Supplementary Material

Design, Synthesis and Biological Evaluation of Novel 5-Hydroxy-2methyl-4*H*-pyran-4-one Derivatives as Antiglioma Agent

Yi-Bin Li¹, Wen-Hou¹, Hui-Lin, Ping-Hua Sun, Jing Lin*, Wei-Min Chen*

College of Pharmacy, Jinan University, Guangzhou 510632, P. R. China

¹ These authors contributed equally to this work.

*Corresponding authors. Tel.: +86 20 8522 1367(J. Lin), +86 20 8522 4497 (W.-M. Chen).

Fax: +86 20 8522 4766.

E-mail address: linjing_jnu@163.com (J. Lin), twmchen@jnu.edu.cn(W.-M. Chen)

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1. Experimental Section

1.1. Chemistry

All chemicals were purchased from Alfa Aesar or Sigma Aldrich. The synthetic route to 5-hydroxy-2-methyl-4*H*-pyran-4-one derivatives is shown in **Scheme 1**. All the compounds were fully identified through ¹HNMR and ¹³CNMR spectroscopy, and mass spectrometry (MS) and high resolution mass spectrometry (HRMS) before biological testing. Unless otherwise specified, proton (¹H) and carbon (¹³C) NMR spectra were recorded at 18 °C in base-filtered CDCl₃ with a Varian spectrometer operating at 300 MHz for ¹H and 75 MHz for ¹³C. Low resolution electrospray ionization (ESI) mass spectra were recorded on a Finnigan LCQ Advantage MAX mass spectrometer fitted with a 4000 Q TRAP or Agilent 6130 Quadruple LC/MS. High resolution mass spectra were obtained on an Agilent 6210 series LC/MSD TOF mass spectrometer. Melting points were determined with a digital melting point apparatus (Briwell/GH60) and are uncorrected.

1.1.1. General procedure for the reaction of **2a-2h** (procedure A)

A mixture of allomaltol (5-hydroxy-2-methyl-4H-pyran-4-one, 1) (7.90 mmol) and an appropriate aldehyde (9.48 mmol) was stirred in the presence of DABCO (9.48 mmol) in dioxane:H₂O (1:1, 20 mL) at room temperature for 12 h. The product was extracted with EtOAc (3×20 ml) when the reaction finished. The combined organic layer was dried with anhydrous Na₂SO₄, concentrated *in* vacuo, and purified by column chromatography (MeOH/DCM =0.5:9.5) to afford the white solid.

1.1.1.1. 3-Hydroxy-2-(hydroxy(phenyl)methyl)-6-methyl-4H-pyran-4-one (2a)

The reaction was performed according to the general procedure A, using **1** (1.00 g, 7.90 mmol) and benzaldehyde (0.97 mL, 9.48 mmol). The crude product was purified by column chromatography (MeOH:DCM=0.5:9.5) to afford the white solid **2a**. (1.56 g, 85%): mp 170.3-171.1 °C; ¹HNMR (300 MHz, DMSO- d_6) δ 2.18 (s, 3H, CH₃), 6.00 (s, 1H, CH-OH), 6.20 (s, 1H, C=CH), 7.27-7.43 (m, 5H, Ph-H); ¹³CNMR (75 MHz, DMSO- d_6) δ 174.28, 164.98, 150.97, 141.81, 141.00, 128.69, 127.81, 126.39, 111.58, 66.26, 19.71; ESI-MS m/z: 233.0 [M+H]⁺; ESI-HRMS m/z: 233.0804 [M+H]⁺, calcd for C₁₃H₁₃O₄ 233.0808.

The reaction was performed according to the general procedure A, using **1** (1.00 g, 7.90 mmol) and *p*-fluorobenzaldehyde (1.00 mL, 9.48 mmol). The crude product was purified by column chromatography (MeOH:DCM=0.5:9.5) to afford the white solid **2b**. (1.79 g, 90%): mp 151.4-152.2 °C; ¹HNMR (300 MHz, DMSO-*d*₆) δ 2.19 (s, 3H, CH₃), 6.00 (s, 1H, C*H*-OH), 6.20(s, 1H, C=CH), 7.14-7.20 (t, *J* = 9.0 Hz, 2H, Ph-H), 7.41-7.46 (dd, *J* = 8.4 Hz, *J* = 5.4 Hz, 2H, Ph-H); ¹³CNMR (75 MHz, DMSO-*d*₆) δ 174.33, 164.98, 150.68, 141.08, 137.96, 128.42, 115.61, 115.33, 111.59, 65.74, 19.69; ESI-MS m/z: 251.0 [M+H]⁺; ESI-HRMS m/z: 251.0710 [M+H]⁺, calcd for C₁₃H₁₃FO₄ 251.0714.

1.1.1.3. 2-((4-Chlorophenyl)(hydroxy)methyl)-3-hydroxy-6-methyl-4H-pyran-4-one (2c)

The reaction was performed according to the general procedure A, using **1** (1.00 g, 7.90 mmol) and 4-chlorobenzaldehyde (1.21 g, 9.48 mmol). The crude product was purified by column chromatography (MeOH:DCM=0.5:9.5) to afford the white solid **2c**. (1.85 g, 87%): mp 152.5-153.0 °C; ¹HNMR (300 MHz, DMSO- d_6) δ 2.18 (s, 3H, CH₃), 6.00 (s, 1H, CH-OH), 6.20 (s, 1H, C=CH), 7.41 (s, , 4H, Ph-H); ¹³CNMR (75 MHz, DMSO- d_6) δ 174.34, 165.02, 150.46, 141.22, 140.82, 132.34, 128.69, 128.23, 111.61, 65.74, 19.69; ESI-MS m/z: 267.0 [M+H]⁺; ESI-HRMS m/z: 267.0418 [M+H]⁺, calcd for C₁₃H₁₂ClO₄ 267.0419.

1.1.1.4. 3-Hydroxy-2-(hydroxy(4-(trifluoromethyl)phenyl)methyl)-6-methyl-4H-pyran-4-one (2d)

The reaction was performed according to the general procedure A, using **1** (1.00 g, 7.90 mmol) and 4-(trifluoromethyl)benzaldehyde (1.30 ml, 9.48 mmol). The crude product was purified by column chromatography (MeOH:DCM=0.5:9.5) to afford the white solid **2d**. (2.05 g, 89%): mp 179.7-180.6 °C; ¹HNMR (300 MHz, DMSO- d_6) δ 2.17 (s, 3H, CH₃), 6.10 (s, 1H, CH-OH), 6.21(s, 1H, C=CH), 7.60-7.63 (d, *J* = 8.4 Hz, 2H, Ph-H), 7.71-7.74 (d, *J* = 8.4 Hz, 2H, Ph-H); ¹³CNMR (75 MHz, DMSO- d_6) δ 174.30, 165.15, 150.13, 146.46, 141.37, 128.64, 127.11, 126.51, 125.63, 111.66, 65.89, 19.67; ESI-MS m/z: 301.0 [M+H]⁺; ESI-HRMS m/z: 301.068 [M+H]⁺, calcd for

$C_{14}H_{12}F_3O_4$ 301.0682.

1.1.1.5. 2-((4-(Benzyloxy)phenyl)(hydroxy)methyl)-3-hydroxy-6-methyl-4H-pyran-4-one(2e)

The reaction was performed according to the general procedure A, using **1** (1.00 g, 7.90 mmol) and 4-(benzyloxy)benzaldehyde (2.00 g, 9.48 mmol). The crude product was purified by column chromatography (MeOH:DCM=0.5:9.5) to afford the white solid **2e.** (1.92 g, 63%): mp 180.2-180.7 °C, ¹HNMR (300 MHz, DMSO-*d*₆) δ 2.20 (s, 3H, CH₃), 5.08(s, 2H, OCH₂-Ph), 6.06 (s, 2H, CH-OH), 6.19 (s, 1H, C=CH), 6.99 (d, *J* = 8.4 Hz, 2H, Ph-H), 7.31-7.45 (m, 7H, Ph-H); ¹³CNMR (75 MHz, DMSO-d₆) δ 174.26, 164.91, 158.12, 151.19, 140.77, 137.54, 134.05, 128.87, 128.09, 127.69, 114.97, 111.54, 69.64, 65.95, 19.72; ESI-MS m/z: 339.1 [M+H]⁺; ESI-HRMS m/z: 339.1226 [M+H]⁺, calcd for C₂₀H₁₉O₅ 339.1227.

1.1.2. General procedure B for the reaction of 3a-3h

After dissolving 1.0 equiv of the **2a-2h** in dry DCM, 5.0 equiv of triethylsilane was added at 0 °C. The homogeneous mixture was treated with 5.0 equiv of trifluoroacetic acid, and stirred at 0 °C for 45 min and at room temperature overnight. The reaction mixture were extracted with DCM, and dried over anhydrous MgSO₄, provided the crude product which was purified by chromatography¹.

1.1.2.1. 2-Benzyl-3-hydroxy-6-methyl-4H-pyran-4-one (3a)

According to procedure B, **2a** (1.16 g, 5.00 mmol) was treated with triethylsilane (2.91 g, 25.00 mmol). The crude product was purified by chromatography to provide 1.00 g (92%) of **3a** as white solid: mp 141.6-142.7 °C: ¹HNMR (300 MHz, CDCl₃) δ 2.26 (s, 3H, CH₃), 4.03 (s, 2H, CH₂-Ph), 6.22 (s, 1H, C=CH), 7.26-7.34 (m, 5H, Ph-H); ¹³CNMR (75 MHz, CDCl₃) δ 165.40, 149.42, 136.52, 128.84, 128.68, 126.93, 110.79, 34.40, 20.07; ESI-MS m/z: 217.0 [M+H]⁺; ESI-HRMS m/z: 217.0857 [M+H]⁺, calcd for C₁₃H₁₃O₃ 217.0859.

1.1.2.2. 2-(4-Fluorobenzyl)-3-hydroxy-6-methyl-4H-pyran-4-one (3b)

According to procedure B, **2b** (1.25 g, 5.00 mmol) was treated with triethylsilane (2.91 g, 25.00 mmol). The crude product was purified by chromatography to provide 1.10 g (94%) of **3b** as white solid: mp 158.7-159.6 °C: ¹HNMR (300 MHz, CDCl₃) δ

2.26 (s, 3H, CH₃), 4.00 (s, 2H, CH₂-Ph), 6.22 (s, 1H, C=CH), 7.00 (t, J = 8.7 Hz, 2H, Ph-H), 7.27 (dd, J = 8.7 Hz, J = 6.4 Hz, 2H, Ph-H); ¹³CNMR (75 MHz, CDCl₃) δ 165.40, 163.52, 160.27, 149.21, 132.20, 130.28, 115.64, 115.36, 110.80, 33.59, 20.04; ESI-MS m/z: 235.0 [M+H]⁺; ESI-HRMS m/z: 235.0757 [M+H]⁺, calcd for C₁₃H₁₂FO₃ 235.0765.

1.1.2.3. 2-(4-Chlorobenzyl)-3-hydroxy-6-methyl-4H-pyran-4-one (3c)

According to procedure B, **2c** (1.33 g, 5.00 mmol) was treated with triethylsilane (2.91 g, 25.00 mmol). The crude product was purified by chromatography to provide 1.24 g (93%) of **3c** as white solid: mp 170.1-171.0 °C: ¹HNMR (300 MHz, CDCl₃) δ 2.25 (s, 3H, CH₃), 3.99 (s, 2H, CH₂-Ph), 6.22 (s, 1H, C=CH), 7.23-7.30 (m, 4H, Ph-H); ¹³CNMR (75 MHz, CDCl₃) δ 165.39, 149.05, 141.55, 135.00, 132.82, 130.20, 128.79, 110.91, 33.74, 20.03; ESI-MS m/z: 251.0 [M+H]⁺; ESI-HRMS m/z: 251.0460 [M+H]⁺, calcd for C₁₃H₁₂ClO₃ 251.0469.

1.1.2.4. 3-Hydroxy-6-methyl-2-(4-(trifluoromethyl)benzyl)-4H-pyran-4-one (3d)

According to procedure B, **2d** (1.50 g, 5.00 mmol) was treated with triethylsilane (2.91 g, 25.00 mmol). The crude product was purified by chromatography to provide 1.35 g (90%) of **3d** as white solid: mp 159.8-161.0 °C: ¹HNMR (300 MHz, CDCl₃) δ 2.27 (s, 3H, CH₃), 4.08 (s, 2H, CH₂-Ph), 6.24 (s, 1H, C=CH), 7.43 (d, *J* = 8.1 Hz, 2H, Ph-H), 7.57 (d, *J* = 8.1 Hz, 2H, Ph-H); ¹³CNMR (75 MHz, CDCl₃) δ 165.51, 148.47, 141.73, 140.57, 129.18, 125.64, 122.32, 110.96, 34.18, 20.02; ESI-MS m/z: 285.0731 [M+H]⁺; ESI-HRMS m/z: 285.0731 [M+H]⁺, calcd for C₁₄H₁₂F₃O₃ 285.0733.

1.1.2.5. 2-(4-(Benzyloxy)benzyl)-3-hydroxy-6-methyl-4H-pyran-4-one (3e)

According to procedure B, **2e** (1.69 g, 5.00 mmol) was treated with triethylsilane (2.91 g, 25.00 mmol). The crude product was purified by chromatography to provide 1.35 g (90%) of **3e** as white solid: mp 188.7-189.7 °C: ¹HNMR (300 MHz, CDCl₃) δ 2.27 (s, 3H, CH₃), 3.96 (s, 2H, CH₂-Ph), 5.07 (s, 2H, OCH₂-Ph), 6.21 (s, 1H, C=CH), 6.95 (d, *J* = 8.4 Hz, 2H, OCH₂Ph-H), 7.26 (t, *J* = 8.4 Hz, 3H, OCH₂Ph-H), 7.34-7.46 (m, 5H, Ph-H); ¹³CNMR (75 MHz, CDCl₃) δ 165.39, 157.83, 149.42, 136.96, 129.87, 128.58, 127.97, 127.45, 115.04, 110.47, 33.59, 20.08; ESI-MS m/z: 323.1 [M+H]⁺; ESI-HRMS m/z: 323.1276 [M+H]⁺, calcd for C₂₀H₁₉O₅ 323.1278.

1.1.3. General procedure for the reaction of 4a-4f (Procedure C)

Allomaltol (5-hydroxy-2-methyl-4H-pyran-4-one, 1) (1 eq) and triethylenediamine (DABCO, 1.2 eq) were dissolved in dioxane:H₂O (1:1) and stirred for 15 min. Afterwards, the aldehyde (0.5 eq) was added dropwise to the reaction mixture. The solution was stirred at 50 °C for 24 h. The reaction mixture was then cooled to 0 °C and the resulting precipitate was collected by filtration. If no precipitation occurred, the reaction mixture was extracted with EtOAc (3×30 mL). The combined organic layers were washed twice with saturated NaHCO₃ (30 mL) and water (30 mL), dried over Na₂SO₄, filtered and concentrated in vacuo. The crude product was purified by recrystallization.

1.1.3.1. 6,6'-((4-(Benzyloxy)phenyl)methylene)bis(5-hydroxy-2-methyl-4H-pyran-4one) (4a)

The reaction was performed according to the general procedure C, using **1** (1.00 g, 7.90 mmol) and 4-(benzyloxy)benzaldehyde (0.84 g, 3.95 mmol).² The crude product was recrystallized from isopropanol affording a white powder **4a** (1.53 g, 87%): mp 261.4-262.1 °C; ¹HNMR (300 MHz, DMSO- d_6) δ 2.22 (s, 6H, CH₃), 5.08 (s, 3H, OCH₂-Ph), 5.96 (s, 1H, CH-Ar), 6.25 (s, 2H, C=CH), , 7.01 (d, *J* = 9.0 Hz, 2H, Ar-H3'/H5'), 7.22 (d, *J* = 9.0 Hz, 2H, Ar-H2'/H6'), 7.31-7.45 (m, 5H, Ph-H); ¹³CNMR (75 MHz, DMSO- d_6) δ 173.95, 165.08, 158.12, 151.20, 147.68, 142.19, 140.77, 137.42, 129.87, 128.91, 128.16, 127.69, 115.46, 114.97, 111.74, 69.69, 19.63; ESI-MS m/z: 447.1 [M+H]⁺; ESI-HRMS m/z: 447.1438 [M+H]⁺, calcd for C₂₆H₂₃O₇ 447.1438.

1.1.3.2. 6,6'-((4-((4-Methoxybenzyl)oxy)phenyl)methylene)bis(5-hydroxy-2-methyl-4H-pyran-4-one)(**4b**)

The reaction was performed according to the general procedure using **1** (1.00 g, 7.90 mmol) and 4-((4-methoxybenzyl)oxy)benzaldehyde (0.96 g, 3.95 mmol)³. The crude product was recrystallized from isopropanol affording **4b** as a white powder (1.50 g, 80%): mp 241.6-242.1 °C; ¹HNMR (300 MHz, DMSO-*d*₆) δ 2.22 (s, 6H, CH₃), 3.75 (s, 3H, OCH₃), 4.99 (s, 2H, OCH₂-Ph), 5.96 (s, 1H, CH-Ar), 6.25 (s, 2H, CH), 6.93 (d, *J* = 9.0 Hz, 2H, Ar-H3'/H5'), 6.99 (d, *J* = 9.0 Hz, 2H, OCH₂Ar-H3'/H5'), 7.20 (d, *J* = 9.0 Hz, 2H, Ar-H2'/H6'), 7.37 (d, *J* = 9.0 Hz, 2H, OCH₂Ar-H2'/H6'), 9.22(s, 2H, C=C-

OH); ¹³CNMR (75 MHz, DMSO-*d*₆) δ 173.94, 165.07, 159.46, 158.18, 147.69, 142.19, 129.69, 129.83, 129.28, 128.90, 115.47, 114.27, 111.74, 69.45, 55.53, 19.63; ESI-MS m/z:477.1 [M+H]⁺; ESI-HRMS m/z: 477.1537 [M+H]⁺, calcd for C₂₇H₂₅O₈ 447.1544 *1.1.3.3*. *6*,6'-((4-((4-Nitrobenzyl)oxy)phenyl)methylene)bis(5-hydroxy-2-methyl-4H-pyran-4-one)(**4c**).

The reaction was performed according to the general procedure C, using **1** (1.00 g, 7.90 mmol) and 4-((4-nitrobenzyl)oxy)benzaldehyde (1.01 g, 3.95 mmol).¹¹ The crude product was recrystallized from isopropanol affording **4c** as a white powder (1.45 g, 75%): mp 226.3-226.8 °C; ¹HNMR (300 MHz, DMSO- d_6) δ 2.21 (s, 6H, CH₃), 5.27 (s, 2H, OCH₂-Ph), 5.93 (s, 1H, CH-Ar), 6.24 (s, 2H, C=CH), 7.01 (d, *J* = 9.0 Hz, 2H, Ar-H3'/H5'), 7.24 (d, *J* = 9.0 Hz, 2H, Ar-H2'/H6'), 7.70(d, *J* = 9.0 Hz, 2H, OCH₂Ar-H3'/H5'), 8.25 (d, *J* = 9.0 Hz, 2H, OCH₂Ar-H2'/H6'); ¹³CNMR (75 MHz, DMSO- d_6) δ 174.27, 164.94, 157.64, 147.77, 147.43, 145.46, 142.60, 129.97, 129.43, 128.68, 124.07, 115.50, 111.76, 68.50, 19.63; ESI-MS m/z: 492.1292 [M+H]⁺, calcd for C₂₆H₂₂NO₉492.1289.

1.1.3.4. 6,6'-((4-Phenethylphenyl)methylene)bis(5-hydroxy-2-methyl-4H-pyran-4one)(4d)

The reaction was performed according to the general procedure C, using **1** (1.00 g, 7.90 mmol) and 4-phenethylbenzaldehyde (0.83 g, 3.95 mmol). ¹² The crude product was recrystallized from isopropanol affording **4d** as a white powder (1.38 g, 79%): mp 202.5-202.9 °C; ¹HNMR (300 MHz, DMSO-*d*₆) δ 2.22 (s, 6H, CH₃), 2.86 (s, 4H, PhCH₂-CH₂Ph), 6.00 (s, 1H, CH-Ar), 6.26 (s, 2H, C=CH), 7.01 (d, *J* = 9.0 Hz, 2H, Ar-H3'/H5'), 7.24 (d, *J* = 9.0 Hz, 2H, Ar-H2'/H6'), 7.14-7.22 (m, 9H,Ar-H), 9.22 (s, 2H, C=C-OH); ¹³CNMR (75 MHz, DMSO-*d*₆) δ 173.91, 165.08, 147.49, 142.35, 141.91, 141.22, 141.22, 134.40, 129.22, 128.78, 128.67, 128.53, 126.29, 111.76, 37.38, 37.63, 19.63; ESI-MS m/z: 445.1 [M+H]⁺; ESI-HRMS m/z: 445.1646 [M+H]⁺, calcd for C₂₇H₂₅O₆ 445.1646.

1.1.3.5.

6, 6'-((4-Propoxyphenyl)methylene)bis(5-hydroxy-2-methyl-4H-pyran-4-one)(4e)

The reaction was performed according to the general procedure C, using 1 (1.00

g, 7.90 mmol) and *p*-butoxybenzaldehyde (0.70 mL, 3.95 mmol). The crude product was recrystallized from isopropanol affording **4e** as a white powder (1.40 g, 89%): mp 252.3-252.7 °C; ¹HNMR (300 MHz, DMSO- d_6) δ 0.98 (t, 3H, J = 7.5 Hz, CH₂CH₃), 1.65-1.77 (m, 2H, OCH₂CH₂CH₃), 2.22 (s, 6H, CH₃), 3.89 (t, 2H, J = 6.6 Hz, OCH₂CH₂), 5.95 (s, 1H, CH-Ar), 6.25 (s, 2H, C=CH), 6.92 (d, J = 8.7 Hz, 2H, Ar-H3'/H5'), 7.20 (d, J = 8.7 Hz, 2H, Ar-H2'/H6'), 9.21 (s, 2H, C=C-OH); ¹³CNMR (75 MHz, DMSO- d_6) δ 173.93, 165.05, 158.42, 147.71, 142.18, 129.81, 128.64, 115.09, 111.73, 69.35, 22.46, 19.63, 10.86; ESI-MS m/z: 399.1 [M+H]⁺; ESI-HRMS m/z: 399.1442 [M+H]⁺, calcd for C₂₂H₂₃O₇ 397.1438.

1.1.3.6.

6,6'-((4-(Allyloxy)phenyl)methylene)bis(5-hydroxy-2-methyl-4H-pyran-4-one)(4f)

The reaction was performed according to the general procedure C, using **1** (1.00 g, 7.90 mmol) and p-(allyloxy)benzaldehyde (0.65 mL, 3.95 mmol). The crude product was recrystallized from isopropanol affording **4f** as a white powder (1.30 g, 84%): mp 232.4-232.8 °C; ¹HNMR (300 MHz, DMSO- d_6) δ 2.22 (s, 6H, CH₃), 4.54 (d, 2H, J = 5.1 Hz, ArOC H_2 -CH=CH₂), 5.25 (dd, 1H, J = 9.0 Hz, J = 5.1 Hz, CH=CH₂), 5.39 (dd, 1H, J = 9.0 Hz, J = 5.1 Hz, CH=CH₂), 5.39 (dd, 1H, J = 9.0 Hz, J = 5.1 Hz, CH=CH₂), 6.25 (s, 2H, C=CH), 6.94 (d, J = 9.0 Hz, 2H, Ar-H3'/H5'), 7.22 (d, J = 9.0 Hz, 2H, Ar-H2'/H6'), 9.21 (s, 2H, C=C-OH); ¹³CNMR (75 MHz, DMSO- d_6) δ 173.93, 165.06, 157.93, 147.67, 142.18, 134.12, 129.82, 128.93, 117.94, 115.34, 111.74, 68.63, 19.63; ESI-MS m/z: 397.1 [M+H]+; ESI-HRMS m/z: 397.1281 [M+H]+, calcd for C₂₂H₂₁O₇ 397.1282

1.2. Bioactivity

1.2.1. Antiproliferation assay

First, the cytotoxicity against HT1080 cell line (obtained from ATCC) and U87 cell line (obtained from ATCC) of the synthetic compounds was studied at concentration gradients. The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine and 100 units/mL penicillin/streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. Cells were

seeded at a density of 5000 cell per well in a 96 well plate for 12 h. Each dose was prepared in DMEM medium by $1000 \times$ dilution of the test compound which was prepared in DMSO solution ensuring that the DMSO concentration is less than 0.1%. Control experiments showed that 0.1% DMSO had no effect on cytotoxicity. Viability was assessed on the basis of cellular conversion of MTT into a formazan product for 4 h. Absorbance was detected by a Gen5 Reader (Bioteck/ Synergy TM HT) at 562 nm. The experiment was performed in 4 replicate wells for each compound or concentration with at least three experimental runs.

1.2.2. Inhibition of D-2HG Production in HT1080 Cells

The D-2HG production inhibition assay followed our previous protocol. In brief, 1 \times 10⁵ HT1080 cells (obtained from ATCC) per well were seeded into wells of a sixwell plate and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% dialyzed fetal bovine serum at 37 °C in a 5% CO₂ atmosphere overnight for cell attachment. Cells were treated with an increasing concentration of compounds in 2 mL of culture medium for 48 h. Cells were collected and washed with PBS thrice. After homogenizing the cells, the mixture was centrifuged to remove any precipitate, and the supernatant was subjected to HPLC-MS (ABI/Sciex API 5000) to separate and quantitate the D-2HG. HPLC was run using a C₁₈ column (COSMOIL) with 0.1% formic acid-water solution and MeOH as an eluent at a flow rate of 0.5 mL/min. Mobile phase buffer A was 0.1% formic acid-water solution. Buffer B was MeOH. HPLC Gradient steps were: 0 min, 10% A, 90% B; 2 min, 50% A, 50% B; 5 min, 50% A, 50% B; 10 min, 95% A, 5% B; 15 min, 95% A, 5% B; 15.1 min, 10% A, 90% B; 25 min, 10% A, 90% B. A single ion monitoring (SIM) for 147 Da was used to detect and quantitate the amount of D-2HG (parameters: interface voltage, -4.2 kV; detector voltage, 1.3 kV; nebulizing gas, 1.5 L/min; drying gas, 15 L/min; desolvation line temperature, 250 °C; heat block temperature, 200 °C; Pirani gauge vacuum, 10² Pa; ion gauge vacuum: 5×10^{-4} Pa). Authentic *D*-2HG purchased from Sigma-Aldrich was used to validate and calibrate the HPLC-MS assay conditions before measuring the D-2HG concentrations secreted by the HT1080 cells untreated or treated with 4a. The injection volume of control group is 1 μ L, while the injection volume of compound 4a

and AGI-5198 groups are 5 μ L. Quantitative calculations are based on the peak area and the standard curve line (Fig. S3 in SI).

1.2.3. Measurement of cell migration

A scratch assay was used to assess the difference in migration distance between non-treated and treated cells. Briefly, 2.5×10^5 cells per well were seeded on a 6-well plate. Two linear scratches were made in each well using a 200 µL sterile pipette tip after removal of medium. Wells were washed with PBS to remove any cellular debris created by the scratch. Cells were then treated with a specific concentration of compound **4a** or DMSO in 2 mL media. A microscope system was used to take pictures in four different fields in each sample every 6 h or 12 h. The scratch area were measured using Image J software.

1.2.4. Measurement of colony-forming ability

For colony forming assays, 200 cells per well were seeded on a 6-well plate in 2 mL media and treated with different concentration of DMSO (control), AGI-5198 (5 μ M and 20 μ M) compound 4a (0.5 μ M and 1 μ M) for 8 days. Then the medium was removed and the cells were washed thrice with pre-cooled PBS. Finally the cells were fixed in 95% pre-cooled MeOH for 15 min with subsequent Giemsa-Wright staining. Colonies were eye-counted at least three times.

1.2.5. Expression/Purification of Homodimeric Mutant IDH1

Mutant human IDH1 genes bearing a His-tag were expressed using Escherichia coli. The genes were subcloned into pET28a to produce the expression constructs. The expressed in Rosetta (DE3)induced by isopropyl-Denzyme was thiogalactopyranoside (1 mM). The cleaved proteins were separated through a nickel-NTA column (GE Healthcare) and eluted with a step gradient of imidazole Buffer (50, 100, 250, and 500 mM). The purified protein fractions were filtered and stored in 50 mM Tris,50 mM NaCl, 1 mM DTT, 0.5 mM EDTA, and 10% glycerol, pH 7.5. The purities were verified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Fig. **S1**)

1.2.6. Mutant IDH1 inhibitory assay.

Reaction buffer (50 mM Tris, 200 mM NaCl , 5 mM β -mercaptoethanol, 2 mM

MgSO₄, 10% glycerinum), compounds and enzyme were added to a 96 well plate to a total volume of 240 μ L for an incubation lasting 30 min at room temperature. Then 96 mM MgCl₂, 1 mg/ μ L NADPH, and 3.51 μ g/ μ L α -KG were subsequently added for another incubation lasting 1 h at 37 °C. Finally, absorption at 340 nm was detected using a microplate-reader (Biotek/ Synergy TM HT). Parallel triplicate repetition for each dose was made.

References

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- (a) Kantam, M. L.; Chakravarti, R.; Chintareddy, V. R.; Sreedhar, B.; Bhargava, S., Adv. Synth. Catal., 2008, **350**, 2544-2550; (b) Luo, B.; Wang, J.; Ge, D.; Li, X.; Cao, X.; Pan, Y.; Gu, H., Sci. China Chem., 2014, **57**, 1310-1314.
- (a) Moreno, L.; Parraga, J.; Galan, A.; Cabedo, N.; Primo, J.; Cortes, D., Bioorg. Med. Chem. 2012, 20, 6589-6597; (b) Kurosawa, W.; Kan, T.; Fukuyama, T., J. Am. Chem. Soc. 2003, 125, 8112-8113.

¹H NMR spectrum of compound 2a



¹³C NMR spectrum of compound 2a



220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -1 f1 (ppm)

LRMS of compound 2a



HRMS of compound 2a



¹H NMR spectrum of compound 2b



¹³C NMR spectrum of compound 2b



LRMS of compound 2b



HRMS of compound 2b

	Best	Formula (M)	Ion Formula	Score	Cross Score	Calc m/z	Diff (ppm)	Mass Match	Abund Match	Spacing Match
		C13 H11 F O4	C13 H12 F O4	86.16		251.0714	1.53	98.75	93.1	52.64
	Isotope	Abund Sum%	Calc Abund Sum%	m/z	Calc m/z /	Diff (ppm)				
	1	83.74	86.11	251.071	251.0714	1.65				
	2	14.29	12.36	252.0808	252.0748	-23.81				
	3	1.96	1.53	253.0835	253.077	-25.6				
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¹H NMR spectrum of compound 2c



¹³C NMR spectrum of compound 2c



LRMS of compound 2c



HRMS of compound 2c

	Best	Formula (M)	Ion Formula	Score	Cross Score	Calc m/z	Diff (ppm)	Mass Match	Abund Match	Spacing Match
	V	C13 H11 CI O4	C13 H12 CI O4	86.4		267.0419	0.63	99.77	98.29	45.37
	Isotope	Abund Sum%	Calc Abund Sum%	m/z	Calc m/z /	Diff (ppm)				
	1	64.88	65.41	267.0418	267.0419	0.13				
	2	9.33	9.39	268.0415	268.0453	13.98				
	3	22.9	22.09	269.0364	269.0394	10.93				
	4	2.9	3.11	270.0406	270.0426	7.36				
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¹H NMR spectrum of compound 2d



¹³C NMR spectrum of compound 2d



220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -1 f1 (ppm)

LRMS of compound 2d



HRMS of compound 2d



¹H NMR spectrum of compound 2e



¹³C NMR spectrum of compound 2e



LRMS of compound 2e



HRMS of compound 2e



¹H NMR spectrum of compound 3a

¹³C NMR spectrum of compound 3a

LRMS of compound 3a

HRMS of compound 3a

	Best	Formula (M)	Ion Formula	Score	Cross Score	Calc m/z	Diff (ppm)	Mass Match	Abund Match	Spacing Match
		C13 H12 O3	C13 H13 O3	91.36		217.0859	1.15	99.4	97.73	67.61
	Isotope	Abund Sum%	Calc Abund Sum%	m/z	Calc m/z /	Diff (ppm)				
	1	86.84	86.29	217.0857	217.0859	1.12				
	2	11.33	12.36	218.0862	218.0893	14.38				
	3	1.84	1.35	219.0909	219.0917	3.86				
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¹H NMR spectrum of compound 3b

¹³C NMR spectrum of compound 3b

LRMS of compound 3b

HRMS of compound 3b

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¹H NMR spectrum of compound 3c

¹³C NMR spectrum of compound 3c

LRMS of compound 3c

HRMS of compound 3c

	Best	Formula (M)	Ion Formula	Score	Cross Score	Calc m/z	Diff (ppm)	Mass Match	Abund Match	Spacing Match
-		C13 H11 CI O3	C13 H12 CI O3	95.89		251.0469	3.76	92.68	98.82	98.79
	Isotope	Abund Sum%	Calc Abund Sum%	m/z	Calc m/z	Diff (ppm)				
	1	64.86	65.32	251.046	251.0469	3.65				
	2	8.84	9.35	252.0494	252.0503	3.85				
	3	21.99	21.92	253.0429	253.0444	6.07				
	4	3.47	3.07	254.0458	254.0476	7.05				
	5	0.83	0.33	255.054	255.0499	-15.96				
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¹H NMR spectrum of compound 3d

¹³C NMR spectrum of compound 3d

LRMS of compound 3d

HRMS of compound 3d

Best	Formula (M)	Ion Formula	Score	Cross Score	Calc m/z	Diff (ppm)	Mass Match	Abund Match	Spacing Ma
	C14 H11 F3 O3	C14 H12 F3 O3	83.76		285.0733	0.61	99.77	75.36	6
Isotope	Abund Sum%	Calc Abund Sum%	m/z	Calc m/z /	Diff (ppm)				
. 1	79.9	85.39	285.0731	285.0733	0.58				
2	16.49	13.14	286.0755	286.0767	4.29				
3	3.62	1.47	287.0894	287.0792	-35.73				
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¹H NMR spectrum of compound 3e

¹³C NMR spectrum of compound 3e

LRMS of compound 3e

HRMS of compound 3e

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	323.1276	(M+H)+	C20 H19 O4	22674.5						
	Best	Formula (M)	Ion Formula	Score	Cross Score	Calc m/z	Diff (ppm)	Mass Match	Abund Match	Spacing Match
		C20 H18 O4	C20 H19 O4	47.51		323.1278	0.58	99.76	0	0
	Isotope	Abund Sum%	Calc Abund Sum%	m/z	Calc m/z /	Diff (ppm)				
	1	100	100	323.1276	323.1278	0.57				
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¹H NMR spectrum of compound 4a

¹³C NMR spectrum of compound 4a

LRMS of compound 4a

HRMS of compound 4a

	Best	Formula (M)	Ion Formula /	Score	Cross Score	Calc m/z	Diff (ppm)	Mass Match	Abund Match	Spacing Match
-		C26 H22 O7	C26 H23 O7	71.36		447.1438	-0.05	100	41.47	49.95
	Isotope	Abund Sum%	Calc Abund Sum%	m/z	Calc m/z /	Diff (ppm)				
	1	72.85	77.73	447.1438	447.1438	-0.04				
	2	27.15	22.27	448.1473	448.1472	-0.28				
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¹H NMR spectrum of compound**2b**

¹³C NMR spectrum of compound**2b**

LRMS of compound 2b

HRMSof compound2b

	Best	Formula (M)	Ion Formula	Score	Cross Score	Calc m/z	Diff (ppm)	Mass Match	Abund Match	Spacing Match
3	V	C27 H24 O8	C27 H25 O8	93.96		477.1544	1.63	97.29	88.64	93.7
	Isotope	Abund Sum%	Calc Abund Sum%	m/z	Calc m/z /	Diff (ppm)				
	1	71.43	73.68	477.1537	477.1544	1.54				
	2	24.33	21.95	478.156	478.1578	3.76				
	3	4.24	4.37	479.1577	479.1605	5.72				
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¹H NMR spectrum of compound 4c

¹³C NMR spectrum of compound 4c

HRMS of compound 4c

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Best	Formula (M)	Ion Formula	Score	Cross Score	Calc m/z	Diff (ppm)	Mass Match	Abund Match	Spacing Match
	C26 H21 N O9	C26 H22 N O9	88.21		492.1289	-0.2	99.96	97.58	53.45
Isotope	Abund Sum%	Calc Abund Sum%	m/z	Calc m/z /	Diff (ppm)				
1	71.97	73.58	492.1292	492.1289	-0.58				
2	22.47	21.4	493.1262	493.1322	12.16				
3	4.5	4.36	494.1314	494.1348	6.89				
4	1.05	0.66	495.1414	495.1374	-8.03				

¹H NMR spectrum of compound 4d

¹³C NMR spectrum of compound 4d

LRMS of compound 4d

HRMS of compound 4d

¹H NMR spectrum of compound 4e

¹³C NMR spectrum of compound 4e

LRMS of compound 4e

HRMS of compound 4e

¹H NMR spectrum of compound 4f

¹³C NMR spectrum of compound 4f

LRMS of compound 4f

HRMS of compound 4f

Gan S Formula Results: + Scan (0.710 min) Ion Formula Formula (M) Diff(ppm) Mass Match Abund Match Spacing Match 0.15 99.98 84.38 48.16 Best Score Cross Score Calc m/z C22 H20 O7 C22 H21 O7 83.19 ė V 397.1282 48.16 Isotope Abund Sum% Calc Abund Sum% Calc m/z Diff (ppm) m/z 74.49 77.45 397.1281 397.1282 0.24 18.25 18.82 398.1332 398.1316 -4.17 2 3 6.29 3.3 399.1476 399.1341 -33.7 🗿 Method Editor: Generate Formulas 🖓 MS Formula Results: + Scan (0.710 min) 🛛 Mass Calculator 🔥 Chromatogram Results III SSpectrum Results ư ↔ ‡ | Q 🛨 🚧 🕊 🛧 🛝 🔊 C ₃ 🔹 👖 隊 % 🧏 🎒 +ESI Scan (0.710 min) Frag=175.0V YB-125.d x10¹ 397. 1281 3 (M+H) + 2.8-2.6-2.4 2.2-2 1.8-1.6-1.4-1.2 398, 1332 0.8-(M+H) + 0.6 0.4 0.2 401 402 403 404 405 406 407 408 409 410 411 412 Counts (%) vs. Mass-to-Charge (m/z) 0-413 396 395 397 398 399 400 393 394

Figure S1. Protein electrophoresis of purified mutant IDH1. 10 μ l for each fraction to test by SDS-PAGE gel

Figure S2. Enzyme activity of purified mutant IDH1 (R132H). Determination of the activity is based on the consumption of NADPH (OD_{340}) in the reaction.

Figure S3. Standard curve line of *D*-2HG. The The concentration of the standard samples are 4.8 µg/ml, 48µg/ml, 105.6µg/ml, 232.3µg/ml, and 511.104µg/ml.