either inhibit or stimulate adenylate cyclase activity. For example, A_{2A} and A_{2B}ARs are Gs-coupled receptors that can stimulate adenylate cyclase while A_1 and A_3ARs are coupled to Gi protein that can inhibit adenylate cyclase (Fredholm et al. 2001).

Extracellular adenosine concentrations can be rapidly increased from 300 nM to 1200 nM in response to cellular damage to protect tissue from damage due to hypoxia and ischemia (Liang and Jacobson 1999; Haskó 2004). Extracellular adenosine concentration is also dramatically increased during renal hypoxia and ischemia compared to that in normal kidneys (Rabadi and Lee 2015). It has been recently reported that adenosine deaminase (ADA) knockout mice show tubulointerstitial fibrosis, indicating that adenosine might play an important role in controlling chronic kidney disease (Dai et al. 2011). It has also been reported that all four subtypes of ARs are up-regulated in obstructed kidneys which is a well characterized model of tubulointerstital fibrosis (Lee et al. 2012). We have reported the structure–activity relationship of truncated 4′-oxo- and 4′-thionucleosides **1**–**8** as potent and selective A_3AR antagonists (Jeong et al. 2007, 2008; Pal et al. 2009). These compounds have been found to be orally active and species-independent A_3AR antagonists suitable for efficacy determination in animal model. Among these compounds, truncated 2-chloro-N^6-(3-iodobenzyl)-4′-thioadenosine (**1**, X = S) with *K*i values 4.16 nm and 3.91 nM for human (h) A_3ARs and rat (r) A_3ARs, respectively, exhibited highly potent therapeutic effects on UUO-induced renal fibrosis. Compound **1** inhibited UUO-induced renal fibrosis and collagen I upregulation in a dose-dependent manner. It also showed potent anti-tubulointerstitial fibrosis effect as losartan at dose of 10 mg/kg, suggesting that compound **1** might be a good candidate for treating chronic kidney disease (Lee et al. 2013).

Jinha Yu and Gyudong Kim contributed equally to this work.

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Based on these findings, we wanted to establish the relationship between binding affinity for A_3AR and anti-renal fibrosis activity of truncated 4′-thio- and 4′-oxoadenosine derivatives acting as A_3AR antagonists (Fig. 1). Herein, we report therapeutic potentials of A_3AR antagonists on renal tubulointerstital fibrosis.

Materials and methods

^1H NMR Spectra ($CDCl_3$, CD_3OD or DMSO-d_6) were recorded with Varian Unity Invoa 400 MHz Spectrometer. ^1H NMR data were reported as peak multiplicities: s for singlet, d for doublet, dd for doublet of doublets, t for triplet, q for quartet, br s for broad singlet, and m for multiplet. Coupling constants were reported in Hertz. ^{13}C NMR spectra ($CDCl_3$, CD_3OD or DMSO-d_6) were recorded with Varian Unity Inova 100 MHz Spectrometer. Chemical shifts were reported as parts per million (δ) relative to solvent peak. Optical rotations were determined with Jasco III in appropriate solvent. UV spectra were recorded on U-3000 (Hitachi) in methanol or CH_2Cl_2. Flash column chromatography was performed using silica gel 60 (230–400 mesh, Merck). All anhydrous solvents were distilled over CaH_2, P_2O_5, or sodium/benzophenone prior to reaction.

Synthesis

Designed compounds **1–8** were synthesized as depicted in Schemes 1, 2 and 3 following published procedures (Jeong et al. 2007, 2008; Pal et al. 2009).

*General procedure for Vorbruggen condensation: synthesis of compounds **17** and **18***

2,6-Dichloropurine (2 equiv), ammonium sulfate (0.3 equiv), and bis(trimethylsilyl)amine (20 mL) were refluxed under inert and dry conditions until clear solution was obtained. The solution was evaporated under high vacuum. The resulting solid was dissolved in 1,2-dichloroethane (10 mL) at 0 °C. The solution of compound **14** or **16** (1 equiv) in 1,2-dichloroethane (10 mL) was added dropwise to this mixture. Trimethylsilyl trifluoromethanesulfonate (2 equiv) was added dropwise to the mixture. The mixture was stirred at 0 °C for 30 min, warmed to room temperature with stirring for 1 h, and then heated with stirring at 80 °C for 2 h. The mixture was cooled, diluted with CH_2Cl_2, and washed with saturated $NaHCO_3$ solution. The organic layer was dried with anhydrous $MgSO_4$ and evaporated. The residue was subjected to a flash silica gel column chromatography (CH_2Cl_2:MeOH = 50:1) to give compound **17** or **18**.

*2,6-Dichloro-9-((3aR,4R,6aS)-2,2-dimethyltetrahydrothieno[3,4-d][1,3]dioxol-4-yl)-9H-purine (**17**)* Yield: 79%; UV (CH_2Cl_2): λ_{max} = 275.0 nm; ^1H NMR ($CDCl_3$): δ 8.17 (s, 1H), 5.87 (s, 1H), 5.32 (pseudo t, 1H, J = 4.8 Hz), 5.21 (d, 1H, J = 5.6 Hz), 3.79 (dd, 1H, J = 4.4, 12.8 Hz), 3.26 (d, 1H, J = 13.2 Hz), 1.59 (s, 3H), 1.36 (s, 3H); ^{13}C NMR ($CDCl_3$): δ 153.3, 152.5, 152.4, 145.0, 131.8, 112.3, 89.8, 84.6, 70.6, 41.2, 26.6, 24.8; $[\alpha]_D^{25}$ − 42.04 (c 0.16, CH_2Cl_2); FAB-MS: m/z 347 $[M + H]^+$; Anal. Calcd for $C_{12}H_{12}Cl_2N_4O_2S$: C, 41.51; H, 3.48; N, 16.14; S, 9.24. Found: C, 41.84; H, 3.78; N, 15.99; S, 8.98.

*2,6-Dichloro-9-((3aR,4R,6aS)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)-9H-purine (**18**)* Yield: 69%; UV (MeOH): λ_{max} = 276.5 nm; ^1H NMR ($CDCl_3$): δ 8.15 (s, 1H), 6.07 (s, 1H), 5.41 (d, 1H, J = 6.0 Hz), 5.29–5.26 (m, 1H), 4.31–4.25 (m, 2H), 1.57 (s, 3H), 1.41 (s, 3H); $[\alpha]_D^{25}$ − 21.00 (c 0.10, DMSO); FAB-MS: m/z 331 $[M + H]^+$; Anal. Calcd for $C_{12}H_{12}Cl_2N_4O_3$: C, 43.52; H, 3.65; N, 16.92. Found: C, 43.08; H, 3.61; N, 16.70.

*General procedure for hydrolysis: synthesis of compounds **19** and **20***

A mixture of compound **17** or **18** (10 mmol) in THF (20 mL) and 2 N HCl (10 mL) was stirred at room temperature for 15 h. The mixture was neutralized with 1 N NaOH solution. Volatiles were then carefully evaporated under reduced pressure. The mixture was purified by flash

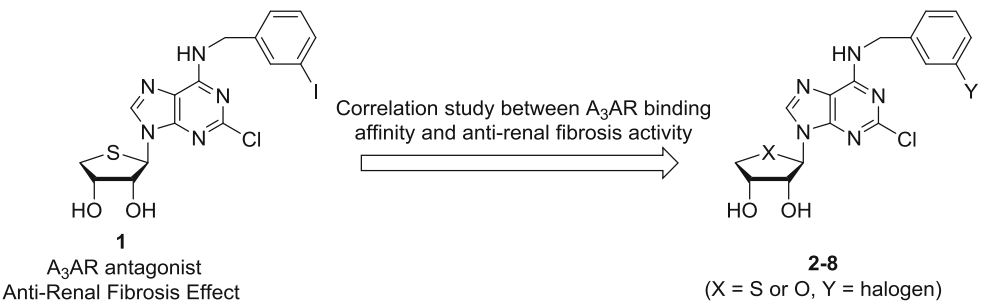
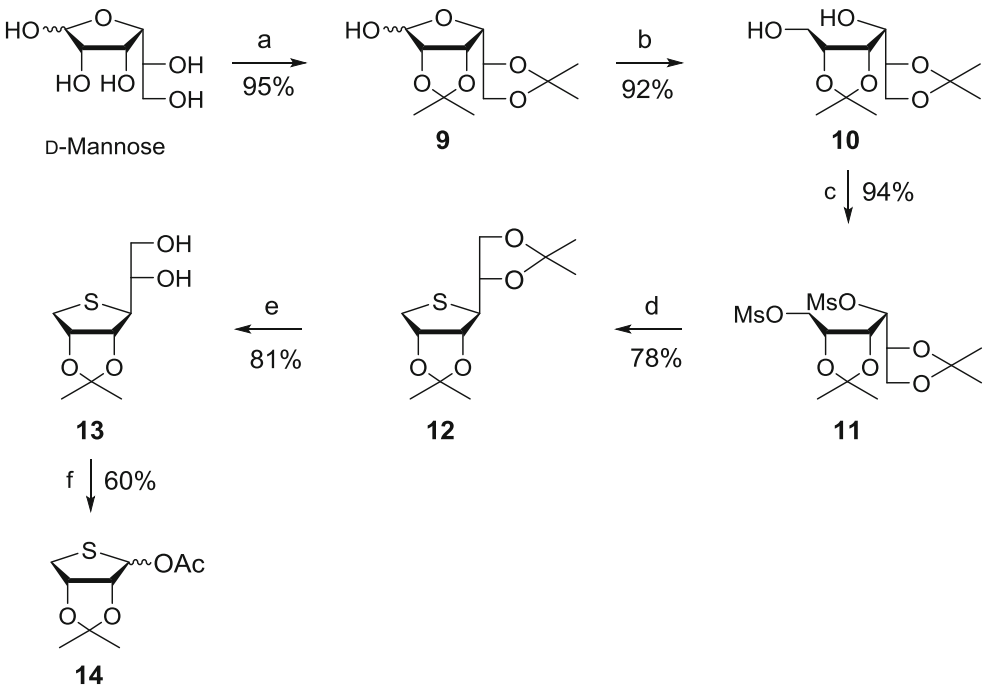


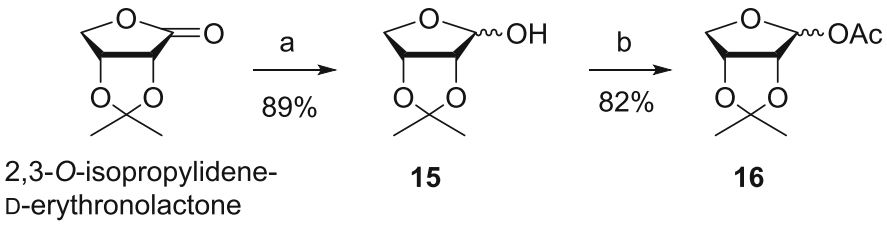
Fig. 1 A correlation study between A_3AR binding affinity and anti-renal fibrosis activity



Reagents and Conditions: a) 2,2-dimethoxypropane, camphosulfonic acid, acetone, rt, 15 h; b) $NaBH_4$, EtOH, rt, 2 h; c) MsCl, Et_3N, CH_2Cl_2, rt, 1 h; d) Na_2S, DMF, 80 °C, 15 h; e) 60% AcOH, rt, 2 h; f) $Pb(OAc)_4$, EtOAc, rt, 15 h.

Scheme 1 Synthesis of 4′-thioglycosyl donor **14**

Scheme 2 Synthesis of 4′-oxoglycosyl donor **16**



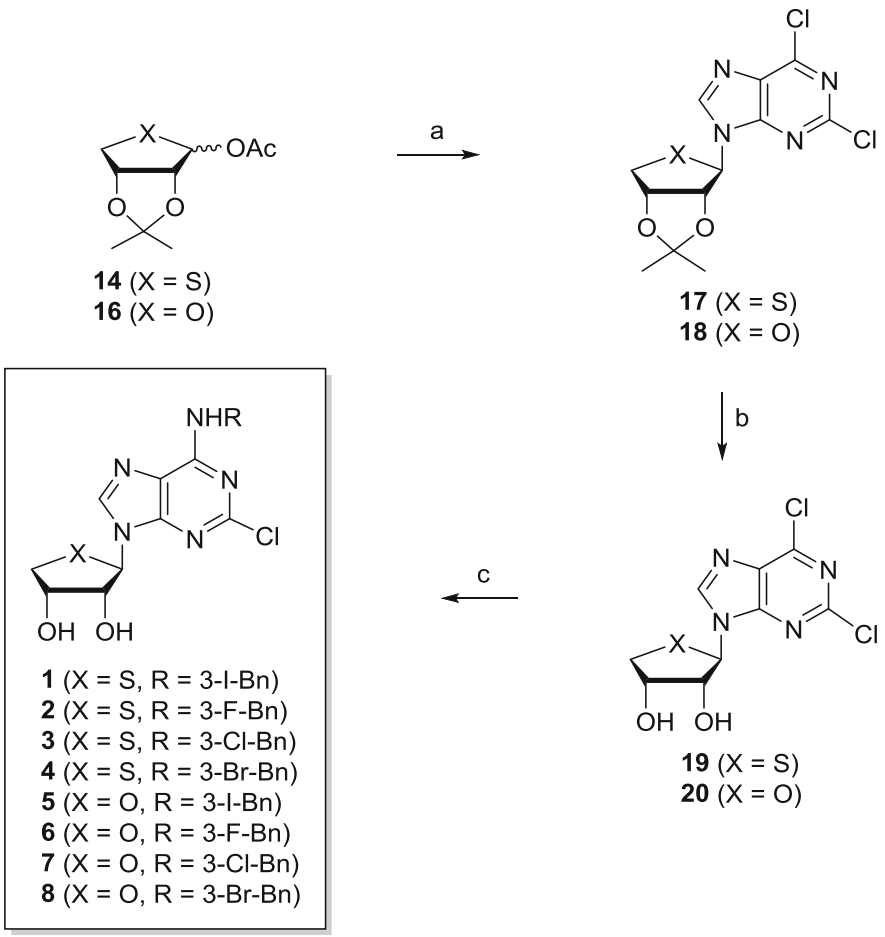
Reagents and Conditions: a) DIBAL, toluene, -78 °C, 30 min; b) Ac_2O, pyridine, rt, 3 h.

silica gel column chromatography (CH_2Cl_2:MeOH = 20:1) to give compound **19** or **20**.

*(2R,3R,4S)-2-(2,6-Dichloro-9H-purin-9-yl)-tetrahydrothiophene-3,4-diol (**19**)* Yield: 96%; mp: 198.3–200.3 °C; UV (MeOH): λ_{max} = 275.0 nm; ^{1}H NMR (CD_3OD) δ 8.87 (s, 1H), 6.08 (d, 1H, *J* = 6.8 Hz), 4.69 (q, 1H, *J* = 3.2 Hz), 4.48 (q, 1H, *J* = 3.6 Hz), 3.56 (dd, 1H, *J* = 4.4, 11.2 Hz), 2.97 (dd, 1H, *J* = 3.4, 11.2 Hz); ^{13}C NMR (DMSO-d_6) 153.7, 151.1, 149.8, 147.5, 130.9, 78.8, 71.9, 62.4, 34.7; $[\alpha]_D^{25}$ − 50.43 (*c* 0.12, DMSO); FAB-MS: *m/z* 307 $[M + H]^+$; Anal. Calcd for $C_9H_8Cl_2N_4O_2S$: C, 35.19; H, 2.63; N, 18.24; S, 10.44. Found: C, 35.48; H, 2.40; N, 18.65; S, 10.84.

*(2R,3R,4S)-2-(2,6-Dichloro-9H-purin-9-yl)-tetrahydrofuran-3,4-diol (**20**)* Yield: 57%; mp: 122.7–123.4 °C; UV (MeOH): λ_{max} = 276.5 nm; ^{1}H NMR (DMSO-d_6): δ 8.98 (s, 1H), 5.96 (d, 1H, *J* = 6.4 Hz), 5.57 (d, 1H, *J* = 6.0 Hz), 5.32 (d, 1H, *J* = 4.0 Hz), 4.74–4.69 (m, 1H), 4.41 (dd, 1H, *J* = 3.6, 9.2 Hz), 4.32–4.29 (m, 1H), 3.87 (dd, 1H, *J* = 2.0, 9.6 Hz); ^{13}C NMR (DMSO-d_6) δ 153.2, 151.2, 150.0, 147.1, 131.2, 88.4, 74.8, 74.1, 70.1; $[\alpha]_D^{25}$ − 68.09 (*c* 0.14, DMSO); FAB-MS: *m/z* 291 $[M + H]^+$; Anal. Calcd for $C_9H_8Cl_2N_4O_3$: C, 37.13; H, 2.77; N, 19.25. Found: C, 37.23; H, 3.11; N, 19.45.

Scheme 3 Synthesis of truncated N^6-(3-halobenzyl)amino derivatives **1–8**



Reagents and Conditions: a) 2,6-dichloropurine, HMDS, $(NH_4)_2SO_4$, TMSOTf, $ClCH_2CH_2Cl$, 0 to 80 °C, 2 h; b) 1 *N* HCl, rt, 15 h; c) 3-halobenzylamine, EtOH, rt, 2 h - 3 d.

*General procedure for N^6-amination: synthesis of **1–8***

Appropriate amine (1.5 equiv) was added to a solution of compound **19** or **20** in EtOH (5 mL) at room temperature. The mixture was stirred at room temperature for 2 h to 3 d and evaporated. The residue was purified by a flash silica gel column chromatography (CH_2Cl_2:MeOH = 20:1) to give compounds **1–8**.

*(2R,3R,4S)-2-(2-Chloro-6-(3-iodobenzylamino)-9H-purin-9-yl)-tetrahydrothiophene-3,4-diol (**1**)* Yield: 84%; mp: 198.7–199.9 °C; UV (MeOH): λ_{max} = 274.0 nm; ^{1}H NMR (DMSO-d_6): δ 8.90 (t, 1H –NH, *J* = 6.4 Hz), 8.51 (s, 1H), 7.74 (s, 1H), 7.60 (d, 1H, *J* = 7.6 Hz), 7.35 (d, 1H, *J* = 7.6 Hz), 7.13 (t, 1H, *J* = 8.0 Hz), 5.82 (d, 1H, *J* = 7.6 Hz), 5.56 (d, 1H, *J* = 6.4 Hz), 5.37 (d, 1H, *J* = 4.0 Hz), 4.60 (d, 3H, *J* = 4.4 Hz), 4.34 (brs, 1H), 3.38 (dd, 1H, *J* = 4.0, 10.8 Hz), 2.80 (dd, 1H, *J* = 4.0, 10.8 Hz); ^{13}C NMR (DMSO-d_6) δ 154.8, 153.0, 150.3, 141.9, 140.6, 136.0, 135.6, 130.6, 126.8, 118.4, 94.7, 78.6, 72.1, 61.5, 42.5, 34.4; $[\alpha]_D^{25}$ − 78.91 (*c* 0.13, DMSO); FAB-MS: *m/z* 504 $[M + H]^+$; Anal. Calcd for $C_{16}H_{15}ClIN_5O_2S$: C, 38.15; H, 3.00; N, 13.90; S, 6.37 Found: C, 38.31; H, 2.96; N, 13.98; S, 6.21.

*(2R,3R,4S)-2-(2-Chloro-6-(3-fluorobenzylamino)-9H-purin-9-yl) tetrahydrothiophene-3,4-diol (**2**)* Yield: 80%; mp: 183.2–183.5 °C; UV (MeOH): λ_{max} = 275.0 nm; ^{1}H NMR (DMSO-d_6): δ 8.91 (t, 1H-NH, *J* = 5.8 Hz), 8.51 (s, 1H), 7.33–7.39 (m, 1H), 7.13–7.18 (m, 2 H), 7.06 (dt, 1H, *J* = 2.8, 11.6 Hz), 5.82 (d, 1H, *J* = 7.2 Hz), 5.56 (d, 1H-OH, *J* = 6.0 Hz), 5. 37 (d, 1H-OH, *J* = 4.4 Hz), 4.65 (d, 1H, *J* = 6.0 Hz), 4.60 (m, 1H), 4.33–4.35 (m, 1H), 3.41 (dd, 1H, *J* = 4.0, 10.8 Hz), 2.79 (dd, 1H, *J* = 2.8, 10.8 Hz); ^{13}C NMR (DMSO-d_6): δ 182.8, 165.3, 162.9, 156.6, 155.3, 142.5, 132.4 (*J* = 8.0 Hz; *C*F), 125.1, 119.9, 115.9, 115.6, 80.5, 73.9, 63.4, 44.5, 36.1; $[\alpha]_D^{25}$ − 96.21 (*c* 0.12, DMSO); FAB-MS: *m/z* 396 $[M + H]^+$; Anal. Calcd for

$C_{16}H_{15}ClFN_5O_2S$: C, 48.55; H, 3.82; N, 17.69; S, 8.10. Found: C, 48.47; H, 3.75; N, 17.57; S, 7.70.

(2R,3R,4S)-2-(2-Chloro-6-(3-chlorobenzylamino)-9H-purin-9-yl)-tetrahydrothiophene-3,4-diol ***(3)*** Yield: 82%; mp: 163.3–165.3 °C; UV (MeOH): λ_{max} = 274.5 nm; ^{1}H NMR (CD_3OD): δ 8.34 (s, 1H), 7.41 (s, 1H), 7.24–7.34 (m, 3 H), 5.94 (d, 1H, *J* = 6.4 Hz), 4.75 (brs, 2 H), 4.61 (q, 1H, *J* = 3.2 Hz), 4.45 (q, 1H, *J* = 4.0 Hz), 3.51 (dd, 1H, *J* = 4.8, 11.2 Hz), 2.95 (dd, 1H, *J* = 3.6, 10.8 Hz); ^{13}C NMR (CD_3OD): δ 141.8, 135.5, 131.2, 128.9, 128.5, 127.3, 80.9, 74.5, 64.2, 44.7, 35.3; $[\alpha]_D^{25}$ − 69.92 (*c* 0.13, DMSO); FABMS: *m/z* 411 $[M]^+$; Anal. Calcd for $C_{16}H_{15}Cl_2N_5O_2S$: C, 46.61; H, 3.67; N, 16.99; S, 7.78. Found: C, 46.65; H, 3.67; N, 16.74; S, 7.39.

(2R,3R,4S)-2-(2-Chloro-6-(3-bromobenzylamino)-9H-purin-9-yl)-tetrahydrothiophene-3,4-diol ***(4)*** Yield: 83%; mp: 184.0–185.0 °C; UV (MeOH): λ_{max} = 274.0 nm; ^{1}H NMR (DMSO-d_6): δ 8.91 (brs, 1H –NH), 8.51 (s, 1H), 7.55 (s, 1H), 7.43 (d, 1H, *J* = 7.6 Hz), 7.33–7.35 (m, 1H), 7.26–7.30 (m, 1H), 5.82 (d, 1H, *J* = 7.2 Hz), 5.57 (d, 1H –OH, *J* = 6.0 Hz), 5.38 (d, 1H –OH, *J* = 4.0 Hz), 4.60–4.63 (m, 3 H), 4.34 (s, 1H), 3.41 (dd, 1H, *J* = 4.4, 11.2 Hz), 2.80 (dd, 1H, *J* = 2.8, 10.8 Hz); ^{13}C NMR (DMSO-d_6): δ 154.8, 153.0, 150.3, 142.1, 140.6, 130.6, 130.1, 129.8, 126.4, 121.6, 118.5, 78.6, 72.1, 61.5, 42.6, 34.5; $[\alpha]_D^{25}$ − 83.60 (*c* 0.13, DMSO); FAB-MS: *m/z* 456 $[M + H]^+$; Anal. Calcd for $C_{16}H_{15}BrClN_5O_2S$: C, 42.07; H, 3.31; N, 15.33; S, 7.02 Found: C, 42.23; H, 3.37; N, 15.19; S, 6.98.

(2R,3R,4R)-2-(2-Chloro-6-(3-iodobenzylamino)-9H-purin-9-yl)-tetrahydrofuran-3,4-diol ***(5)*** Yield: 78%; mp: 195.5–195.8 °C; UV (MeOH): λ_{max} = 274.0 nm; ^{1}H NMR (DMSO-d_6): δ 8.91 (t, 1H, *J* = 6.4 Hz), 8.44 (s, 1H), 7.75 (s, 1H), 7.61 (d, 1H, *J* = 8.0 Hz), 7.36 (d, 1H, *J* = 7.6 Hz), 7.13 (t, 1H, *J* = 4.0 Hz), 5.81 (d, 1H, *J* = 6.8 Hz), 5.47 (d, 1H, *J* = 6.8 Hz), 5.23 (d, 1H, *J* = 4.0 Hz), 4.72 (dd, 1H, *J* = 6.4, 10.8 Hz), 4.61 (d, 1H, *J* = 6.0 Hz), 4.34 (dd, 1H, *J* = 3.6, 9.2 Hz), 3.81 (dd, 1H, *J* = 1.2, 9.2 Hz); ^{13}C NMR (DMSO-d_6): δ 154.8, 153.2, 149.9, 141.9, 140.7, 136.0, 135.6, 130.6, 126.8, 118.8, 94.8, 87.5, 74.4, 73.7, 70.2, 42.5; $[\alpha]_D^{25}$ − 68.07 (*c* 0.12, DMSO); FAB-MS: *m/z* 488 $[M + H]^+$; Anal. Calcd for $C_{16}H_{15}ClIN_5O_3$: C, 39.41; H, 3.10; N, 14.36. Found: C, 39.66; H, 3.08; N, 14.53.

(2R,3R,4R)-2-(2-Chloro-6-(3-fluorobenzylamino)-9H-purin-9-yl)-tetrahydrofuran-3,4-diol ***(6)*** Yield: 83%; mp: 187.0–187.9 °C; UV (MeOH): λ_{max} = 271.0 nm; ^{1}H NMR (DMSO-d_6): δ 8.92 (t, 1H, *J* = 6.0 Hz), 8.43 (s, 1H), 7.39–7.33 (m, 1H), 7.18–7.13 (m, 2 H), 7.06 (dt, 1H, *J* = 2.0, 8.4 Hz), 5.81 (d, 1H, *J* = 6.8 Hz), 5.47 (d, 1H, *J* = 6.0 Hz), 5.22 (d, 1H, *J* = 4.0 Hz), 4.74–4.64 (m, 3 H), 4.41 (dd, 1H, *J* = 3.6, 9.2 Hz), 4.25 (br s, 1H), 3.80 (dd, 1H, *J* = 1.2, 9.6 Hz); ^{13}C NMR (DMSO-d_6): δ 163.4, 163.9, 154.9, 153.1, 149.9, 142.2 (d, *J* = 26.8 Hz), 140.7, 130.3 (d, *J* = 34.8 Hz; *C*F), 123.2, 118.8, 113.8 (dd, *J* = 82.4, 122.0 Hz; *C*-F), 87.5, 74.4, 73.7, 70.2, 42.7; $[\alpha]_D^{25}$ − 73.40 (*c* 0.094, DMSO); FABMS: *m/z* 380 $[M + H]^+$; Anal. Calcd for $C_{16}H_{15}ClFN_5O_3$: C, 50.60; H, 3.98; N, 18.44. Found: C, 50.30; H, 4.01; N, 18.04.

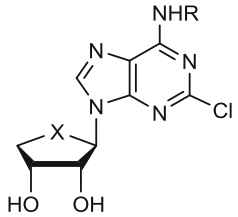
(2R,3R,4R)-2-(2-Chloro-6-(3-chlorobenzylamino)-9H-purin-9-yl)-tetrahydrofuran-3,4-diol ***(7)*** Yield: 76%; mp: 196.4–197.0 °C; UV (MeOH): λ_{max} = 271.5 nm; ^{1}H NMR (DMSO-d_6): δ 8.92 (t, 1H, *J* = 6.0 Hz), 8.43 (s, 1H), 7.39 (s, 1H), 7.37–7.28 (m, 3H), 5.80 (d, 1 H, *J* = 6.8 Hz), 5.47 (d, 1H, *J* = 6.4 Hz), 5.22 (d, 1H, *J* = 4.0 Hz), 4.73–4.64 (m, 3H), 4.32 (dd, 1H, *J* = 3.6, 9.2 Hz), 4.24 (br s, 1H), 3.79 (dd, 1H, *J* = 1.6, 9.2 Hz); ^{13}C NMR (DMSO-d_6): δ 154.8, 153.1, 149.9, 141.8, 140.7, 132.9, 130.2, 127.1, 126.8, 125.9, 118.7, 87.5, 74.4, 73.7, 70.2, 42.7; $[\alpha]_D^{25}$ − 78.19 (*c* 0.133, DMSO); FAB-MS: *m/z* 396 $[M + H]^+$; Anal. Calcd for $C_{16}H_{15}Cl_2N_5O_3$: C, 48.50; H, 3.82; N, 17.68. Found: C, 48.56; H, 3.89; N, 17.12.

(2R,3R,4R)-2-(2-Chloro-6-(3-bromobenzylamino)-9H-purin-9-yl)-tetrahydrofuran-3,4-diol ***(8)*** Yield: 81%; mp: 181.5–181.7 °C; UV (MeOH): λ_{max} = 274.5 nm; ^{1}H NMR (DMSO-d_6): δ 8.92 (t, 1H, *J* = 6.0 Hz), 8.43 (s, 1H), 7.55 (s, 1H), 7.44 (d, 1H, *J* = 8.0 Hz), 7.35–7.33 (m, 1H), 7.30–7.26 (m, 1H), 5.81 (d, 1H, *J* = 6.4 Hz), 5.47 (d, 1H, *J* = 6.4 Hz), 5.22 (d, 1H, *J* = 4.0 Hz), 4.69–4.66 (m, 1H), 4.62 (s, 2H), 4.32 (dd, 1H, *J* = 3.6, 9.2 Hz), 4.25 (br s, 1H), 3.80 (dd, 1H, *J* = 1.6, 9.2 Hz); ^{13}C NMR (DMSO-d_6): δ 154.9, 153.2, 149.9, 142.1, 140.7, 130.6, 130.1, 129.8, 126.4, 121.6, 118.8, 87.5, 74.4, 73.7, 70.2, 42.6; $[\alpha]_D^{25}$ − 62.75 (*c* 0.10, DMSO); FAB-MS: *m/z* 440 $[M + H]^+$; Anal. Calcd for $C_{16}H_{15}ClBrN_5O_3$: C, 43.61; H, 3.43; N, 15.89. Found: C, 43.92; H, 3.44; N, 16.05.

Binding assay for hA_3 and rA_3 adenosine receptor

Binding affinities of final nucleosides **1–8** for human (h) A_3AR and rat (r) A_3AR were measured using standard radioligands and membrane preparations (Jeong et al. 2007, 2008). First, hA_3AR or rA_3AR was expressed in Chinese hamster ovary (CHO) cells. [^{125}I]N^6-(4-amino-3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine (I-AB-MECA) was used to measure the binding affinity. Values are expressed as mean ± SEM (*n* = 3–4 after outliers were eliminated). They were normalized against NECA.

Table 1 Binding affinities and anti-renal fibrosis activity of A_3AR antagonists **1–8**



Compound	Affinity, K_i, nM ± SEM		IC_{50} (μM)[a]
	rA_3	hA_3	
1 (X = S, R = 3-iodobenzyl)	3.9 ± 1.1	4.16 ± 0.50	20.9
2 (X = S, R = 3-fluorobenzyl)	36.2 ± 10.7	7.4 ± 1.3	8.8
3 (X = S, R = 3-chlorobenzyl)	6.2 ± 1.8	1.66 ± 0.90	8.1
4 (X = S, R = 3-bromobenzyl)	6.1 ± 1.8	8.99 ± 5.17	25.8
5 (X = O, R = 3-iodobenzyl)	ND	42.9 ± 8.9	73.2
6 (X = O, R = 3-fluorobenzyl)	ND	284 ± 37	> 100
7 (X = O, R = 3-chlorobenzyl)	ND	75.0 ± 11.7	> 100
8 (X = O, R = 3-bromobenzyl)	ND	13.0 ± 6.9	> 100

ND not determined

[a]Concentration to inhibit TGF-β1-induced collagen I mRNA expression by 50%

Anti-fibrosis assay

Anti-fibrosis assay for compound **1–8** was performed following published procedures (Lee et al. 2013). Briefly, immortalized murine proximal tubular cells (mProx24) derived from microdissected proximal tubular segments of C57BL6/J adult mouse kidneys were supplied from Dr. Sugaya at St. Marianna University School of Medicine, Kanagawa, Japan. mProx24 cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS; Gibco), 100 U/ml penicillin, 100 μg/mL streptomycin, and 44 mM $NaHCO_3$ under 5% CO_2 environment at 37 °C. Cells were cultured in 6-well plates for mRNA analysis. On the next day after seeding cells onto 6-well plates, cultured cells were growth-arrested with DMEM medium containing 0.15% FCS for 24 h. Each synthesized compound was dissolved in DMSO to 50 mM and diluted to 20 mM, 10 mM, and 1 mM. After cells were pretreated with the synthesized compound dissolved in DMEM containing 0.15% FCS for 1 h, they were treated with recombinant human transforming growth factor-β1 (hTGF β1, R&D Systems) at 10 ng/mL for 6 h. Total RNA was extracted from mProx24 cells using Trizol (Invitrogen) according to the standard protocol. mRNA expression level was measured by real-time PCR using StepOnePlus (Applied Biosystems) with reaction volume of 20 μL consisting of cDNA transcripts, primer pairs, and SYBR Green PCR Master Mix (Applied Biosystems). Quantifications were normalized to 18S. Mouse α-SMA primers were 5′-GTCCCAGACATCAGGGAGTAA-3′ and 5′-TCGGATACTTCAGCGTCAGGA-3′.

Results

All final compounds **1–8** showed high binding affinities for hA_3AR. They were synthesized as shown in Schemes 1, 2 and 3 according to our previously published procedures (Jeong et al. 2007, 2008; Pal et al. 2009). First, 4′-thioglycosyl donor **14** (Jeong et al. 2007) was synthesized starting from D-mannose in six steps (Scheme 1). Briefly, D-mannose was converted to diacetonide **9** under acidic conditions. Reduction of diacetonide with sodium borohydride gave diol **10** which was mesylated followed by cyclization with anhydrous sodium sulfide to afford 4-thiosugar (**12**). Selective hydrolysis of 5,6-acetonide of 4-thiosugar with 60% aqueous acetic acid followed by treatment of diol with $Pb(OAc)_4$ afforded 4′-thioglycosyl donor **14**. Then 4′-oxoglycosyl donor **16** (Pal et al. 2009) was synthesized, starting from commercially available 2,3-*O*-isopropylidene-D-erythronolactone (Scheme 2). Lactone was reduced to lactol (**15**) which was treated with acetic anhydride to yield 4′-oxoglycosyl donor **16**.

Glycosyl donors **14** and **16** were condensed with silylated 2,6-dichloropurine in the presence of TMSOTf to give 2,6-dichloropurine derivatives **17** and **18** (Scheme 3).

Compounds **17** and **18** were hydrolyzed with 1 *N* HCl to give **19** and **20**, respectively. Then 2,6-dichloropurine derivatives **19** and **20** were treated with 3-halobenzylamine in EtOH at room temperature to yield final nucleosides **1–4** and **5–8**, respectively.

Discussion

All final compounds were potent and selective A_3AR antagonists. They were evaluated for anti-fibrotic renoprotective activity in TGF-β1-treated murine proximal tubular (mProx) cells (Lee et al. 2013). As potent A_3AR antagonists, 4′-thio analogues **1–4** with K_i's of 1.66–8.99 nM strongly inhibited TGF-β1-induced collagen I upregulation in mProx cells, with IC_{50} values of 8.1–25.8 μM (Table 1). The anti-renal fibrosis activities of 4′-thio analogues **1–4** decreased in the following order: 3-chlorobenzyl derivative **3** ≥ 3-fluorobenzyl derivative **2** > 3-iodobenzyl derivative **1** > 3-bromobenzyl derivative **4**, which was correlated with binding affinities at hA_3AR. 3-Chlorobenzyl derivative **3** (K_i = 1.66 nM) which showed the highest binding affinity for hA_3AR exhibited the most potent inhibitory activity against TGF-β1-induced collagen I mRNA expression (IC_{50} = 8.1 μM). On the other hand, 4′-oxo derivatives **5–8** with K_i values of 13.0–284 nM showed much less binding affinity for hA_3AR. They did not inhibit the elevation of TGF-β1-induced collagen I mRNA expression. These results indicate that anti-fibrotic effects of all final compounds **1–8** are associated with A_3AR-dependent pathway.

In conclusion, this study demonstrates that anti-renal fibrosis activities of **1–8** are proportional to their binding affinities for hA_3AR, indicating that A_3AR antagonists might be useful as new therapeutic candidates for treating chronic kidney disease (CKD).

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Compliance with ethical standards

Conflict of interest The authors declared that they have no conflict of interest.

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