SUPPORTING INFORMATION

Umbelliferone-oxindole hybrids as novel apoptosis inducing agents

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- Experimental protocol for MTT assay, AO/EB staining, DAPI staining, cell cycle analysis, mitochondrial membrane potential, Annexin V assay, DCFDA staining and western blotting ................................................................................................................................. 26-28
Experimental Section:

Chemistry

**General.** All melting points were determined on a stuart digital SMP-30 melting point apparatus. IR spectra were recorded on a Perkin Elmer, FT-IR spectrometer using KBr discs. $^1$H and $^{13}$C NMR spectra were recorded on either Bruker AVANCE-I 300 MHz NMR spectrometer, Bruker AVANCE-II 300 MHz and Inova 500 and recorded in DMSO-$d_6$ solvent. Chemical shift were reported in parts per million (ppm) with respect to internal standard tetramethylsilane (TMS). Mass spectra were obtained on Agilent technologies LC/HRMS-TOF spectrometer. TLC experiments were performed on 0.2 mm Merck pre-coated silica gel 60 F$_{254}$ aluminium sheets and the spots were visualized under a UV lamp or by exposure in iodine vapors. Column chromatography was performed using silica gel (60-120mesh) and the column was usually eluted with EtOAc/hexane.

*General experimental procedure for the synthesis of umbelliferone-oxindole derivatives 7(a-r)*

To a stirred solution of oxindole (1 equiv) in ethanol was added 7-hydroxy-4-methyl-2-oxo-2H-chromene-6-carbaldehyde (1 equiv) and piperidine (0.01 equiv) and refluxed for 3h. Completion of the reaction was checked by TLC ($n$-hexane/EtOAc; 3:7). The product was filtered and was recrystallized using ethanol to give pure 7(a-r) in good yields.

**Spectral Data**

*(E)-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7a)*

Yellow solid; yield: 80%; mp 285-287 °C. IR (KBr): 3325, 3061, 1720, 1694, 1670, 1570, 1555, 1316, 1217, 1067, 748, 597 cm$^{-1}$. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta = 10.6$ (s, 1H), 7.75 (d, $J = 8.6$ Hz, 1H), 7.47 (s, 1H), 7.21 (t, $J =7.5$ Hz, 1H), 7.03 (d, $J = 8.8$ Hz, 1H), 6.87-6.68 (m, 3H), 6.19 (s, 1H), 2.41 (s, 3H) ppm. $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta = 168.2$, 159.6, 159.0, 153.7, 152.2, 142.5, 130.4, 129.6, 127.0, 124.7, 123.7, 121.6, 121.1, 112.4, 111.9, 110.2, 109.6, 18.2 ppm. HRMS (ESI): $m/z$ calcd. for C$_{19}$H$_{13}$NO$_4$ [M+H]$^+$ 320.0923; found 320.0912.

*(E)-3-((7-(benzyl)oxy)-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7b)*

Yellow solid; yield: 75%; mp 250-252 °C. IR (KBr): 3323, 3063, 1699, 1587, 1592, 1467, 1380, 1295, 1096, 732, 587 cm$^{-1}$. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta = 10.57$ (s, 1H), 7.85 (d,
J = 8.8 Hz, 1H), 7.42 (s, 1H), 7.31-7.08 (m, 7H), 6.84-6.56 (m, 3H), 6.21 (s, 1H), 5.22 (s, 2H), 2.44 (s, 3H) ppm. $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ = 168.1, 159.4, 158.6, 153.6, 151.4, 142.6, 136.0, 131.1, 130.1, 128.3, 127.9, 127.3, 124.2, 123.3, 121.5, 121.3, 113.6, 111.6, 111.2, 109.8, 109.2, 70.3, 18.3 ppm. HRMS (ESI): $m/z$ calcd. for C$_{26}$H$_{19}$NO$_4$ [M+H]$^+$ 410.1392; found 410.1379.

(E)-5-bromo-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7c)
Yellow solid; yield: 78%; mp 308-310 °C. IR (KBr): 3135, 1698, 1681, 1560, 1537, 1426, 1360, 1311, 1301, 1283, 1279, 1273, 1242, 1233, 1215, 1213, 1136, 1116, 1112, 1098, 1092, 70.3, 18.3 ppm. HRMS (ESI): $m/z$ calcd. for C$_{19}$H$_{12}$BrNO$_4$ [M+H]$^+$ 398.0028; found 398.0038

(E)-5-iodo-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7d)
Brown solid; yield: 76%; mp 289-291 °C. IR (KBr): 3160, 1699, 1679, 1384, 1225, 806, 527 cm$^{-1}$. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ = 10.74 (s, 1H), 7.79 (d, $J$ = 8.6 Hz, 1H), 7.53 (dd, $J$ = 1.3, 8.0 Hz, 1H), 7.06 (d, $J$ = 8.6 Hz, 1H), 7.01 (s, 1H), 6.73 (d, $J$ = 8.2 Hz, 1H), 6.22 (s, 1H), 2.43 (s, 3H) ppm. $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ = 167.6, 159.4, 158.9, 152.5, 141.6, 131.8, 128.8, 127.6, 126.6, 123.7, 112.7, 111.6, 111.4, 110.0, 109.1, 18.2 ppm. HRMS (ESI): $m/z$ calcd. for C$_{19}$H$_{12}$INO$_4$ [M+H]$^+$ 445.9889; found 445.9878

(E)-5-fluoro-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7e)
Yellow solid; yield: 79%; mp 278-280 °C. IR (KBr): 3320, 3087, 1698, 1597, 1568, 1389, 1176, 1068, 768, 658, 603 cm$^{-1}$. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ = 10.61 (s, 1H), 7.74-7.65 (m, 1H), 7.45 (s, 1H), 7.04 (d, $J$ = 8.3 Hz, 2H), 6.76-6.70 (m, 1H), 6.54 (s, 1H), 6.18 (s, 1H), 2.45 (s, 3H) ppm. $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ = 168.5, 160.6, 159.7, 153.9, 152.7, 138.7, 129.2, 127.4, 126.9, 123.0 (d, $J_{C-F}$ = 9.3 Hz), 115.8, 115.5, 113.2, 111.3 (d, $J_{C-F}$ = 9.3 Hz), 111.0, 110.1 (d, $J_{C-F}$ = 7.7 Hz), 109.5, 109.2, 18.2 ppm. HRMS (ESI): $m/z$ calcd. for C$_{19}$H$_{12}$FNO$_4$ [M+H]$^+$ 338.0829; found 338.0819

(E)-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)-5-methylindolin-2-one (7f)
Yellow solid; yield: 80%; mp 282-284 °C. IR (KBr): 3183, 1699, 1679, 1602, 1568, 1387, 1073, 806, 602 cm$^{-1}$. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ = 10.49 (s, 1H), 7.77-7.69 (m, 1H), 7.45 (s, 1H), 7.04 (d, $J$ = 8.3 Hz, 2H), 676-6.70 (m, 1H), 6.54 (s, 1H), 6.18 (s, 1H), 2.45 (s,
(E)-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)-5-nitroindolin-2-one (7g)

Orange solid; yield: 72%; mp 388-390 °C. IR (KBr): 3157, 1706, 1617, 1594, 1569, 1323, 1298, 1076, 843, 746, 631 cm⁻¹. ¹H NMR (300 MHz, DMSO-d₆): δ = 11.60 (bs, 1H), 8.19 (dd, J = 2.0, 8.4 Hz, 1H), 7.79-7.73 (m, 2H), 7.61 (d, J = 2.0 Hz, 1H), 7.08-6.96 (m, 2H), 6.16 (s, 1H), 2.42 (s, 3H) ppm. ¹³C NMR (75 MHz, DMSO-d₆): δ = 168.8, 160.5, 159.6, 153.9, 152.8, 148.2, 141.6, 128.4, 128.0, 127.3, 125.9, 122.1, 119.8, 113.1, 111.4, 109.8, 109.6, 108.9, 18.3 ppm. HRMS (ESI): m/z calcd. for C₁₉H₁₂N₂O₆ [M+H]⁺ 365.0774; found 365.0780

(E)-5-bromo-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)-1-methylindolin-2-one (7h)

Yellow solid; yield: 83%; mp 265-267 °C. IR (KBr): 3321, 3075, 1727, 1679, 1575, 1365, 1115, 1062, 845, 805, 618 cm⁻¹. ¹H NMR (300 MHz, DMSO-d₆): δ = 7.71 (s, 1H), 7.64 (d, J = 8.8 Hz, 1H), 7.46 (dd, J = 1.8, 8.3 Hz, 1H), 7.01 (d, J = 8.4 Hz, 1H), 6.89 (d, J = 1.8 Hz, 1H), 6.84 (d, J = 8.8 Hz, 1H), 6.02 (s, 1H), 3.25 (s, 3H), 2.34 (s, 3H) ppm. ¹³C NMR (75 MHz, DMSO-d₆): δ = 166.9, 163.2, 160.0, 154.0, 153.2, 142.5, 131.2, 128.4, 127.5, 126.7, 126.6, 123.3, 114.2, 113.4, 110.0, 109.3, 108.5, 26.0, 18.3 ppm. HRMS (ESI): m/z calcd. for C₂₀H₁₄BrNO₄ [M+H]⁺ 412.0199; found 412.0184

(E)-5-chloro-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)-1-methylindolin-2-one (7i)

Yellow solid; yield: 85%; mp 302-304 °C. IR (KBr): 3327, 3061, 1699, 1681, 1593, 1370, 1109, 1064, 844, 753, 627 cm⁻¹. ¹H NMR (300 MHz, DMSO-d₆): δ = 7.77 (d, J = 8.6 Hz, 1H), 7.67 (s, 1H), 7.39 (dd, J = 2.0, 8.3 Hz, 1H), 7.09-7.00 (m, 2H), 6.73 (d, J = 1.7 Hz, 1H), 6.18 (s, 1H), 3.22 (s, 3H), 2.42 (s, 3H) ppm. ¹³C NMR (75 MHz, DMSO-d₆): δ = 166.6, 159.5, 159.2, 153.8, 152.5, 142.5, 129.0, 128.1, 127.1, 125.6, 123.4, 122.5, 112.6, 111.9, 110.2, 109.8, 109.0, 26.0, 18.2 ppm. HRMS (ESI): m/z calcd. for C₂₀H₁₄ClNO₄ [M+H]⁺ 368.0678; found 368.0690

(E)-5-acetyl-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7j)

Yellow solid; yield: 73%; mp 179-181 °C. IR (KBr): 3315, 2948, 1693, 1611, 1570, 1381, 1174, 1067, 747, 439 cm⁻¹. ¹H NMR (500 MHz, DMSO-d₆): δ = 10.29 (bs, 1H), 7.80-7.76 (m, 1H), 7.69 (s, 1H), 7.62 (d, J = 8.8 Hz, 1H), 6.98-6.95 (m, 2H), 6.79 (d, J = 8.8 Hz, 1H), 2.08 (s, 3H) ppm. ¹³C NMR (75 MHz, DMSO-d₆): δ = 168.3, 159.6, 159.2, 153.7, 152.3, 140.2, 130.4, 129.9, 129.5, 127.0, 124.5, 124.4, 121.7, 112.5, 111.7, 110.0, 109.6, 109.2, 20.7, 18.2 ppm. HRMS (ESI): m/z calcd. for C₂₀H₁₅NO₄ [M+H]⁺ 334.1079; found 334.1070
5.98 (s, 1H), 2.58 (s, 3H), 2.37 (s, 3H) ppm. $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta = 198.8, 169.0, 160.0, 153.8, 153.5, 141.6, 129.6, 129.6, 128.9, 128.3, 127.1, 123.6, 120.6, 120.6, 118.0, 114.9, 114.9, 109.4, 107.5, 27.2, 18.2 ppm. HRMS (ESI): $m/z$ calcd. for C$_{23}$H$_{15}$NO$_5$ [M+H]$^+$ 362.1028; found 362.0914

(E)-5-benzoyl-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7k)

Yellow solid; yield: 74%; mp 162-164 °C. IR (KBr): 3194, 1698, 1637, 1597, 1569, 1384, 1178, 1067, 741, 671 cm$^{-1}$. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta = 11.06$ (s, 1H), 7.77 (d, $J = 8.0$ Hz, 1H), 7.62-7.55 (m, 3H), 7.55-7.51 (m, 2H), 7.49-7.42 (m, 2H), 7.19 (s, 1H), 7.03 (d, $J = 8.0$ Hz, 1H), 6.89 (d, $J = 8.8$ Hz, 1H), 6.08 (s, 1H), 2.34 (s, 3H) ppm. $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta = 194.1, 168.7, 159.6, 153.5, 152.4, 146.4, 137.4, 132.2, 131.5, 129.6, 128.6, 128.5, 128.5, 128.2, 127.1, 126.8, 126.5, 121.5, 113.0, 111.0, 109.6, 109.5, 109.1, 18.2 ppm. HRMS (ESI): $m/z$ calcd. for C$_{26}$H$_{17}$NO$_5$ [M+H]$^+$ 424.1185; found 424.1180

(E)-5-benzhydryl-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7l)

Yellow solid; yield: 76%; mp 300-302 °C. IR (KBr): 3274, 3024, 1715, 1603, 1563, 1509, 1357, 1242, 1172, 1067, 850, 594 cm$^{-1}$. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta = 11.15$ (bs, 1H), 10.58 (s, 1H), 7.65 (d, $J = 8.6$ Hz, 1H), 7.36 (s, 1H), 7.20-7.08 (m, 6H), 7.02-6.97 (m, 1H), 6.90-6.76 (m, 6H), 6.33 (s, 1H), 6.07 (s, 1H), 5.35 (s, 1H), 2.38 (s, 3H) ppm. $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta = 168.3, 159.5, 158.9, 153.4, 151.5, 143.5, 140.7, 136.4, 130.8, 130.6, 128.6, 128.0, 126.8, 125.9, 124.8, 123.8, 121.4, 112.1, 111.9, 110.4, 109.5, 109.4, 55.2, 18.3 ppm. HRMS (ESI): $m/z$ calcd. for C$_{32}$H$_{23}$NO$_4$ [M+H]$^+$ 486.1705; found 486.1688

(E)-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)-5-(4-methoxybenzyl)indolin-2-one (7m)

Yellow solid; yield: 79%; mp 148-150 °C. IR (KBr): 3319, 2950, 1689, 1567, 1509, 1381, 1242, 1172, 1066, 808, 438 cm$^{-1}$. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta = 10.48$ (bs, 1H), 7.54 (s, 1H), 7.48 (d, $J = 8.8$ Hz, 1H), 6.97-6.86 (m, 3H), 6.74-6.62 (m, 3H), 6.54 (s, 1H), 5.85 (s, 1H), 3.67 (s, 3H), 3.59 (s, 2H), 2.34 (s, 3H) ppm. $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta = 169.0, 165.8, 160.3, 157.3, 153.8, 153.2, 140.1, 134.0, 132.9, 129.4, 128.9, 128.3, 127.1, 126.6, 124.6, 122.5, 114.9, 113.6, 109.9, 109.0, 108.7, 107.2, 59.8, 54.9, 18.3 ppm. HRMS (ESI): $m/z$ calcd. for C$_{27}$H$_{21}$NO$_5$ [M+H]$^+$ 440.1498; found 440.1500
(E)-5-(1,3-diphenylprop-2-yn-1-yl)-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7n)

Yellow solid; yield: 71%; mp 235-237 °C. IR (KBr): 3322, 2924, 1713, 1676, 1564, 1360, 1311, 1064, 816, 594 cm⁻¹. ¹H NMR (300 MHz, DMSO-d₆): δ = 10.45 (s, 1H), 10.11 (s, 1H), 7.68-7.60 (m, 1H), 7.38-7.09 (m, 12H), 7.01-6.70 (m, 3H), 5.95 (s, 1H), 4.98 (s, 1H), 2.26 (s, 3H) ppm. ¹³C NMR (75 MHz, CDCl₃+DMSO-d₆): δ = 168.3, 159.4, 158.9, 153.2, 151.8, 141.8, 141.3, 134.4, 131.1, 130.4, 129.2, 128.4, 128.3, 128.1, 127.2, 126.8, 126.5, 125.0, 122.5, 122.3, 121.9, 112.2, 111.8, 110.4, 109.5, 90.2, 83.9, 41.9, 18.1 ppm. HRMS (ESI): m/z calcd. for C₃₄H₂₃NO₄ [M+H]⁺ 510.1705; found 510.1688

(E)-5-(2-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)prop-2-yn-1-yl)indolin-2-one (7o)

Yellow solid; yield: 73%; mp 228-230 °C. IR (KBr): 3324, 2921, 1714, 1676, 1563, 1359, 1311, 1065, 817, 753, 595 cm⁻¹. ¹H NMR (300 MHz, DMSO-d₆): δ = 11.06 (bs, 1H), 10.59 (s, 1H), 7.60 (d, J = 8.6 Hz, 1H), 7.44 (s, 1H), 7.40-7.27 (m, 3H), 7.22-7.02 (m, 7H), 6.97-6.88 (m, 2H), 6.82-6.76 (m, 1H), 6.09 (s, 1H), 5.12 (s, 1H), 2.29-2.22 (m, 6H) ppm. ¹³C NMR (75 MHz, CDCl₃+DMSO-d₆): δ = 168.2, 159.4, 158.9, 153.3, 151.8, 141.2, 138.9, 135.6, 134.6, 131.1, 130.4, 129.2, 129.1, 129.0, 128.4, 128.1, 127.1, 126.9, 124.9, 122.4, 122.3, 121.8, 112.2, 111.9, 110.3, 109.5, 90.5, 83.8, 41.4, 20.5, 18.0 ppm. HRMS (ESI): m/z calcd. for C₃₅H₂₅NO₄ [M+H]⁺ 524.1862; found 524.1849

(E)-5-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)-3-(1-(4-methoxyphenyl)-3-phenylprop-2-yn-1-yl)indolin-2-one (7p)

Yellow solid; yield: 75%; mp 224-226 °C. IR (KBr): 3330, 2988, 1713, 1677, 1594, 1563, 1310, 1247, 1065, 755, 595 cm⁻¹. ¹H NMR (300 MHz, DMSO-d₆): δ = 10.55 (s, 1H), 10.02 (s, 1H), 7.86-7.60 (m, 1H), 7.38-7.09 (m, 9H), 6.96-6.87 (m, 2H), 6.82-6.74 (m, 3H), 5.95 (s, 1H), 4.93 (s, 1H), 3.78 (s, 3H), 2.26 (s, 3H) ppm. ¹³C NMR (75 MHz, CDCl₃+DMSO-d₆): δ = 168.3, 159.4, 158.9, 157.8, 153.2, 151.8, 141.2, 134.7, 133.8, 131.0, 130.5, 129.1, 128.3, 128.2, 128.0, 126.7, 124.9, 122.4, 121.8, 113.8, 112.2, 111.8, 110.3, 109.5, 109.4, 90.6, 83.7, 54.9, 41.0, 18.0 ppm. HRMS (ESI): m/z calcd. for C₃₅H₂₅NO₅ [M+H]⁺ 540.1181; found 540.1795

(E)-5-(1-(4-fluorophenyl)-3-phenylprop-2-yn-1-yl)indolin-2-one (7q)

Yellow solid; yield: 70%; mp 245-247 °C. IR (KBr): 3325, 2920, 1715, 1674, 1595, 1214, 1062, 848, 711 cm⁻¹. ¹H NMR (500 MHz, DMSO-d₆): δ = 10.44-10.24 (m, 1H), 10.02 (s,
1H), 7.71-7.60 (m, 1H), 7.57 (s, 1H), 7.38-7.08 (m, 8H), 7.03-6.72 (m, 5H), 5.95 (s, 1H), 4.98 (s, 1H), 2.26 (s, 3H) ppm. $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta = 168.2, 159.4, 158.9, 153.4, 151.8, 141.3, 138.0$ (d, $J_{C-F} = 3.6$ Hz), 134.3, 131.1, 130.4, 129.2, 129.1 (d, $J_{C-F} = 8.1$ Hz), 128.4, 128.3, 126.9, 126.5, 125.0, 122.3, 122.2, 121.9, 115.3 (d, $J_{C-F} = 20.8$ Hz), 112.2 111.8, 110.3, 109.6, 109.4, 90.1, 84.1, 40.8, 18.0 ppm. HRMS (ESI): m/z calcd. for $C_{34}H_{22}FNO_4 [M+H]^+$ 528.1611; found 528.1605.

(E)-5-(1-(2,3-dichlorophenyl)-3-phenylprop-2-yn-1-yl)-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7r)

Orange solid; yield: 73%; mp 268-270 °C. IR (KBr): 3333, 3062, 1715, 1675, 1566, 1566, 1314, 1214, 1063, 785, 754, 690 cm$^{-1}$. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta = 10.61$ (s, 1H), 7.65 (dd, $J = 2.6, 6.7$ Hz, 1H), 7.56-7.46 (m, 2H), 7.39-7.28 (m, 5H), 7.25-7.19 (m, 2H), 6.87-6.73 (m, 3H), 6.62-6.56 (m, 1H), 5.90 (s, 1H), 5.54 (s, 1H), 2.25 (s, 3H) ppm. $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta = 168.6, 159.8, 159.7, 153.5, 153.2, 152.5, 152.1, 141.7, 141.3, 141.2, 132.1, 131.9, 131.8, 131.2, 129.9, 129.2, 128.7, 128.4, 127.0, 123.0, 122.4, 121.9, 121.7, 113.9, 113.4, 109.7, 109.6, 109.4, 88.8, 84.2, 43.8, 18.2 ppm. HRMS (ESI): m/z calcd. for $C_{34}H_{21}Cl_2NO_4 [M+H]^+$ 578.0926; found 578.0914.
$^1$H and $^{13}$C Spectra of the synthesized compounds

1. (E)-3-(((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7a)

![Chemical Structure](image)

$^1$H NMR

$^{13}$C NMR
2. \((E)-3-((7-\text{benzyloxy})-4\text{-methyl}-2\text{-oxo}-2\text{H}-\text{chromen}-8\text{-yl})\text{methylene})\text{indolin-2-one} \(7b\)
3. (E)-5-bromo-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7c)
4. (E)-5-ido-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7d)
5. (E)-5-fluoro-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7e)
6. (E)-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)-5-methylindolin-2-one (7f)
7. (E)-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)-5-nitroindolin-2-one (7g)

$\begin{align*}
\text{H NMR} \\
\text{C NMR}
\end{align*}$
8. (E)-5-bromo-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)-1-methylindolin-2-one (7h)
9. (E)-5-chloro-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)-1-methylindolin-2-one (7i)
10. \((E)-5\text{-acetyl}-3\text{-}((7\text{-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl})\text{methylene})\text{indolin-2-one} \quad \text{(7j)}\)
11. (E)-5-benzoyl-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7k)
12. (E)-5-benzhydryl-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7I)
13. (E)-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)-5-(4-methoxybenzyl)indolin-2-one (7m)
14. (E)-5-(1,3-diphenylprop-2-yn-1-yl)-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7n)

![Chemical Structure]

**1H NMR**

![NMR Spectrum]

**13C NMR**

![NMR Spectrum]
15. (E)-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)-5-(3-phenyl-1-(p-tolyl)prop-2-yn-1-yl)indolin-2-one (7o)

1H NMR

13C NMR
16. (E)-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)-5-(1-(4-methoxyphenyl)-3-phenylprop-2-yn-1-yl)indolin-2-one (7p)
17. (E)-5-(1-(4-fluorophenyl)-3-phenylprop-2-yn-1-yl)-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7q)
18. (E)-5-(1-(2,3-dichlorophenyl)-3-phenylprop-2-yn-1-yl)-3-((7-hydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methylene)indolin-2-one (7r)
Biological evaluation

Cytotoxicity activity of compounds (MTT assay):

The cytotoxicity of the newly synthesized compounds was assessed by employing MTT assay that converts yellow tetrazolium salt MTT into the purple formazan crystals by metabolically active cells. Prostate (PC-3, DU145), Breast (MDA-MB-231, MCF-7), Lung (A549) cancer cells and RWPE-1 prostate normal epithelial cells were seeded at $5 \times 10^3$ cells per well in 96 well plates and allowed to attach overnight in incubator. All the compounds were screened at 50 μM conc. and the compounds that showed 50 % of inhibition in cell viability were selected for further dose response analysis. Cells were treated with 3.125, 6.25, 12.5, 25, 50 and 100 μM conc. of selected compounds and incubated for 48 h. After treatment period, 100 μl of MTT in medium (0.5 mg/ml) was added and incubated for 4 h. At the end of incubation, medium was aspirated; purple formazan crystals were dissolved in 200 μl of DMSO and read at 570 nm using spectrophotometer (Spectramax M4, Molecular devices, USA).

Acridine Orange/Ethidium Bromide (AO/EB) staining:

To determine morphological features of treated cells, Acridine orange & Ethidium Bromide Dual staining was performed. Briefly PC-3, DU145 cells were treated with designated conc. of 7q and 7r for 48 h and treated with dyes 10 μg/ml acridine orange and 10 μg/ml ethidium bromide. Morphological changes were observed with fluorescent microscope with excitation at 488 nm and emission at 550 nm. The photographs were taken with an inverted phase contrast microscope (Model: Nikon, Japan) at 200X magnification.

DAPI staining:

Nuclear morphological changes were observed through DAPI staining. After treatment with 7q and 7r for 48 h in PC-3 and DU145 cells, cells were washed with PBS and permeabilized with 0.1 % Tween 20 for 10 min followed by staining with 1μM DAPI. Control and Treated cells were observed with fluorescence microscope (Model: Nikon, Japan) with excitation at 359 nm and emission at 461 nm using DAPI filter at 200X magnification.

Cell Cycle Analysis (FACS):

This assay was performed using method given by UV Mallavadhani et al., with slight variations. (Mallavadhani et al.,2014). PC-3, DU145 cells were seeded in 12 well plate (1×10^5 cells /ml) and grown overnight at 37 °C. Cells were exposed to different to conc. of 7q and 7r for 24 h and cells were collected and washed twice with PBS and fixed in ice cold 70% ethanol at -20 °C. Then cells were washed with ice cold PBS and pelleted and suspended in 100 μl of PBS and incubated with cell cycle reagent (PI-2.5μg/ml, RNAse 4 μg/ml for 20 min in dark and analysed by flowcytometry (FACS verse, Becton Dickinson, US).

Flow cytometric determination of mitochondrial membrane potential using JC-1 dye
The changes in the mitochondrial membrane potential (MMP) induced by \textit{7q} and \textit{7r} were analyzed using JC-1 probe by flow cytometry (FACS verse, Becton Dickinson, US). JC-1 is lipophilic cationic dye with selectivity towards mitochondrial membrane potential and displays potential dependent accumulation in the mitochondria resulting in fluorescence shift from green to red. In cells with healthy mitochondria JC-1 accumulates in mitochondria and forms JC-1 aggregates and exhibit red fluorescence. However, JC-1 remains as monomers emitting green fluorescence in the cells with depolarized mitochondria. Thus loss of mitochondrial membrane potential was determined by increase in green to red fluorescence ratio (Wong Y H et al., 2011) (2). Briefly, PC-3, DU145 cells were treated with designated conc. of \textit{7q} and \textit{7r} for 24 h. The medium was removed and washed with PBS and incubated with 2 μM JC-1 for 45 min in incubator at 37°C and 5% CO₂. Later cells were trypsinized, washed with PBS and resuspended in 500μl of PBS and analyzed by flow cytometry in green and red channels (FACS verse, Becton Dickinson, US).

\textit{Annexin-FITC assay}

Annexin V-FITC, PI dual staining was carried out as described previously by Kloesch et al., with minor modifications. Briefly, to confirm apoptosis induced by \textit{7q} and \textit{7r} in PC-3, DU-145 cells, cells were collected after treatment with \textit{7q} and \textit{7r} for 24 h, washed twice with 1X PBS and stained with BD Annexin V –FITC /PI reagent as per manufacturer’s instructions (Becton Dickinson, US) and analysed by flow cytometry (FACS verse, Becton Dickinson, US). Live, Early apoptotic, late apoptotic & necrotic cell populations were identified by quadrant statistics with annexin-FITC -ve and PI -ve, annexin-FITC +ve and PI –ve cells, annexin-FITC +ve and PI -ve cells, annexin-FITC +ve and PI +ve cells, annexin-FITC +ve and PI -ve cell populations.

\textit{Measurement of intracellular ROS generation by DCFDA staining:}

The intracellular reactive oxygen species (ROS) generation was assessed by DCFDA staining as per the method by Eruslanov and Kusmartsev et al., 2009 with slight modifications. H2DCFDA is a cell permeable non fluorescent chemical that upon entering cell cleaved by intracellular esterases into precursor of fluorescent product DCF. This DCF later gets oxidized by intracellular ROS into highly fluorescent DCF. PC-3, DU145 cells (1 ×10⁵) were seeded in 12 well plates, cultured overnight and treated with indicated conc. of \textit{7q} and \textit{7r} for 3 h either with or without pretreatment with antioxidant 3 mM N-acetyl cysteine (NAC). After treatment period, cells were incubated with 2 μM DCFDA for 15 min at dark in incubator at 37 °C and 5% CO₂. Cells were washed with PBS twice, trypsinized and resuspended in 500μl of PBS and analyzed by measuring emission at 530 nm using excitation at 488 nm laser by flow cytometry (FACS verse, Becton Dickinson, US). At least 10,000 events were analyzed.
Preparation of whole cell and cytoplasmic protein extracts:

Whole cell protein extract was prepared from cells from different treatment groups using ice-cold RIPA buffer (sigma Aldrich) with protease inhibitor cocktail (Sigma Aldrich). Cytoplasmic fraction was separated by using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific Inc., Rockford, IL, USA) containing 1% protease inhibitor cocktail (Sigma Aldrich) according to the manufacturer's protocol and used for analysis of cytochrome c.

Western blotting:

The modulatory effect of most active compounds \(7q\) and \(7r\) on apoptotic protein expression in PC-3 cells was assessed through western blotting as per Shrivastava et al., 2014 method with minor modifications. Briefly, after treatment with indicated conc. of \(7q\) and \(7r\) for 48 h in PC-3, medium was removed, washed twice with PBS, cells were lysed with RIPA buffer mixture along with protease and phosphatase inhibitors cocktails and proteins were separated after centrifugation at 12000 g for 15min. Equal amounts of proteins were loaded and separated through SDS-PAGE, and transferred to PVDF membrane. Subsequent to blocking with 5% non-fat milk, membranes were probed with various primary antibodies at 4 °C overnight followed by incubation with appropriate secondary antibodies. Primary antibodies include cytochrome C (14 kDa), Cleaved PARP (89 kDa), \(\beta\)-actin (45 kDa) of monoclonal nature were used in 1: 1000 ratio. Anti–rabbit Horse radish peroxidase- linked secondary antibody was diluted and used in 1:20000 ratios. Membranes were visualized with ECL chemiluminescence substrate (Biorad) using FUSION FX (Vilber Lourmat, Marne-la-Vallée, France) and further the protein bands were quantified using Image J software. \(\beta\)-Actin protein was used as an internal control to ensure equal loading. Each Western blot shown is a representative of at least three independent blots.

Statistical analysis:

All results were expressed as mean ± SEM. The variation between different experimental groups was measured by one way analysis of variance (ANOVA) using the Graph Pad Prism, version 5.0. The comparison between different experimental groups was performed by “Bonferroni’s Multiple Comparison Test”. Results were described as statistically significant when P<0.05.