Supporting Information

Discovery and SAR research for Anti-virus activity of novel butenolide on influenza A virus H1N1 in vitro and in Vivo

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Outline

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1. General informations for chemistry

Reagents and solvents were purchased from Bide Pharmatech Ltd, Aladdin, Sinopharm Chemical Reagent Co, Ltd. with purities of at least 97%. 1H NMR (400 MHz) and 13C NMR (100 MHz) spectra were recorded with a Bruker spectrometer. HRMS was carried on Thermo Q Exactive Ultimate 3000. The melting points for the synthetic compounds were determined onXT5A Micro digital melting point measuring instrument(Beijing Electrotoptic Instrument Company). All reactions were monitored by thin-layer chromatography (TLC) on 25.4 mm × 76.2 mm silica gel platesGF-254) and UPLC-Mass on Waters ACQUITY UPLC H-Class. The silica gel used for column chromatography was 200-300 mesh or recrystallization with solvents specified in the corresponding experiments.HPLC was used to determine the purity of the active compound with more than 96% on Waters e2695 Separations Module 2489 UV/Vis Deteco (Chromatographycolumn: Agilent columnC18, 4 μM, 4.6 mm x 250 mm); Mobile phase: methanol/water(50/50); The detection wavelength: 254nm; The flow rate: 1mL/Min; The column temperature:30°C.

1.1 General Synthesis Procedure for 2-ethoxy-2-oxoethyl-2-phenylacetate (3)

Triethylamine (1.79 g,17.6 mmol) and ethyl chloroacetate (2.16 g, 17.6 mmol) were added to the solution of phenylacetic acid (2.00 g,14.7 mmol) in dry THF (10 mL). The reaction mixture was stirred for 6-7 hour at about 75°C. The reaction was monitored using TLC. After reaction completed, the solid was filtered off, and the solvent was removed on rotary evaporator. The residue was then diluted with H2O (30 ml) and extracted by diethyl ether (3*15 mL). The organic phase was dried over anhydrous Na2SO4, and the solvent removed. The crude product was not purified and applied directly to the next step.

1.2 General Synthesis Procedure for 4-hydroxy-3-phenylfuran-2(5H)-ones (4-12)

Potassium tert-butoxide (2.2 g, 28.8 mmol) was added into the solution of compound 3 (3.20 g,14.4 mmol) in dry DMF (15 mL) in six portions over 0.5 hours at °C. The reaction mixture was then warmed to room temperature, stirred for another 2-4 hours. After the reaction completed, the mixture was added 15 mL water and acidified by diluted hydrochloric acid to pH of 3-4. Then 200 mL water was added to the solution, the precipitate yielded and was filtered and dried to give the target compounds.

1.3 General Synthesis Procedure for 4-methoxy-3-phenylfuran-2(5H)-ones (13-21) with compound 13 as sample

To the solution of compound 4 (150 g,8.52mmol), inacetone (15 ml), anhydrous potassium carbonate (1.525 g, 11.1 mmol) was mixed at room temperature. Then a solution of dimethyl sulfate (1.29 g,10.24mmol) in acetone (10 mL) was added in dropwise. The reaction was monitored by TLC. After the reaction completed, the solvent was removed under reduced pressure. Then 25 mL water was added to dissolve the excess anhydrous potassium carbonate, the precipitate was filtered of and the solvent was removed. The residue was purified by column chromatography (PE: EA=4:1) to give the title compounds

1.4 General Synthesis Procedure for 3,4-dimethoxy-4-methyl-butenolides (22~30) with compound 22 as sample

To a solution of compound 13 (2.10 mmol) in CH2OH (2 mL) was added (CH3)2O (37.8 mg, 1.26 mmol) and CH3ONa (34.05 mg, 0.630 mmol). The mixture system was heated at 75°C. (CH3)2O (18.9 mg, 0.63 mmol) and 0.3N CH3ONa (34.05 mg, 0.630 mmol) were added into the reaction after 8 hours. The reaction was monitored by TLC. After the reaction completed, then volatiles were removed under reduced pressure, and the residue was partitioned between ethyl acetate (3*15 mL) and water (20 mL). The organic phase was dried over anhydrous Na2SO4, and the solvent removed. The crude product was not purified and applied directly to the next step.

1.5 3,4-dimethoxy-4-methyl-2-phenylbutenolide (22)

Yield 40.3% as a white solid. m.p.185.7-186.8°C; 1H NMR (400 MHz, CDCl3) δ 7.53 (m, 2H), 7.41 – 7.33 (m, 3H), 3.86 (s, 3H), 3.34 (s, 3H), 1.76 (s, 3H). 13C NMR (100 MHz, CDCl3) δ 169.86,169.68, 129.90, 128.86, 128.39, 128.21, 105.71, 103.24, 60.08, 51.00, 22.80. HRMS: Calcd for C13H15O4 [M+H]+: 235.09703, found:235.09483.

1.6 2-(4-fluorophenyl)-3, 4-dimethoxy-4-butenolide (23)

Yield 50.9% as a white solid. m.p.174.3-174.8°C. 1H NMR (400 MHz, CDCl3) δ 7.58 – 7.56 (m, 2H), 7.11–7.07 (m, 2H), 3.95 (s, 3H), 3.34 (s, 3H), 1.77 (s, 3H). 13C NMR (100 MHz, CDCl3) δ 169.86,169.45, 162.5 (d, J=247 Hz). 131.42 (d, J=8.1Hz), 124.82 (d, J=3.4 Hz). 115.31(d, J = 22 Hz), 104.76, 103,21, 59.81, 51.05, 22.87. HRMS: Calcd for C13H14FO3 [M+H]+: 253.08761, found: 253.08660.

1.7 2-(4-chlorophenyl)-3, 4-dimethoxy-4-butenolide (24)

Yield 49.8% as a white solid. m.p.189.6-190.7°C. 1H NMR (400 MHz, DMSO) δ 7.62 (d, J = 8.1 Hz, 2H), δ 7.49 (d, J = 8.1 Hz, 2H), 3.93 (s, 3H), 3.24 (s, 3H), 1.72 (s, 3H). 13C NMR (100 MHz, DMSO) δ 170.11, 168.52, 132.73, 131.25, 128.05, 127.86, 103.42, 103.00, 60.10, 50.65, 22.60. HRMS: Calcd for C13H14ClO4 [M+H]+: 269.05806/271.05511, found:269.05825/271.05410.

S2
1.8 2-(4-bromophenyl)-3, 4-dimethoxy-4-methylbutenolide (25)

Yield 50.2 % as a white solid; m.p. 251.9-253.3°C; 1H NMR (400 MHz, CDCl3) δ 7.53 (d, J = 8.1 Hz, 2H), δ 7.49 (d, J = 8.1 Hz, 2H), 3.97 (s, 3H), 3.33 (s, 3H), 1.77 (s, 3H). 13C NMR (100 MHz, CDCl3) δ 170.21, 169.07, 131.43, 131.06, 127.79, 122.55, 104.62, 103.19, 59.85, 51.09, 22.88. HRMS: Calcd for C19H14BrO4[M+H]+: 313.00755/315.00550, found:313.00497/315.00262.

1.9 3, 4-dimethoxy-4-methyl-2-pentanone (26)

Yield 52.4 % as a white solid; m.p. 212.6-213.1°C; 1H NMR (400 MHz, DMSO) δ 7.42 (d, J = 8.1 Hz, 2H), 7.22 (d, J = 8.1 Hz, 2H), 3.86 (s, 3H), 3.33 (s, 3H), 1.76 (s, 3H). 13C NMR (100 MHz, DMSO) δ 169.30, 168.97, 134.75, 129.62, 125.82, 125.97, 104.62, 102.86, 59.97, 50.48, 22.61, 20.79.

1.10 3, 4-dimethoxy-4-phenylpentanone (27)

Yield 48.6 % as a white solid; m.p. 165.8-166.4°C; 1H NMR (400 MHz, CDCl3) δ 7.47 (d, J = 8.7 Hz, 2H), 6.93 (d, J = 8.8 Hz, 2H), 3.91 (s, 3H), 3.82 (s, 3H), 3.33 (s, 3H), 1.75 (s, 3H). 13C NMR (100 MHz, CDCl3) δ 169.97, 169.19, 159.56, 131.03, 120.97,113.72, 105.46, 103.19, 59.83, 55.30, 50.97, 22.86. HRMS: Calcd for C19H14O4[M+H]+: 265.10760, found:265.10519.

1.11 2-(3-fluorophenyl)-3, 4-dimethoxy-4-methylbutenolide (28)

Yield 46.2 % as a white solid; m.p. 198.1-198.8°C; 1H NMR (400 MHz, CDCl3) δ 7.43 – 7.35 (m, 3H), 7.04 (t, J=8-Hz, 1H), 4.00 (s, 3H), 3.34 (s, 3H), 1.79 (s, 3H). 13C NMR (100 MHz, CDCl3) δ 170.41, 168.97, 162.40 (d, J=244 Hz), 130.85 (d, J=8.6 Hz), 129.73 (d, J=8.4 Hz), 125.23 (d, J=3 Hz), 116.47 (d, J=22 Hz), 115.32 (d, J=21 Hz), 104.59 (d, J=2 Hz), 103.12, 59.82, 51.10, 22.93. HRMS: Calcd for C19H14F3O4[M+H]+: 253.08761, found: 253.08662.

1.12 2-(2-fluorophenyl)-3, 4-dimethoxy-4-methylbutenolide (29)

Yield 47.5 % as light yellow solid; m.p.176.7-177.9°C; 1H NMR (400 MHz, DMSO) δ 7.52 – 7.47 (m, 2H), 7.33 – 7.26 (m, 2H), 3.77 (s, 3H), 3.23 (s, 3H), 1.66 (s, 3H). 13C NMR (100 MHz, DMSO) δ 171.00, 168.33, 159.92 (d, J=244 Hz), 132.97 (d, J=2.2 Hz), 131.07 (d, J=8.1 Hz), 124.21 (d, J=3.4 Hz), 116.84 (d, J=16 Hz), 115.34 (d, J=21 Hz), 103.15, 98.4, 59.9, 50.4, 22.3. HRMS: Calcd for C19H14F3O4[M+H]+: 253.08761, found: 253.08662.

1.13 2-(3,4-difluorophenyl)-3, 4-dimethoxy-4-methylbutenolide (30)

Yield 44.3 % as light yellow solid; m.p.187.3-188.6°C; 1H NMR(400 MHz,CDCl3) δ 7.60–7.55 (m,1H), 7.49–7.46 (m, 1H), 7.21–7.15 (m, 1H), 3.97 (s, 3H), 3.36 (s, 3H), 1.79 (s, 3H). 13C NMR (100 MHz, CDCl3) δ 170.30, 168.75, 151.39–148.62(overlap,2c), 125.76–125.48 (overlap, 2c),118.31–117.01 (overlap, 2c), 103.79, 103.04, 59.53, 51.15, 23.04. HRMS: Calcd for C19H12F4O4[M+H]+:271.07819, found: 271.07595.

1.14 Synthetic procedure for 3-chloro-2-(4-fluorophenyl)butenolide (31)

Compound 9 (7.73mmol) was dissolved in DMF (5 mL) and CH2Cl2 (5 mL) at 0°C. 1.18 g (6.44 mmol) Oxaly chloride was added dropwise to the reaction system, and then the mixture was stirred for 7 h at room temperature. After the reaction completed, the system was alkalized to neutral via NaHCO3 solution, and the reaction system was partitioned between ethyl acetate (20 mL) and water (20 mL). The organic phase was dried over anhydrous Na2SO4, filtered, and concentrated under reduced pressure. The residue was subjected to silica gel column chromatography to yield 95% of compound 31 as a red solid.

1.15 Synthetic Procedure for 2-(4-methoxyphenyl)-3-morpholinobutenolide (32)

Compound 31 was dissolved in THF (2 mL), and K2CO3 (778 mg, 5.64 mmol) was added to the solution at room temperature. Then 492 mg (5.64 mmol) morpholine was added dropwise to reaction system, and the mixture was stirred for 3h. Then volatiles were removed under reduced pressure, and the residue was partitioned between ethyl acetate (20 mL) and water (20 mL). The organic phase was dried over anhydrous Na2SO4, filtered, and concentrated under reduced pressure. The residue was subjected to silica gel column chromatography to yield 32 as a white solid.

1.16 Synthetic Procedure for 2-(4-bromophenyl)-4-methyl-3-morpholinobutenolide (37)

To a solution of 32 (1.52 mmol) in CH3OH (2 mL) was added (CH3)2O (27.3 mg, 0.91 mmol) and CH3ONa (24.6 mg, 0.45 mmol). The mixture system was heated at about 75°C. Then (CH3)2O (13.6 mg, 0.45 mmol) and CH3ONa (24.6 mg, 0.45 mmol) were added into the reaction after 8 hours. The reaction was monitored by TLC. After the reaction completed, then volatiles were removed under reduced pressure, and the residue was partitioned between ethyl acetate (3*15 ml) and water (20 ml). The organic phase was dried over anhydrous Na2SO4, and the solvent was removed. The residue was purified by column chromatography to give compound 36 in 38.9% yield as a white solid. m.p. 153.6-154.0°C; 1H NMR (400 MHz, CDCl3) δ 7.20 (d, J = 8.6 Hz, 2H), 6.91 (d, J = 8.7 Hz, 2H), 3.82 (s, 3H), 3.71 – 3.60 (m, 4H), 3.54-3.45 (m, 2H), 3.35 (s, 3H), 3.32-3.27 (m, 2H), 1.79 (s, 3H). 13C NMR (100 MHz, CDCl3) δ 170.74,
3.53 - 3.47 (m, 2H), 3.35 (s, 3H), 3.33 - 3.27 (m, 2H), 2.37 (s, 3H), 1.80 (s, 1H). 13C NMR (100 MHz, CDCl3) δ 170.74, 159.04, 137.47, 130.30, 128.98, 128.83, 103.56, 99.23, 66.75, 50.83, 48.26, 24.93, 21.23. HRMS: Calcd for C12H14O3N+ [M+H]+: 304.15488, found: 304.15685

2-(3-fluorophenyl)-4-methoxy-4-methyl-3-morpholinobutenolide (60)

m.p.168.3-169.4°C; 1H NMR (400 MHz, CDCl3) δ 7.28-7.23 (overlapping, 1H), 3.72-3.62 (m, 4H), 3.53-3.47 (m, 2H), 3.35 (s, 3H), 3.33-3.27 (m, 2H), 2.37 (s, 3H), 1.80 (s, 1H). 13C NMR (100 MHz, CDCl3) δ 170.74, 159.04, 137.47, 130.30, 128.98, 128.83, 103.56, 99.23, 66.75, 50.83, 48.26, 24.93, 21.23. HRMS: Calcd for C12H14O3N+ [M+H]+: 304.15488, found: 304.15685

1.16 3-(4-chlorophenyl)-5-((4-chlorophenyl)(hydroxy)methyl)-4-methoxy-4-methylbutenolide (45)

To solution of compound 44 (300 mg, 0.9 mmol) in CH2OH (2 mL) was added CH2ONa (58.84 mg, 1.01 mmol) and 4-chlorobenzaldehyde (136.5 mg, 1.1 mmol) at 0°C. The resulting mixture was stirred at 0°C for 30 min, then warmed to room temperature for 4 h. After workup, chromatography with EA in PE gave compound 45 as a white solid (45.4%). 1H NMR (400 MHz, CDCl3) δ 7.51 (d, J = 8.2 Hz, 2H), 7.42 (d, J = 8 Hz, 2H), 7.36 (d, J = 8 Hz, 2H), 7.14 (d, J = 8.2 Hz, 2H), 4.81 (s, 1H), 3.76 (s, 3H), 2.42 (s, 1H), 1.41 (s, 3H). 13C NMR (100 MHz, CDCl3) δ 175.40, 136.94, 134.72, 132.76, 132.16, 131.40, 129.25, 128.67, 122.67, 118.34, 84.61, 76.86, 61.50, 61.11, 30.91, 20.43. UPLC-MS (ESI) Calcd for C19H16ClNO4 [M+H]+: 379.0503/381.04744, found:379.0403/381.04745

1.17 2-(4-chlorophenyl)-4-(hydroxyp-toly)methyl)-3-methoxy-4-methylbutenolide (46)

Compound 46 as a white solid was prepared with yield of 48.6% from compound 44 (800 mg, 3.35 mmol) and p-toluualdehyde (370.6 mg, 4.02 mmol) by the same procedure as described for compound 45. 1H NMR (400 MHz, CDCl3) δ 7.53 (d, J = 8.5 Hz, 2H), 7.43 (d, J = 8.5 Hz, 2H), 7.38-7.35 (m, 2H), 7.10 (t, J = 8.6 Hz, 2H), 5.63 (s, 1H), 3.80 (s, 3H), 2.71 (s, 3H), 1.35 (s, 3H). 13C NMR (100 MHz, CDCl3) δ 173.32, 171.69, 161.53, 136.32, 132.62, 132.53, 131.50, 130.41, 129.23, 125.17 (d, J = 3.6 Hz), 115.49, 115.28, 104.00, 83.48, 81.39, 61.25, 39.57, 20.14. UPLC-MS (ESI) Calcd for C29H23ClO4 [M+H]+: 359.1050/361.10206, found: 359.1050/361.10209

1.18 2-(4-chlorophenyl)-4-(hydroxy(pyridin-2-yl)methyl)-3-methoxy-4-methylbutenolide (47)

Compound 47 as a white solid (2:1) was prepared with yield of 50.9% from compound 44 (800 mg, 3.35 mmol) and 2-pyridine aldehyde (438.1 mg, 4.02 mmol) by the same procedure as described for compound 45. 1H NMR (400 MHz, CDCl3) δ 8.60 (d, J = 4 Hz, 1.5H), 7.74-7.89 (m, 1.5H), 7.39 (d, J = 7.8 Hz, 1H), 7.33-7.28 (m, 3H), 7.26-7.24 (m, 1H), 7.01 (d, J = 8 Hz, 1H), 6.87(d, J = 12 Hz, 2H), 4.92-4.89 (m, 1.5H), 4.74 (d, J = 12 Hz, 1H), 4.62 (d, J = 8 Hz, 0.5H), 3.74 (s, 1.5H), 3.64 (s, 3H), 1.76 (s, 3H), 1.69 (s, 1.5H). HRMS Calcd for C19H17ClNO5 [M+H]+: 346.08461/348.08166, found:346.08671/348.08691

1.19 2-(4-chlorophenyl)-4-(morpholinophenyl)methyl)-3-methoxy-4-methylbutenolide (48)

Compound 48 as a white solid was prepared with yield of 56.7% (isomers:1:1) from compound 44 (800 mg, 3.35 mmol) and 4-morpholinobenzaldehyde (386.5 mg, 4.02 mmol) by the same procedure as described for compound 45. 1H NMR (400 MHz, CDCl3) δ 7.35 (d, J = 8.4 Hz, 1H), 7.26-7.21 (overlapping, 3H), 7.21 - 7.14 (m, 1H), 7.08 (d, J = 8 Hz, 0.5H), 7.02 (d, J = 12, 4 Hz, 0.5H), 6.91 (t, J = 8 Hz, 1H), 6.80 (d, J = 8.4 Hz, 1H), 4.79-4.74 (overlapping, 1H), 3.92 - 3.84 (overlapping, 4H), 3.76 (s, 1.5H), 3.61 (s, 1.5H), 3.12 - 3.04 (overlapping, 4H), 2.69 (d, J = 4 Hz, 0.5H), 2.38 (d, J = 4 Hz, 0.5H), 1.73 (s, 1.5H), 1.40 (s, 1.5H). 13C NMR (100 MHz, CDCl3) δ 175.60, 175.07, 171.98, 171.42, 156.49, 156.29, 154.04, 153.84, 140.30, 140.22,
| Mass Value | Mass Value | Mass Value | Mass Value | Mass Value | Mass Value | Mass Value | Mass Value | Mass Value | Mass Value | Mass Value | Mass Value | Mass Value | Mass Value | Mass Value | Mass Value | Mass Value | Mass Value | Mass Value |
|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| 140.18     | 140.09     | 134.51     | 134.43     | 133.01     | 132.94     | 132.78     | 132.00     | 131.71     | 128.41     | 128.31     | 128.24     | 128.82     | 125.00     | 124.25     | 124.22     | 122.74     | 122.71     | 118.84     | 118.27     | 115.84     |
| 115.62     | 114.78     | 103.52     | 103.37     | 103.11     | 85.11      | 75.61      | 66.91      | 61.09      | 60.79      | 50.79      | 50.76      | 50.72      | 20.50      | 19.81      |            |            |            |            |            |            |            |

UPLC-MS (ESI)
Calcd for C$_{23}$H$_{25}$ClNO$_{5}$$^{[M+H]^+}$: 430.14213/432.13918, found: 430.14125/432.13591
2. $^1$H NMR, $^{13}$C NMR spectra of synthetic compounds

Figure S1. $^1$H NMR of 3,4-dimethoxy-4-methyl-2-phenylbutenolide (22)

Figure S2. $^{13}$C NMR of 3,4-dimethoxy-4-methyl-2-phenylbutenolide (22)
Figure S3. $^1$H NMR of 2-(4-fluorophenyl)-3, 4-dimethoxy-4-butenolide (23)

Figure S4. $^{13}$C NMR of 2-(4-fluorophenyl)-3, 4-dimethoxy-4-butenolide (23)
Figure S5. $^1$H NMR of 2-(4-chlorophenyl)-3, 4-dimethoxy-4-methylbutenolide(24)

Figure S6. $^{13}$C NMR of 2-(4-chlorophenyl)-3, 4-dimethoxy-4-methylbutenolide(24)
Figure S7. $^1$H NMR of 2-(4-bromophenyl)-3, 4-dimethoxy-4-methylbutenolide(25)

Figure S8. $^{13}$C NMR of 2-(4-bromophenyl)-3, 4-dimethoxy-4-methylbutenolide(25)
Figure S9. $^1$H NMR of 3, 4-dimethoxy-4-methyl-2-(p-tolyl) butenolide(26)

Figure S10. $^{13}$C NMR of 3, 4-dimethoxy-4-methyl-2-(p-tolyl) butenolide(26)
Figure S11. $^1$H NMR of 3, 4-dimethoxy-2-(4-methoxyphenyl)-4-butenolide(27)

Figure S12. $^{13}$C NMR of 3, 4-dimethoxy-2-(4-methoxyphenyl)-4-butenolide(27)
Figure S13. $^1$H NMR of 2-(3-fluorophenyl)-3, 4-dimethoxy-4-methylbutenolide (28)

Figure S14. $^{13}$C NMR of 2-(3-fluorophenyl)-3, 4-dimethoxy-4-methylbutenolide (28)
Figure S15. $^1$H NMR of 2-(2-fluorophenyl)-3, 4-dimethoxy-4-methylbutenolide (29)

Figure S16. $^{13}$C NMR of 2-(2-fluorophenyl)-3, 4-dimethoxy-4-methylbutenolide (29)
Figure S17. $^1$H NMR of 2-(3,4-difluorophenyl)-3,4-dimethoxy-4-methylbutenolide(30)

Figure S18. $^{13}$H NMR of 2-(3,4-difluorophenyl)-3,4-dimethoxy-4-methylbutenolide(30)
Figure S19. $^1$H NMR of 2-(4-methoxyphenyl)-4-methoxy-4-methyl-3-morpholinobutenolide (37)

Figure S20. $^{13}$C NMR of 2-(4-methoxyphenyl)-4-methoxy-4-methyl-3-morpholinobutenolide (37)
Figure S21. $^1$H NMR OF 4-methoxy-2-(4-methoxyphenyl)-4-methyl-3-thiomorpholinobutenolide(38)

Figure S22. $^{13}$C NMR OF 4-methoxy-2-(4-methoxyphenyl)-4-methyl-3-thiomorpholinobutenolide(38)
Figure S23. $^1$H NMR of 3-(4-hydroxypiperidin-1-yl)-4-methoxy-2-(4-methoxyphenyl)-4-methylbutenolide (39)

Figure S24. $^{13}$C NMR of 3-(4-hydroxypiperidin-1-yl)-4-methoxy-2-(4-methoxyphenyl)-4-methylbutenolide (39)
Figure S25. $^1$H NMR of 4-(3-hydroxypiperidin-1-yl)-5-methoxy-3-(4-methoxyphenyl)-5-methyl butenolide (40)

Figure S26. $^{13}$C NMR of 4-(3-hydroxypiperidin-1-yl)-5-methoxy-3-(4-methoxyphenyl)-5-methyl butenolide (40)
Figure S27. $^1$H NMR of 4-methoxy-2-(4-methoxyphenyl)-4-methyl-3-((4-methylbenzyl)amino)butenolide (41)

Figure S28. $^{13}$C NMR of 4-methoxy-2-(4-methoxyphenyl)-4-methyl-3-((4-methylbenzyl)amino)butenolide (41)
Figure S29. $^1$H NMR of 3-(4-chlorophenyl)-5-((4-chlorophenyl)(hydroxy)methyl)-4-methoxy-4-methyl butenolide (45)

Figure S30. $^{13}$C NMR of 3-(4-chlorophenyl)-5-((4-chlorophenyl)(hydroxy)methyl)-4-methoxy-4-methylbutenolide (45)
Figure S31. $^1$H NMR of 2-(4-chlorophenyl)-4-(hydroxy(p-tolyl)methyl)-3-methoxy-4-methylbutenolide (46)

Figure S32. $^{13}$C NMR of 2-(4-chlorophenyl)-4-(hydroxy(p-tolyl)methyl)-3-methoxy-4-methylbutenolide (46)
Figure S33. $^1$H NMR of 2-(4-chlorophenyl)-4-(hydroxy(pyridin-2-yl)methyl)-3-methoxy-4-methylbutenolide (47)

Figure S34. $^{13}$C NMR of 2-(4-chlorophenyl)-4-(hydroxy(pyridin-2-yl)methyl)-3-methoxy-4-methylbutenolide (47)
Figure S35. $^1$H NMR of 2-(4-chlorophenyl)-4-(hydroxy(4-morpholinophenyl)methyl)-3-methoxy-4-methylbutenolide (48)

Figure S36. $^{13}$C NMR of 2-(4-chlorophenyl)-4-(hydroxy(4-morpholinophenyl)methyl)-3-methoxy-4-methylbutenolide (48)
Figure S37. $^1$H NMR of 2-(4-fluorophenyl)-4-methoxy-4-methyl-3-morpholinobutenolide (57)

Figure S38. $^{13}$C NMR of 2-(4-fluorophenyl)-4-methoxy-4-methyl-3-morpholinobutenolide (57)
Figure S39. $^{1}$H NMR of 2-(4-chlorophenyl)-4-methoxy-4-methyl-3-morpholinobutenolide(58)

Figure S40. $^{13}$C NMR of 2-(4-chlorophenyl)-4-methoxy-4-methyl-3-morpholinobutenolide(58)
Figure S41. $^1$H NMR of 4-methoxy-4-methyl-3-morpholino-3-(p-tolyl)butenolide (59)

Figure S42. $^{13}$C NMR of 4-methoxy-4-methyl-3-morpholino-3-(p-tolyl)butenolide (59)
Figure S43. $^1$H NMR of 2-(3-fluorophenyl)-4-methoxy-4-methyl-3-morpholinobutenolide (60)

Figure S44. $^{13}$C NMR of 2-(3-fluorophenyl)-4-methoxy-4-methyl-3-morpholinobutenolide (60)
3. Biology methods for anti influenza H1N1 activity in vitro and in vivo.

3.1 Virus

Influenza A virus (H1N1) was used for challenge infections, it was purchased from China Center for Type Culture Collection. Viral stocks were produced in Madin-Darby canine kidney (MDCK).

3.2 Cell Lines

MDCK used in this study was purchased from ATCC. It was maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) supplemented with 10% Fetal bovine serum (GBICO, USA) and 1% penicillin/streptomycin (Solarbio, Beijing, China).

3.3 Virus propagation

Viral stocks were produced in Madin-Darby canine kidney (MDCK) cells. 90% confluent cells were infected with the virus in 10mL medium of a moi of 0.01 and incubated for five days until an overall cytopathic effect (CPE) could be observed. Virus containing cell culture supernatants were collected and frozen in aliquots from -20 to -80 °C. The results of all TCID\textsubscript{50} assays were calculated according to the Reed and Muench method.

3.5 Cytotoxicity assay

An MTT assay was performed to evaluate the cytotoxic effects of all compounds on MDCK cells. Cell monolayers were washed after the 24h cultivation and a series of concentrations of compounds (0–800 μM) was added to the cells. After incubation at 37°C in a CO\textsubscript{2} incubator for 72h, 20μL MTT was added to each well and the plates were further incubated for 3h. Solubilisation of the formazan crystals formed during this period was achieved by the addition of dimethyl sulfoxide (DMSO). The absorbance (OD) at 490 nm was measured using a microplate reader. Cell survival rate was expressed as the percentage of compounds treated average OD value / control average OD value.

3.5 Antiviral activity assay

MDCK cells were placed in 24-well cell culture plates. After 24h, washing with PBS for three times, cells were incubated with H1N1 in DMEM medium for 2h in 0.01 moi. Then different concentrations of all compounds ranging from 5 to 100 μM was added to cells. RI (100μM) and Ose (3μM) were used as positive control. All cultures were incubated at 37°C for 72 h. Inhibition rate (%) = [(the average OD value of drug-treatment - the average OD value of virus controls)/(the average OD value of cell controls - the average OD value of virus controls)] × 100%.

3.6 NA inhibition assay

The influenza A virus have two types of glycoprotein of hemagglutinin (HA) and neuraminidase (NA). A fluorescence-based NA inhibition assay was used to determine whether the compounds have the abilities to suppress NA protein. The assay using the Neuraminidase Inhibitors Screen Kit (Beyotime, China) according to the manufacturer's instructions. Adding 70μL NA loading buffer, moderate NA, sample and water to black 96-well microplate for 2min at 37°C. Next, 10μL NA fluorogenic substrate was added and the plates were incubated at 37°C for 30min. Fluorescence was measured at Ex = 322 nm and Em = 450nm.
3.7 Animals and experimental setup

SPF KM mice (5 weeks-old, 18-22g) were purchased from Animal Experimental Center, Zhengzhou University, China [NO. SCXK (YU) 2015-0004 ]. All animals experimentation was approved by the local animal welfare authority. Mice (n=40) were raised at School of Pharmacy of Zhengzhou University and the air temperature was maintained at 22±2°C, on 12h light/dark cycle.

Mice were randomly kept in groups of 4 (n=8). There were normal control group, the group of viruses, high dose group of compound **compound 37** (200mg/kg/d), low-dose group of Compound **compound 37** (50mg/kg/d). After seven days feeding, the other group were inoculated by the intranasal route with 50μL of H1N1 with a titer of 1×10^{-4.5} influenza virus apart from the control group. The compounds were administered according to body weight by oral gavage once daily for 7 days after 2 hours of virus infection. Mice were sacrificed to calculate lung index and so on after day 5 post-infection.

3.8 Clinical follow-up and sampling

During the experiment, mice were clinically monitored daily for the development of clinical signs including fever, fatigue, anorexia, dyspnea and cough. Body weights and body temperature were recorded daily. Removal of the eye of mice and the blood were kept in heparinized tubes and processed into serum after the last administration. Lungs were taken and weighed, a part of lung tissue were prepared for histological analysis. The rest of lung tissue was stored at -80 °C for further use.

3.9 Gross pathology and Histopathology

At necropsy, the lungs immediately examined macroscopically and photographs taken for further analysis. A small sections of lung were fixed in 10% buffered formalin for histopathology. Fix tissues were dehydrated, embedded in paraffin and 5μM sections were cut for histological examination. Lung sections from the portion most consistently affected by gross lesions (well-demarcated purple dark red areas of tissue consolidation) were stained using a hematoxylin/eosin (HE) standard staining protocol and examined microscopically (this study was done by PhD Yan of school of basic medical sciences of Zhengzhou university).

3.10 Quantitative real-time RT-PCR (qRT-PCR)

The total RNA was extracted from lung tissue using the RNA prepare Tissue Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. The concentration of RNA was detected by NANODROP 2000 (Thermo, USA) and the integrity of RNA was detected trough agarose gel electrophoresis. Then RNA was subjected to reverse transcription using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo, Beijing, China). The reaction setup was incubated for 60 min at 42°C and then 5 min at 70°C. Quantification of IV gene copy numbers was performed using a pan-Influenza A-M1.2 assay by qRT-PCR for with an appropriate *in vitro* transcribed RNA standard. The primers are listed below.

- Front primer: AGATGAGTCTTCTAACCAGGTCG
- Reserve primer: TGCAAAAAACATCTTCAAGTCTCTG
