Supporting Information

(Thio)Chromenones derivatives exhibit anti-metastatic effects through selective inhibition of uPAR in cancer cell lines: Discovery of an uPAR-targeting fluorescent probe

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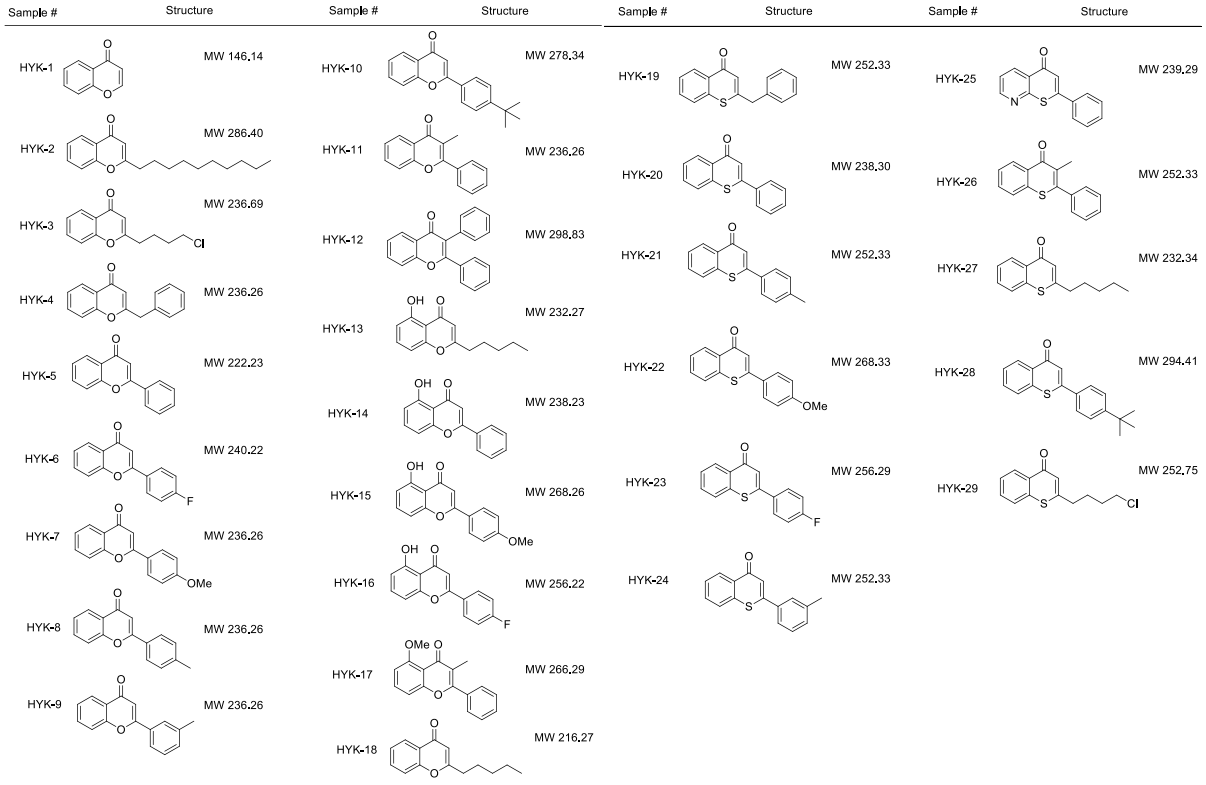
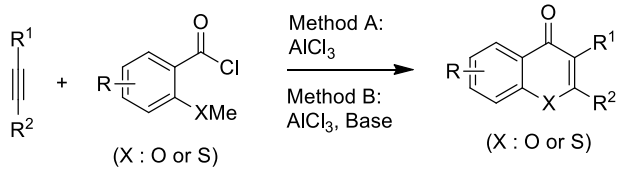
1. List of Screened (Thio)Chromenones



1.1. Preparation of (Thio)Chromenones

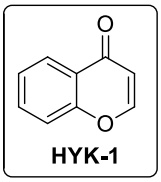
(Thio)Chromenones were prepared by previously reported methods and ^{1}H and ^{13}C NMR spectra of the prepared compounds were compared with literature data.[1] All products were purified by column chromatography and analytically pure by NMR (>98%) without any identifiable impurities.



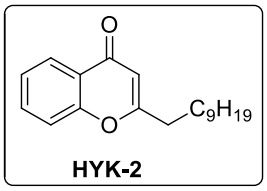
Method A: A dry flask was charged with aluminum chloride (1.25 mmol, 167 mg) under argon. Dry dichloromethane (2.5 mL) was added to this flask and then 2-methoxybenzoyl (or 2-(methylthio) benzoyl) chloride (0.5 mmol) and alkyne (0.5 mmol) were added dropwise at 0 °C. After stirring at this temperature for 30 minutes, the flask was slowly warmed to ambient temperature. The suspension was continued to stir until reaction was complete by TLC. The reaction was then quenched with H_2O (3 mL) at 0 °C, extracted with dichloromethane (5 mL x 3), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The desired products were purified by column chromatography on silica gel (10-20% ethyl acetate in hexanes).

Method B: Aluminum chloride (1.25 mmol, 167 mg) was added to a flame-dried 10 mL flask with dichloromethane (2.5 mL) at 0 °C under argon. 2-Methoxybenzoyl (or 2-(methylthio) benzoyl) chloride (0.5 mmol) and alkyne (0.5 mmol) were slowly added to this flask. After stirring for 30 minutes at 0 °C, the flask was warmed to ambient temperature and continued to stir for 4 hours. The flask was then placed to 0 °C ice bath and additional dichloromethane (2.0 mL) with H_2O (10 drops) were added. After which, triethyl amine (0.75 mmol, 104 μL) and *t*-BuOK (1.5 mmol, 168 mg) were added. The solution was left to stir at ambient temperature until the cyclization was complete by TLC (18 hours). The reaction was then quenched with H_2O (3 mL) at 0 °C, extracted with dichlromethane (5 mL x 3). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purifed by column chromatography on silica gel (10% EtOAc in hexanes).

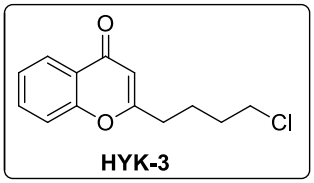
1.2. Characterization of (Thio)Chromenones



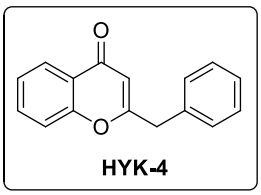
4*H*-Chromen-4-one (**HYK-1**): The product was prepared by the **Method B**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.20 (d, 1H, *J* = 9.6 Hz), 7.84 (d, 1H, *J* = 6.0 Hz), 7.62-7.70 (m, 1H), 7.45 (d, 1H, *J* = 8.4 Hz), 7.40 (t, 1H, *J* = 7.5 Hz), 6.33 (d, 1H, *J* = 6.0 Hz); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 177.5, 156.5, 155.2, 133.7, 125.8, 125.2, 124.8, 118.1, 112.9.



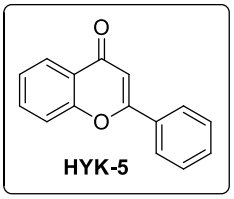
2-Decyl-4*H*-chromen-4-one (**HYK-2**): The product was prepared by the **Method B**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.18 (dd, 1H, *J* = 7.8, 1.8 Hz), 7.62-7.65 (m, 1H), 7.40-7.46 (m, 1H), 7.36-7.38 (m, 1H), 6.17 (s, 1H), 2.53-2.73 (m, 2 H), 1.68-1.80 (m, 2 H), 1.24-1.43 (m, 14H), 0.87 (t, 3H, *J* = 7.8 Hz); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 178.3, 169.7, 156.4, 133.3, 125.6, 124.8, 123.7, 117.8, 109.7, 34.3, 31.8, 29.5, 29.4, 29.2(6), 29.2(4), 28.9, 26.7, 22.6, 14.0.



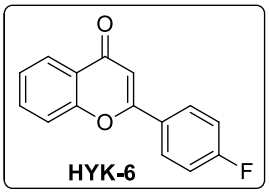
2-(4-Chlorobutyl)-4*H*-chromen-4-one (**HYK-3**): The product was prepared by the **Method B**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.15-8.19 (m, 1H), 7.63-7.66 (m, 1H), 7.42-7.43 (m, 1H), 7.37-7.39 (m, 1H), 6.18 (s, 1H), 3.59 (t, 2H, *J* = 6.8 Hz), 2.67 (t, 2H, *J* = 7.2 Hz), 1.85-1.98 (m, 4H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 178.2, 168.6, 156.4, 133.5, 125.6, 124.9, 123.7, 117.8, 110.0, 44.2, 33.5, 31.6, 24.0.



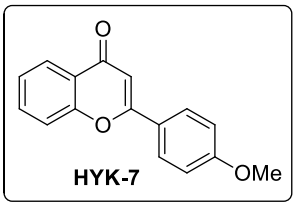
2-Benzyl-4*H*-chromen-4-one (**HYK-4**): The product was prepared by the **Method B**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.17 (dd, 1H, *J* = 7.8, 1.8 Hz), 7.60-7.65 (m, 1H), 7.41 (dd, 1H, *J* = 8.4, 1.2 Hz), 7.26-7.40 (m, 6H), 6.13 (s, 1H), 3.93 (s, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 178.3, 168.0, 156.4, 134.8, 133.5, 129.2, 128.8, 127.4, 125.6, 125.0, 123.6, 117.9, 110.7, 40.6.



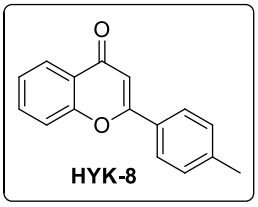
2-Phenyl-4*H*-chromen-4-one (**HYK-5**): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.20-8.26 (m, 1H), 7.91-7.96 (m, 2H), 7.68-7.71 (m, 1H), 7.55-7.58 (m, 1H), 7.50-7.55 (m, 3H), 7.40-7.44 (m, 1H), 6.83 (s, 1H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 178.4, 163.3, 156.2, 133.7, 131.7, 131.5, 129.0, 126.2, 125.7, 125.2, 123.9, 116.0, 107.6.



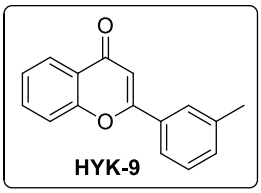
2-(4-Fluorophenyl)-4*H*-chromen-4-one (**HYK-6**): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.20-8.23 (m, 1H), 7.88-7.96 (m, 2H), 7.66-7.73 (m, 1H), 7.54-7.56 (d, 1H, *J* = 8.4 Hz), 7.33-7.46 (m, 1H), 7.17-7.24 (m, 2H), 6.75 (s, 1H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 178.2, 164.5 (d, *J* = 252 Hz), 162.3, 156.1, 133.8, 128.4 (d, *J* = 9.0 Hz), 127.9 (d, *J* = 3.0 Hz), 125.7, 125.2, 123.8, 117.9, 116.2 (d, *J* =21.0 Hz), 107.3 (d, *J* = 1.5 Hz).



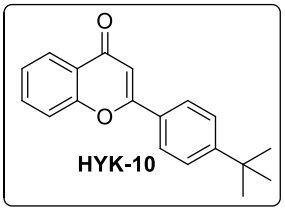
2-(4-Methoxyphenyl)-4*H*-chromen-4-one (**HYK-7**): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.22 (dd, 1H, *J* = 7.8, 1.8 Hz), 7.82-7.94 (m, 2H), 7.60-7.68 (m, 1H), 7.53-7.55 (m, 1H), 7.39-7.41 (m, 1H), 6.96-7.07 (m, 2H), 6.74 (s, 1H), 3.88 (s, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 178.3, 163.3, 162.3, 156.1, 133.5, 127.9, 125.6, 125.0, 124.0, 123.9, 117.9, 114.4, 106.1, 55.4.



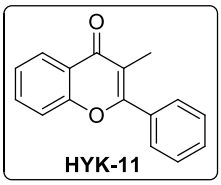
2-(*p*-Tolyl)-4*H*-chromen-4-one (**HYK-8**): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.23 (dd, 1H, *J* = 7.8, 1.8 Hz), 7.79-7.86 (m, 2H), 7.67-7.70 (m, 1H), 7.56 (d, 1H, *J* = 8.4 Hz), 7.40-7.43 (m, 1H), 7.31-7.33 (m, 2H), 6.80 (s, 1H), 2.44 (s, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 178.4, 163.5, 156.2, 142.2, 133.6, 129.7, 128.9, 126.2, 125.6, 125.1, 123.9, 118.0, 106.9, 21.5.



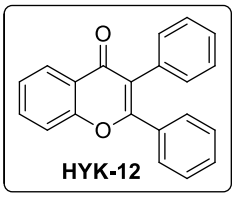
2-(*m*-Tolyl)-4*H*-chromen-4-one (HYK-9): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.24 (dd, 1H, *J* = 7.8, 1.8 Hz), 7.65-7.82 (m, 3H), 7.58 (dd, 1H, *J* = 8.4, 1.2 Hz), 7.38-7.47 (m, 2H), 7.30-7.37 (m, 1H), 6.82 (s, 1H), 2.46 (s, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 178.4, 163.6, 156.2, 138.8, 133.6, 132.3, 131.7, 128.9, 126.8, 125.6, 125.1, 123.9, 123.5, 118.0, 107.5, 21.5.



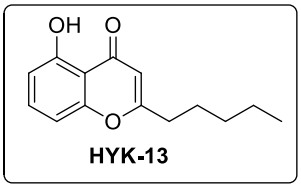
2-(4-(*tert*-Butyl)phenyl)-4*H*-chromen-4-one (**HYK-10**): The product was prepared by **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.23 (dd, 1H, *J* = 7.8, 1.8 Hz), 7.79-7.93 (m, 2H), 7.74-7.76 (m, 1H), 7.44-7.64 (m, 3H), 7.34-7.44 (m, 1H), 6.81 (s, 1H), 1.37 (s, 9H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 178.4, 163.5, 156.2, 155.3, 133.6, 128.9, 126.1, 126.0, 125.6, 125.1, 124.0, 118.0, 107.0, 35.0, 31.1.



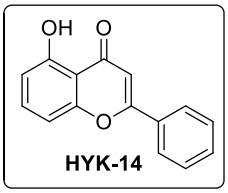
3-Methyl-2-phenyl-4*H*-chromen-4-one (**HYK-11**): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.26 (dd, 1H, *J* = 8.4, 1.8 Hz), 7.62-7.68 (m, 3H), 7.50-7.54 (m, 3H), 7.45 (d, 1H, *J* = 9.0 Hz), 7.37-7.42 (m, 1H), 2.17 (s, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 180.5, 147.8, 137.5, 136.9, 131.0(6), 131.0(4), 130.0, 129.3, 129.1, 128.7, 128.6, 127.2, 125.6, 15.1.



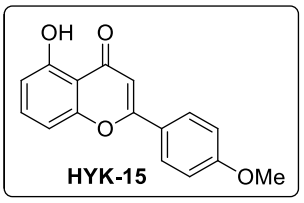
2,3-Diphenyl-4*H*-chromen-4-one (**HYK-12**): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.61-8.65 (m, 1H), 7.60-7.67 (m, 2H), 7.53-7.58 (m, 1H), 7.14-7.29 (m, 8H), 7.07-7.13 (m, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 179.4, 150.2, 137.2, 136.9, 135.7, 135.5, 131.4, 131.3, 131.0, 129.5, 129.2, 128.9, 128.2, 127.6(9), 127.6(0), 127.0, 125.7.



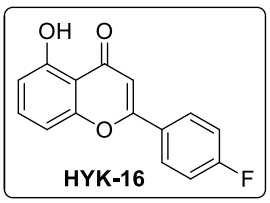
5-Hydroxy-2-pentyl-4*H*-chromen-4-one (**HYK-13**): The product was prepared by the **Method B**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 12.57 (s, 1H), 7.49 (t, 1H, *J* = 8.4 Hz), 6.86 (d, 1H, *J* = 8.4 Hz), 6.77 (d, 1H, *J* = 8.4 Hz), 6.10 (s, 1H), 2.61 (t, 2H, *J* = 7.6 Hz), 1.64-1.82 (m, 2H), 1.30-1.46 (m, 4H), 0.92 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 183.5, 171.2, 160.7, 156.7, 135.0, 111.1, 110.5, 108.3, 106.8, 34.2, 31.0, 26.4, 22.2, 13.8.



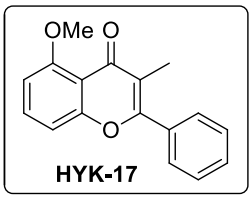
5-Hydroxy-2-phenyl-4*H*-chromen-4-one (**HYK-14**): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 12.57 (s, 1H), 7.91-7.92 (m, 2H), 7.51-7.57 (m, 4H), 7.01 (d, 1H, *J* = 9.2 Hz), 6.82 (d, 1H, *J* = 8.4 Hz), 6.74 (s, 1H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 183.5, 164.5, 160.8, 156.4, 135.3, 132.0, 131.2, 129.1, 126.4, 111.4, 110.8, 107.0, 106.0.



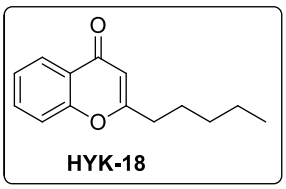
5-Hydroxy-2-(4-methoxyphenyl)-4*H*-chromen-4-one (**HYK-15**): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 12.86 (s, 1H), 7.87 (d, 2H, *J* = 9.0 Hz), 7.53 (t, 1H, *J* = 8.4 Hz), 6.96-6.98 (m, 3H), 6.80 (d, 1H, *J* = 9.0 Hz), 6.65 (s, 1H), 3.90 (s, 3H): δ 183.4, 164.5, 162.7, 160.8, 156.3, 135.1, 128.1, 123.4, 114.5, 113.2, 110.7, 106.9, 104.5, 55.5.



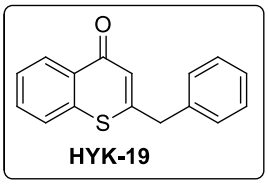
2-(4-Fluorophenyl)-5-hydroxy-4*H*-chromen-4-one (HYK-16): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 12.52 (s, 1H), 7.90-7.93 (m, 2H), 7.55 (t, 1H, *J* = 8.4 Hz), 7.16-7.32 (m, 2H), 6.99 (d, 1H, *J* = 8.4 Hz), 6.82 (d, 1H, *J* = 8.4 Hz), 6.67 (s, 1H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 183.4, 164.9 (d, *J* = 255.0 Hz), 163.5, 160.8, 156.3, 135.4, 128.6 (d, *J* = 36 Hz), 127.4 (d, *J* = 18.0 Hz), 116.4 (d, *J* = 84.2 Hz), 111.5, 110.7, 106.9, 105.8 (d, *J* = 6.0 Hz).



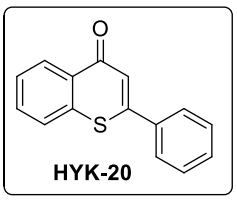
5-Methoxy-3-methyl-2-phenyl-4*H*-chromen-4-one (HYK-17): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.32-7.43 (m, 4H), 7.11 (d, 2H, *J* = 8.4 Hz), 6.98 (d, 1H, *J* = 8.4 Hz), 6.59 (d, 1H, *J* = 8.4 Hz), 3.31 (s, 3H), 1.87 (s, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 162.0, 156.8, 153.5, 149.7, 139.6, 130.8, 127.8, 126.9, 126.6, 122.5, 110.4, 109.5, 106.8, 55.7, 14.4.



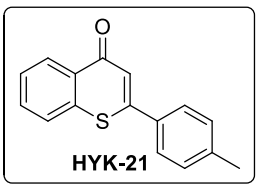
2-Pentyl-4*H*-chromen-4-one (**HYK-18**): The product was prepared by the **Method B**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.43-8.54 (m, 1H), 7.52-7.59 (m, 2H), 7.47-7.52 (m, 1H), 6.85 (s, 1H), 2.55-2.81 (m, 2H), 1.66-1.79 (m, 2H), 1.31-1.41 (m, 4H), 0.90 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 178.3, 169.7, 156.4, 133.3, 125.6, 124.8, 123.7, 117.8, 109.7, 34.2, 31.1, 26.4, 22.3, 13.8.



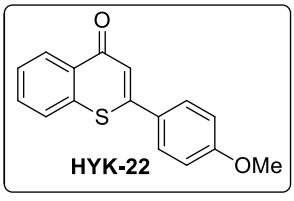
2-Benzyl-4*H*-thiochromen-4-one (HYK-19): The product was prepared by the **Method B**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.46-8.50 (m, 1H), 7.47-7.58 (m, 3H), 7.32-7.38 (m, 2H), 7.28-7.32 (m, 3H), 6.91 (s, 1H), 3.99 (s, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 180.7, 154.9, 137.6, 136.4, 131.4, 130.8, 129.0(5), 129.0(3), 128.9, 128.5, 127.5, 126.2, 124.8, 43.4.



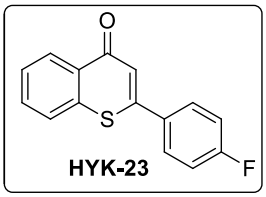
2-Phenyl-4*H*-thiochromen-4-one (**HYK-20**): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.56 (dd, 1H, *J* = 8.2, 1.4 Hz), 7.67-7.71 (m, 2H), 7.59-7.67 (m, 2H), 7.54 (dd, 1H, *J* = 8.2, 1.4 Hz), 7.47-7.52 (m, 3H), 7.24 (s, 1H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 180.8, 153.0, 137.6, 136.5, 131.5, 130.9, 130.7, 129.2, 128.5, 127.7, 126.9, 126.4, 123.4.



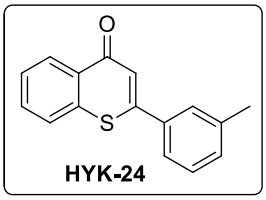
2-(*p*-Tolyl)-4*H*-thiochromen-4-one (**HYK-21**): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.54 (dd, 1H, *J* = 8.0, 1.4 Hz), 7.65 (dd, 1H, *J* = 8.4, 1.2 Hz), 7.57-7.64 (m, 3H), 7.53-7.55 (m, 1H), 7.26-7.34 (m, 2H), 7.23 (s, 1H), 2.42 (s, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 180.8, 153.0, 141.3, 137.7, 133.7, 131.4, 130.9, 129.9, 128.5, 127.6, 126.7, 126.4, 122.8, 21.3.



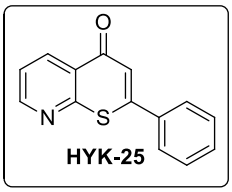
2-(4-Methoxyphenyl)-4*H*-thiochromen-4-one (HYK-22): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.54 (dd, 1H, *J* = 8.0, 1.5 Hz), 7.58-7.75 (m, 4H), 7.52-7.55 (m, 1H), 7.20 (s, 1H), 6.96-7.06 (m, 2H), 3.88 (s, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 180.8, 161.8, 152.6, 137.6, 131.4, 130.9, 128.8, 128.5, 128.3, 127.6, 126.3, 122.2, 114.6, 55.4.



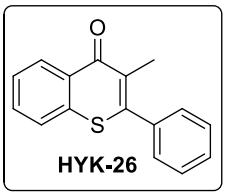
2-(4-Fluorophenyl)-4*H*-thiochromen-4-one (**HYK-23**): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.54 (d, 1H, *J* = 8.4 Hz), 7.66-7.72 (m, 2H), 7.60-7.67 (m, 2H), 7.52-7.58 (m, 1H), 7.15-7.24 (m, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 180.7, 164.3 (d, *J* = 252 Hz), 151.7, 137.4, 132.7 (d, *J* = 3.7 Hz), 131.6, 130.8, 128.9 (d, *J* = 8.9 Hz), 128.6, 127.8, 126.4, 123.4, 116.4 (d, *J* = 22.5 Hz).



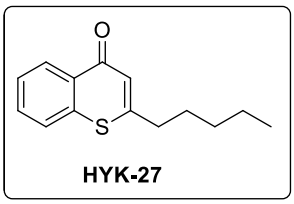
2-(*m*-Tolyl)-4*H*-thiochromen-4-one (**HYK-24**): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.53-8.55 (m, 1H), 7.59-7.67 (m, 2H), 7.53-7.55 (m, 1H), 7.45-7.52 (m, 2H), 7.38 (t, 1H, *J* = 7.8 Hz), 7.31-7.33 (m, 1H), 7.23 (s, 1H), 2.43 (s, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 180.8, 153.2, 139.1, 137.7, 136.5, 131.5(7), 131.5(2), 130.9, 129.1, 128.5, 127.6, 127.5, 126.4, 124.0, 123.2, 21.4.



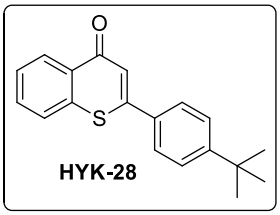
2-Phenyl-4*H*-thiopyrano[2,3-b]pyridin-4-one (**HYK-25**): The product was prepared by the **Method A** (except excess amounts of aluminum chloride (2.25 mmol) was used for this substrate). ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.81 (dd, 1H, *J* = 8.4, 1.8 Hz), 8.77 (d, 1H, *J* = 8.4 Hz), 7.71 (d, 2H, *J* = 9.0 Hz), 7.48-7.57 (m, 4H), 7.26 (s, 1H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 181.3, 159.0, 154.7, 152.7, 136.7, 136.2, 131.1, 129.3, 128.0, 126.9, 123.5, 122.9.



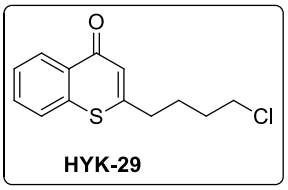
3-Methyl-2-phenyl-4*H*-thiochromen-4-one (**HYK-26**): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.54-8.64 (m, 1H), 7.50-7.63 (m, 3H), 7.46-7.59 (m, 3H), 7.39-7.44 (m, 2H), 2.13 (s, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 180.5, 147.8, 137.5, 136.9, 131.0(6), 131.0(4), 130.0, 129.3, 129.1, 128.7, 128.6(8), 127.2, 125.6, 15.1.



2-Pentyl-4*H*-thiochromen-4-one (**HYK-27**): The product was prepared by the **Method B**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.43-8.54 (m, 1H), 7.52-7.59 (m, 2H), 7.47-7.52 (m, 1H), 6.85 (s, 1H), 2.55-2.81 (m, 2H), 1.66-1.79 (m, 2H), 1.31-1.41 (m, 4H), 0.90 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 178.3, 169.7, 156.4, 133.3 ,125.6, 124.8, 123.7, 117.8, 109.7, 34.2, 31.1, 26.4, 22.3, 13.8.



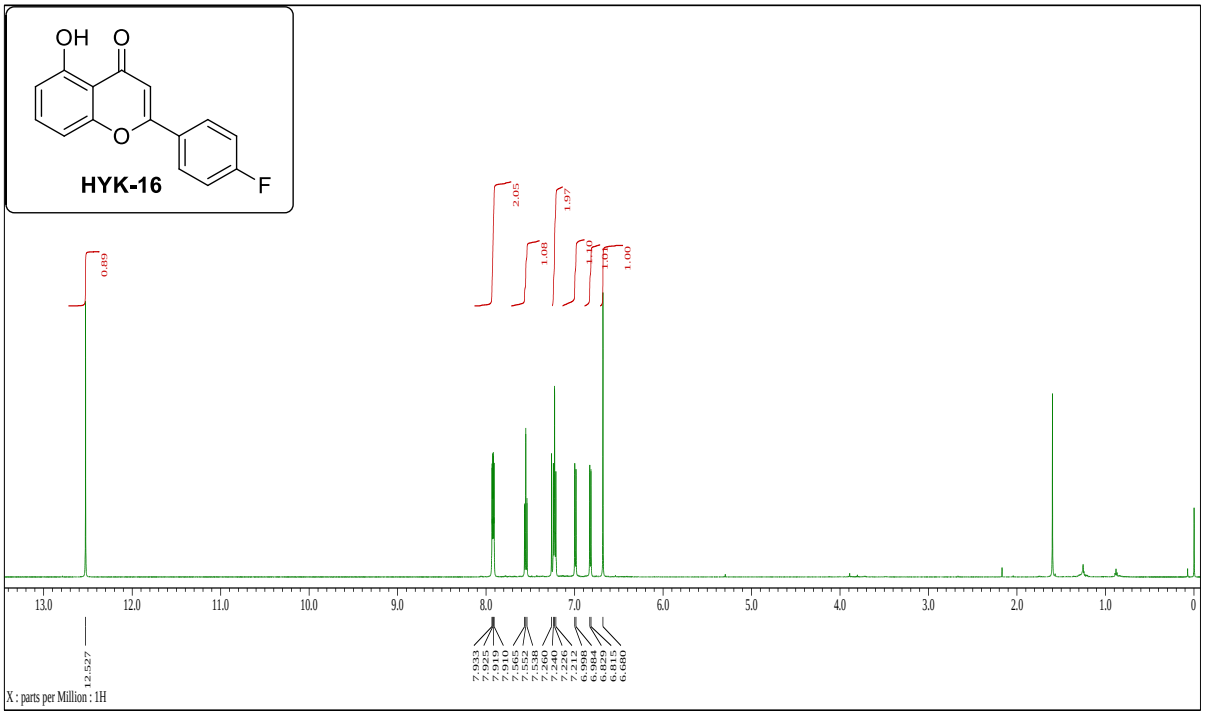
2-(4-(*tert*-Butyl)phenyl)-4*H*-thiochromen-4-one (**HYK-28**): The product was prepared by the **Method A**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.50-8.58 (m, 1H), 7.57-7.66 (m, 4H), 7.47-7.56 (m, 3H), 7.24 (s, 1H), 1.36 (s, 9H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 180.8, 154.4, 152.9, 137.7, 133.6, 131.4, 130.9, 128.5, 127.6, 126.6, 126.4, 126.2, 122.8, 34.9, 31.1.



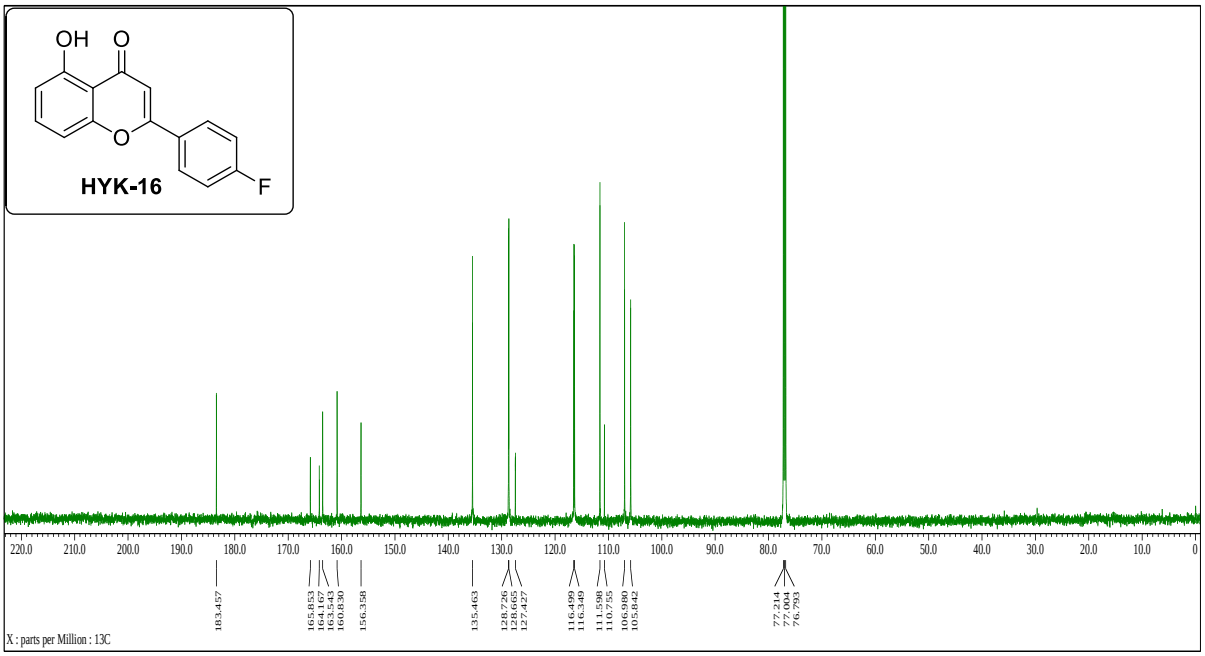
HYK-29

2-(4-Chlorobutyl)-4*H*-thiochromen-4-one (**HYK-29**): The product was prepared by the **Method B**. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.49 (dd, 1H, J = 8.4, 1.2 Hz), 7.54-7.63 (m, 2H), 7.44-7.54 (m, 1H), 6.86 (s, 1H), 3.57 (t, 2H, J = 6.8 Hz), 2.66-2.77 (m, 2H), 1.82-1.97 (m, 4H); ^{13}C NMR ($CDCl_3$, 150 MHz,): δ 180.5, 155.2, 137.4, 131.4, 130.9, 128.5, 127.5, 126.2, 124.3, 44.2, 36.5, 31.4, 26.8.

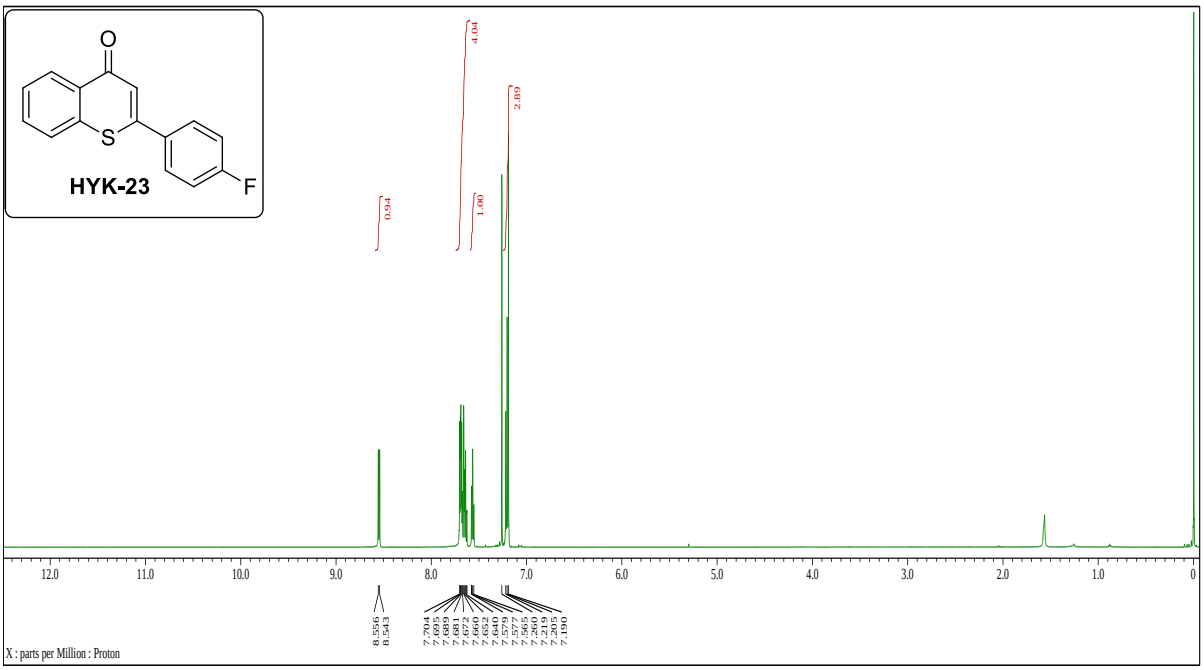
1.3. NMR Spectra of HYK-16 & HYK-23



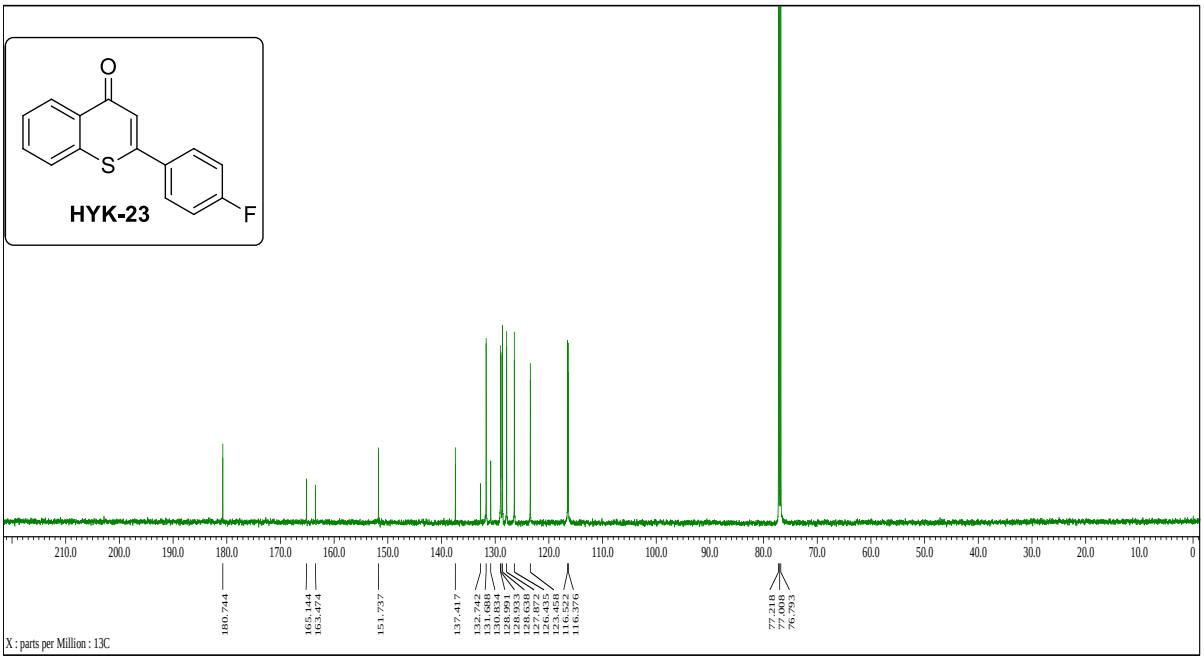
^{1}H NMR spectrum of compound **HYK-16** (600 MHz, $CDCl_3$)



^{13}C NMR spectrum of compound **HYK-16** (150 MHz, $CDCl_3$)



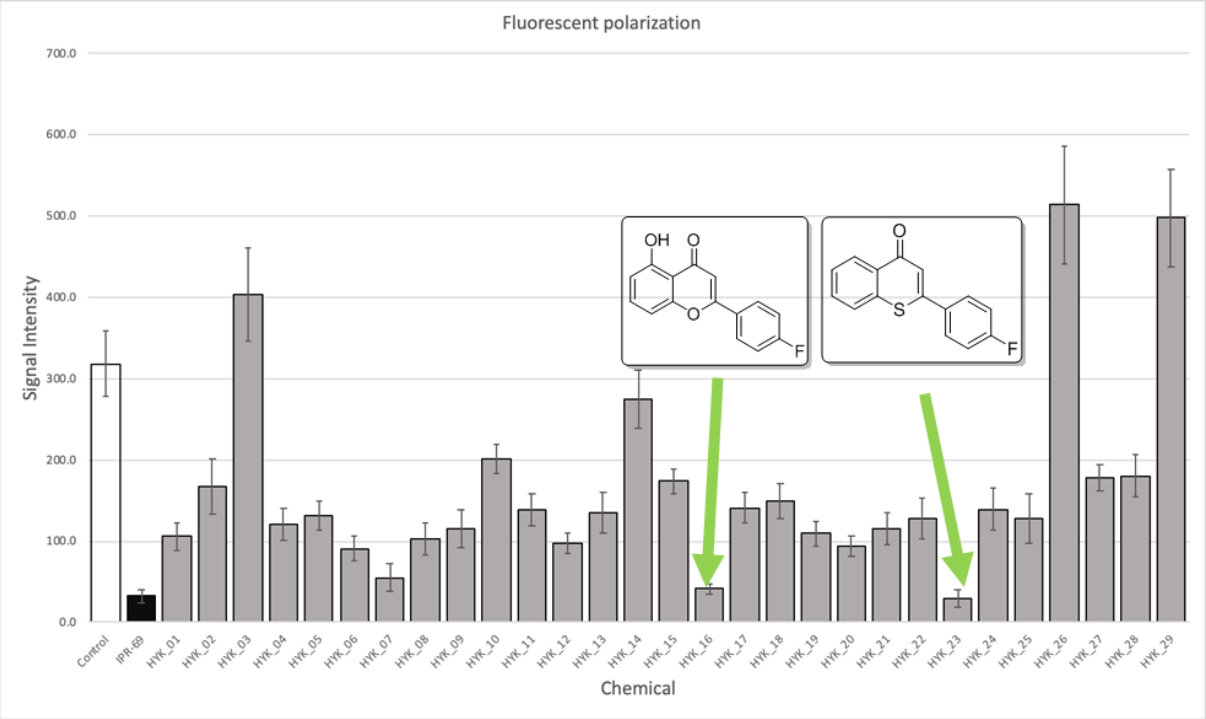
^{1}H NMR spectrum of compound **HYK-23** (600 MHz, $CDCl_3$)



^{13}C NMR spectrum of compound **HYK-23** (150 MHz, $CDCl_3$)

2. Assay Systems

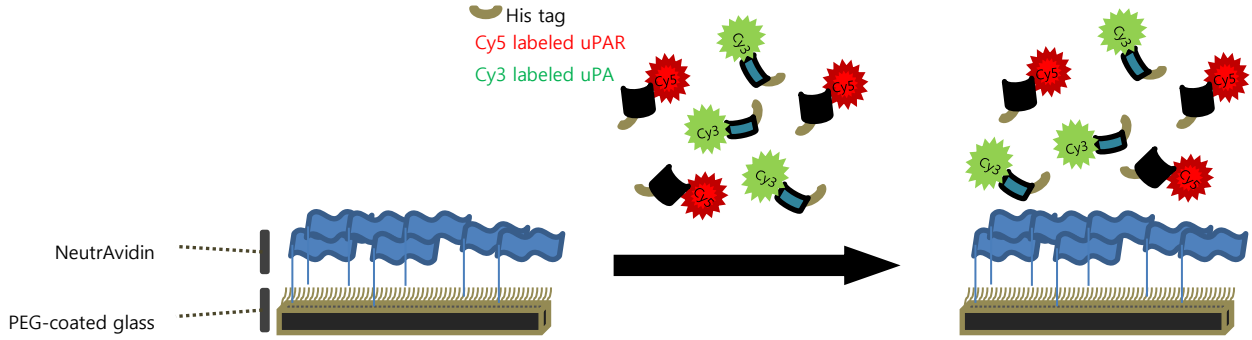
2.1 Fluorescent Polarization (FP) Results (All compounds were analytically pure by NMR (>98%) without any identifiable impurities). The compounds used in the experiments were dissolved in 100% DMSO to prepare stock solutions at a concentration of 100 mM. Serial dilutions were then performed using culture medium to achieve the desired concentrations for the assays. The final concentrations applied to the cells were 0, 10, 20, 40, 80, and 100 µM. The DMSO concentration was kept constant across all samples, and the final DMSO concentration was maintained at a level that does not affect cell viability.

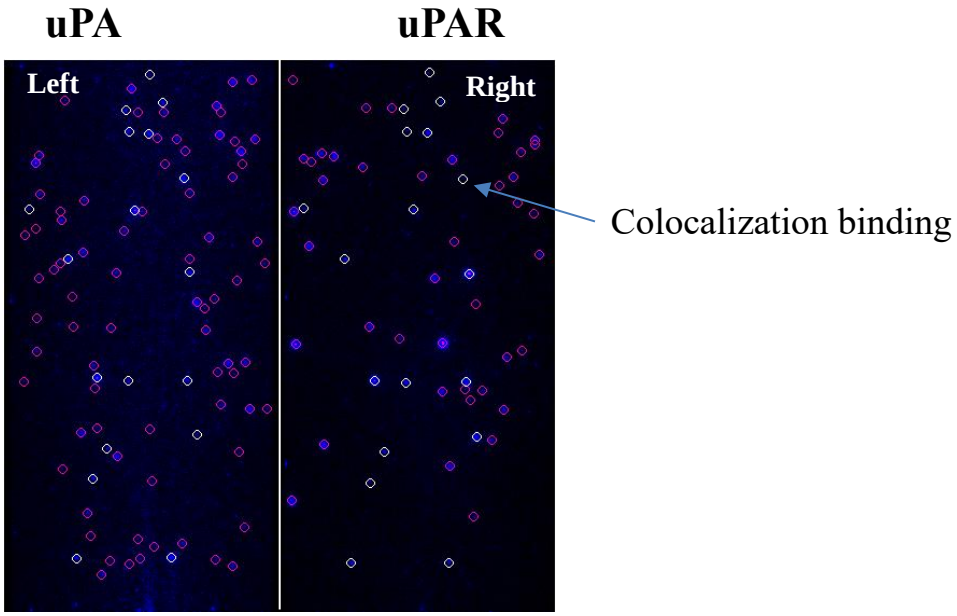


HYK-related chemicals inhibit AE147 peptide and uPAR protein binding using Fluorescence Polarization (FP) analysis: 100 uM of AE147-FAM peptide was incubated with uPAR protein (20 nM) for 30 min and 1 mM of HYK chemicals were treated for 30 min, after which fluorescence polarization (FP) was measured. The data represent FP intensity (mP) measured in every single wells, constituting 6 replicated for each conditions. We used 20 uM of known uPAR inhibitor, IPR-69, as a positive control.

2.2 Single Molecule Binding Assay (SMBA)

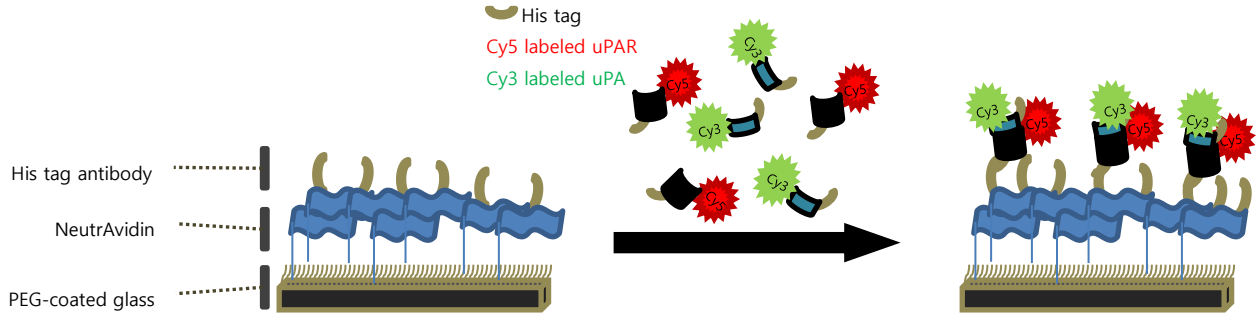
Schematic Illustration of the uPAR and uPA Non-Specific Binding

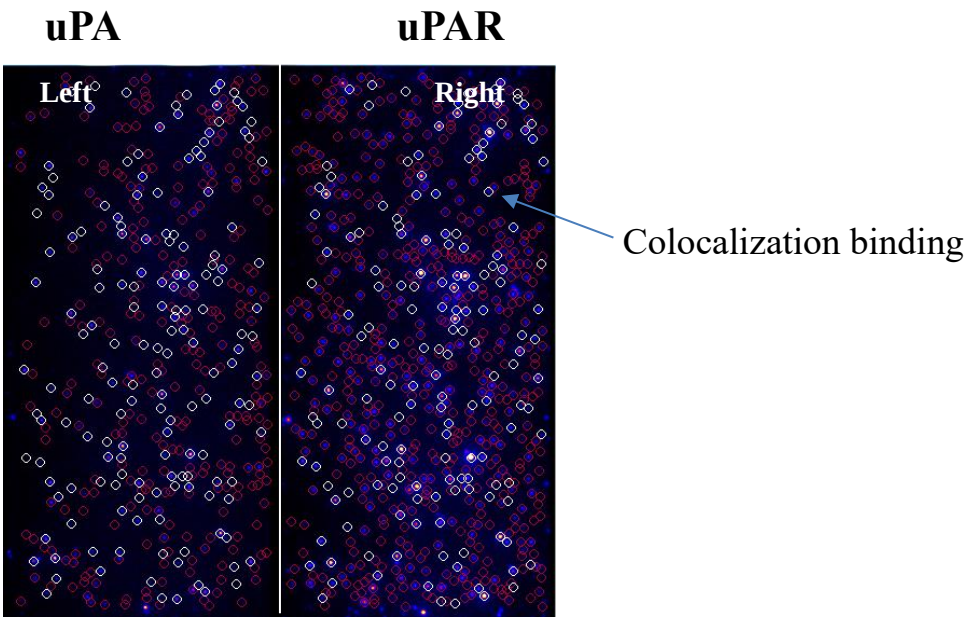




Non-specific of uPAR and uPA

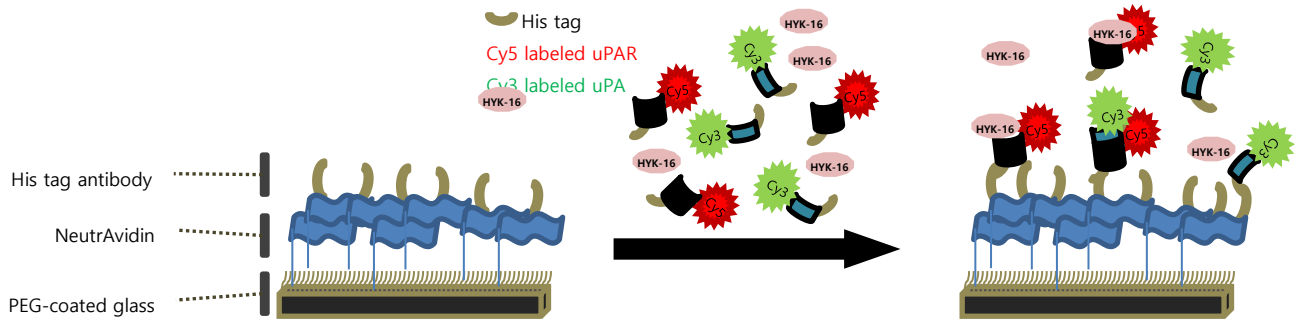
Schematic Illustration of the uPAR and uPA Specific Binding

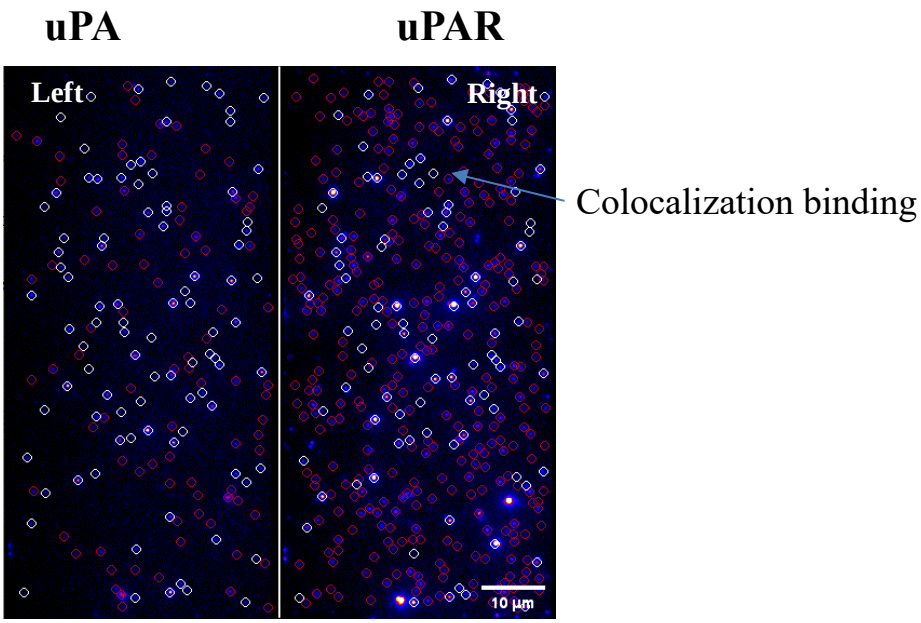




Binding of uPAR and uPA

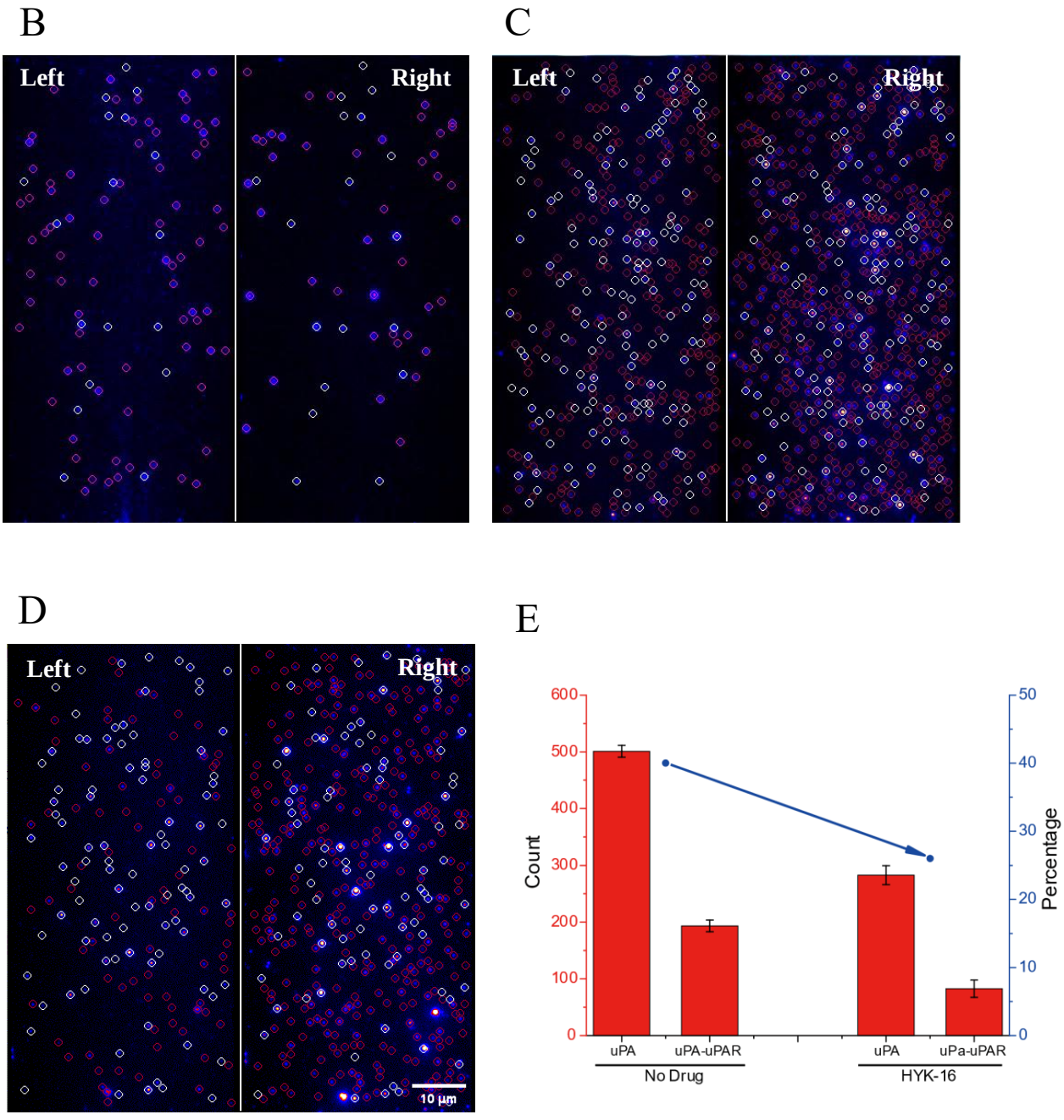
Schematic Illustration of the Inhibition of uPAR and uPA binding





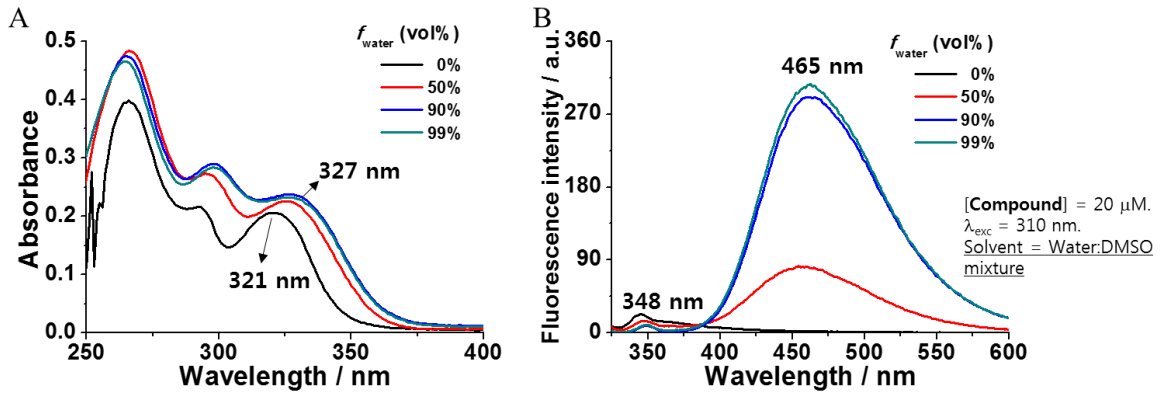
Binding Inhibition of uPA and uPAR

Inhibition of uPAR and uPA by HYK-16



The uPAR-uPA protein binding was confirmed using a single-molecule binding assay. In both panels, the left panel illustrates uPA binding, and the right panel displays uPAR binding, with red circles marking binding spots. The white circles indicate uPAR-uPA colocalization. (B) represents non-specific binding, (C) shows uPAR-uPA binding without HYK-16, and (D) shows binding with HYK-16. (E) Binding of uPAR-uPA was inhibited by approximately 35% at a concentration of 10 nM HYK-16, relative to the binding ratio of uPA binding spots. Scale bar; 10 μm

2.3 Detection of Fluorescence of HYK-16



Determination of excitation and emission wavelength of HYK-16. Absorption (A) and fluorescence emission (B) spectra of 2 in DMSO-water mixtures with different water fraction (f_{water} (vol %); 0, 50, 90, 99%) at 25 °C. Excited at 310 nm. [2] = 20 μM.

2.4 Biological Studies

2.4.1. Cell Culture

Human cancer cell lines MDA-MB-231, MCF-7, T47D, MDA-MB-468, AsPC-1, and PANC-1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Breast cancer cells were cultured in Dulbecco Modified Eagle Medium (DMEM; Gibco BRL, Grand Island, NE, USA) and pancreatic cancer cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco) supplemented with 10% Fetal Bovine Serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C in a humidified atmosphere with 5% CO_2.

2.4.2. Fluorescence Polarization (FP) Assay

FP was measured to assess the inhibition of AE147-FAM binding to uPAR. 20 nM of recombinant uPAR (#10925-H08H, Sino Biological, China) was incubated with 100 μM of AE147-FAM (Bankpeptide Biological Technology, Hefei, Anhui, China) in assay buffer at room temperature for 30 minutes to allow for binding equilibrium. After incubation, 1 mM of HYK compounds were added to the uPAR-AE147-FAM complex, followed by 30 minutes incubation at room temperature. FP was measured using a FlexStation 3 plate reader (Molecular Devices, Sunnyvale, CA, USA) with excitation at 485 nm and emission at 535 nm. 20 μM of IPR-69, a known uPAR inhibitor, was used as a positive control to confirm the inhibition of the uPAR-AE147-FAM interaction.

2.4.3. Cell Viability Assay

Cell viability was assessed using the Quanti-MAX WST-8 Cell viability Assay Kit reagent (Biomax, Seoul, Korea), which is based on the conversion of the tetrazolium salt WST-8 to a water-soluble formazan dye by cellular dehydrogenases in viable cells. The cancer cells were seeded in a 96-well plate at a density of $1x10^4$ cells per well. After 24 hours, the medium was aspirated, and the cells were treated with the HYK-16 or HYK-23 at various concentrations (0, 10, 20, 40, 80, and 100 μM). The cells were incubated with the compounds for 24, 48, and 72 hours under the same culture conditions. At the end of the treatment period, 10 μL of the WST-8 reagent was added to each well and then incubated for an additional 3 hours at 37°C in the CO_2 incubator. After the incubation with WST-8 reagent, the absorbance of the formazan was measured at 450 nm using a FlexStation 3 plate reader.

2.4.4. Single Molecule Binding Assay

To obtain the binding events between uPA and uPAR, we immobilized each protein on a PEGylated quartz slide. The immobilization was carried out by sequentially adding 1 mg/mL biotinylated BSA (Sigma), 0.05 mg/mL NeutrAvidin (Thermo Fisher) and 100 nM biotin-conjugated his-tag antibody (abcam) to the surface. This His-tag antibody was used to capture the uPA or uPAR proteins on the imaging surface.

Before testing uPA and uPAR binding, each protein was labeled with Cy3 or Cy5 dyes. A concentration of 1 nM of each labeled protein was introduced into an image buffer composed of 4 mM Trolox, 10 mM Tris-HCl, pH 8, 50 mM NaCl, 0.2% (w/v) glucose, 0.31 mg/mL Glucose Oxidase, 2170 U/mL catalase. The mixture was pre-incubated on ice for 30 min. Following incubation, the solution was added to the imaging surface and further incubated for 10 min in the dark to allow binding. Binding events were visualized as single fluorescence spots using a Total Internal Reflection Fluorescence (TIRF) microscope. The uPA binding was detected at 532 nm in the left panel, while uPAR binding was observed at 633 nm in the right panel. Fluorescent spots were counted to determine the binding affinity of the drug for the protein.

To assess the inhibition effect of the compound HYK-16, 1 nM of uPA,1 nM of uPAR and 10 nM of HYK-16 were pre-incubated together on ice for 30 min. The subsequent experimental procedure followed the same steps as described above for the binding assay. The binding affinity(f) of uPAR-HYK-16 was calculated using the equation f = [drug] / K_d+[drug]. Although the use of the compound resulted in an overall decrease in the number of binding spots, this reduction did not affect the compound's inhibition effect, suggesting that the reduction may not be essential to the molecule's function.

2.4.5. Confocal Microscopy Analysis

The cells were seeded onto glass coverslips in 24-well plate and allowed to adhere overnight in a humidified atmosphere at 37°C with 5% CO_2. The cells were treated with 40 μM of HYK-16 or HYK-23 for 24 hours. Following the treatment period, the cells were washed with Phosphate Buffered Saline (PBS; Gibco). The cells were then fixed with 4% formaldehyde (Junsei Chemical, Kyoto, Japan) for 15 minutes at room temperature. After fixation, the cells were permeabilized with 0.1% Triton X-100 (Daejung, Kyungkido, Korea) in PBS for 10 minutes and blocked with 1% Bovine Serum Albumin (BSA; Bovogen, Keilor East, Australia) in PBS for 1 hour at room temperature to reduce nonspecific binding. The anti-uPAR antibody (sc-376494, Santa Cruz Biotechnology, CA, USA) was applied to the cells at a dilution of 1:200 and incubated overnight at 4°C. Alexa Fluor™ 594 goat anti-mouse IgG (ThermoFisher Scientific, Waltham, MA, USA) was then applied to the cells and incubated at room temperature for approximately 1 hour. The glass coverslips with adherent cells were mounted onto glass slides using mounting medium (Vector Laboratories, Burlingame, CA, USA) and imaged using a confocal microscope (Zeiss LSM 800, Carl Zeiss Microscopy, Oberkochen, Germany).

2.4.6. Molecular Docking

We used HADDOCK version 2.4 for our protein–ligand docking study[2]. HADDOCK was also employed to refine the docked structures starting from randomly generated initial structures. This process included rigid body docking followed by semi-flexible searching using simulated annealing, particularly at the interface region. The docking process was completed by considering water solvation. During this process, the number of possible docked structures was narrowed down based on the docking scores. The default number of initial structures generated in rigid body docking was 1,000, and the 200 best structures were subjected to the next semi-flexible docking stage. Finally, 200 possible docked structures were obtained for analysis after considering water solvation. For better results, we changed these values to 2,000, 400, and 200, respectively. Regarding the parameters for HADDOCK, we used the default 5.4 version of protein and solvent topologies as implemented in HADDOCK 2.4 throughout the docking procedure. The starting structure of uPAR was obtained from the Protein Data Bank (PDB ID: 2FD6, chain U) and used active site residues (Thr8, Arg25, Thr27, Val29, Arg53, Leu55, Leu66, Glu68, Thr127, Asp141, Leu150, His166, Asp254) with no passive site. For the topology and parameter files of the ligands (HYK-16 and HYK-23), we used the PRODRG server (http://davapc1.bioch.dundee.ac.uk/prodrg). For the analysis of the docked structures, we employed Fraction of Common Contact (FCC) based cluster analysis as incorporated in HADDOCK. In FCC, the structural similarity for clustering was based on atomic contact with a predefined distance as the contact threshold. The structure figures were plotted using the program PyMOL (http://pymol.sourceforge.net).

2.4.7. Wound Healing and Invasion Assay

Each cancer cells were seeded in 6-well plate coated with Collagen Type I (Corning, Bedford, USA) and allowed to grow to confluence. Once the cells reached confluence, a uniform scratch wound was created using a sterile pipette tip, resulting in wound between the cell layers. The cells were treated with HYK-16 or HYK-23 for 24 hours. The cell images of the wounds were captured at 24 hours using a microscope (Best Scope, BS-7000B, China). To invasion assay, the cells were seeded in transwell (Corning) coated with Matrigel Matrix (Corning). After 24 hours, the cells were treated with HYK-16 or HYK-23 for 24 hours. After incubation, invading cells on the membrane were stained with crystal violet (Sigma Aldrich, St. Louis, MO, USA) and visualized under a microscope (Best Scope). 100 nM of phorbol 12-myristate 13-acetate (PMA; Selleckchem, Houston, TX, USA) was treated for stimulation of migration or invasion.

2.4.8. Western Blotting

The cells were lysed using ProEX™ CETi Lysis Buffer (TransLab, Daejeon, Korea). Protein concentrations were determined using the bicinchoninic acid assay kit (Thermo). Equal amounts of protein (20 μg per lane) were separated by 8% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA, USA). Membranes were blocked with 5% BSA in Tris-Buffered Saline with Tween 20 (Bio-Rad,

Hercules, CA, USA) for 1 hour and then incubated overnight at 4°C with anti-uPAR antibody (Santa Cruz) and β-actin (#4967, Cell Signaling Technology, Danvers, MA, USA), both diluted 1:1,000. After washing, membranes were incubated with HRP-conjugated secondary antibody (Cell Signaling) diluted 1:3,000. Protein bands were visualized using an ECL detection system (ATTO, Tokyo, Japan) and captured using a ChemiDoc imaging system (Bio-Rad).

3. Supplementary References

1 H. Y. Kim, E. Song and K. Oh, *Org. Lett.*, 2017, **19**, 312-315.

2 G. C. P. van Zundert, J. P. G. L. M. Rodrigues, M. Trellet, C. Schmitz, P. L. Kastritis, E. Karaca, A. S. J. Melquiond, M. van Dijk, S. J. de Vries and A. M. J. J. Bonvin, *J. Mol. Biol.*, 2016, **428**, 720-725.

Figure Legends

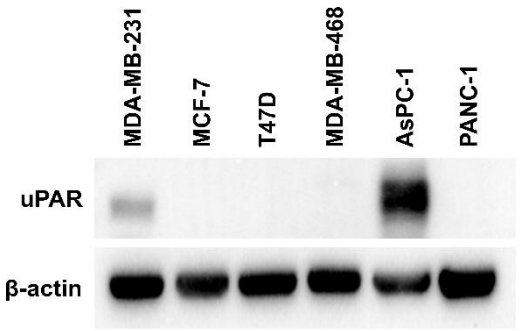


Figure S1. uPAR Protein Expression: uPAR protein expression was analyzed by Western blot, using β-actin as a housekeeping protein.

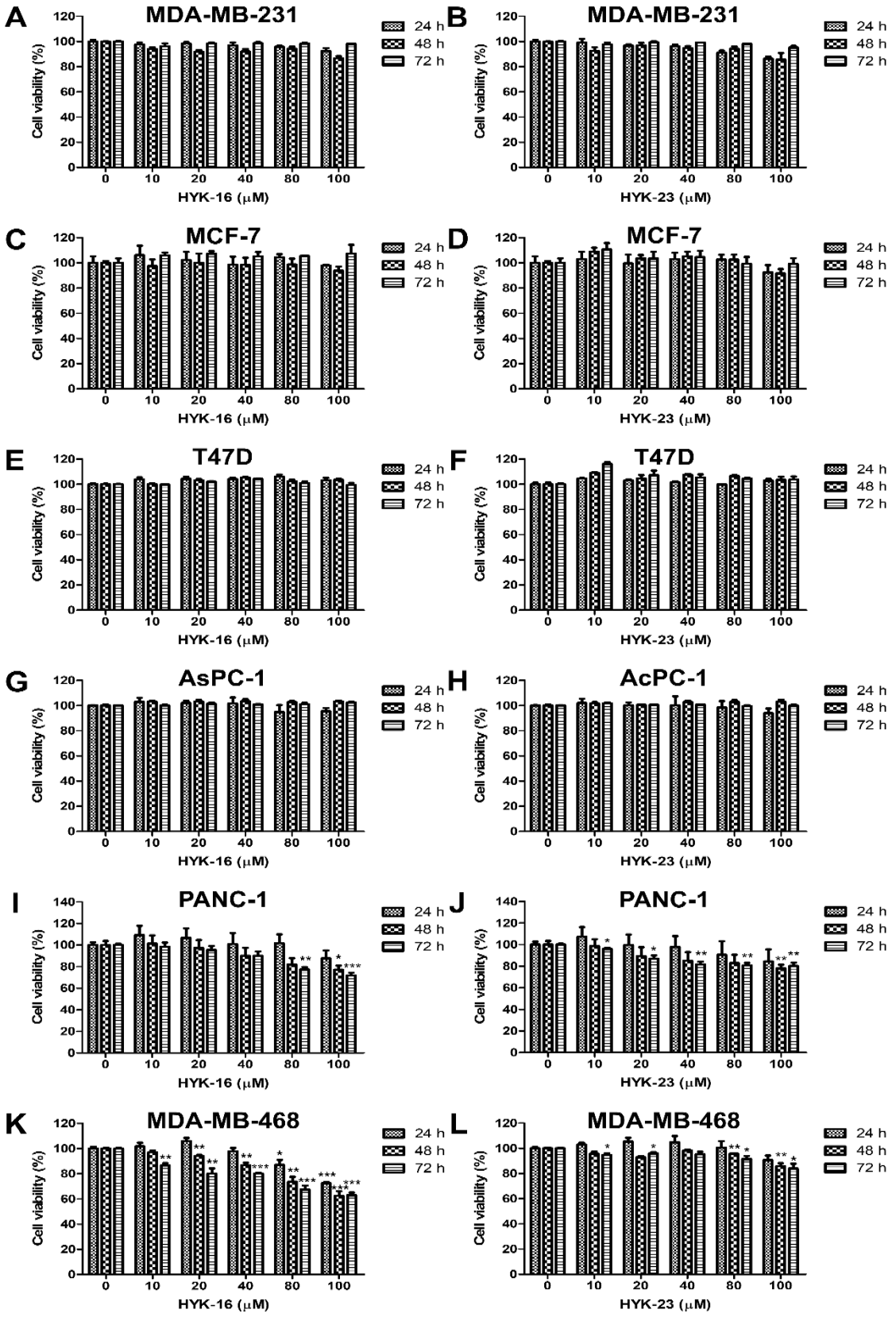

A
MDA-MB-231
Cell viability (%)
HYK-16 (μM)
B
MDA-MB-231
HYK-23 (μM)
C
MCF-7
D
MCF-7
E
T47D
F
T47D
G
AsPC-1
H
AcPC-1
I
PANC-1
J
PANC-1
K
MDA-MB-468
L
MDA-MB-468
24 h
48 h
72 h

Figure S2. Effect of HYK-16 or HYK-23 on Cell Viability in Cancer Cell Lines: Cell viability assessment of (A) HYK-16 or (B) HYK-23 in MDA-MB-231. Cell viability assessment of (C) HYK-16 or (D) HYK-23 in MCF-7. Cell viability assessment of (E) HYK-16 or (F) HYK-23 in T47D. Cell viability assessment of (G) HYK-16 or (H) HYK-23 in AsPC-1. Cell viability assessment of (I) HYK-16 or (J) HYK-23 in PANC-1. Cell viability assessment of (K) HYK-16 or (L) HYK-23 in MDA-MB-468. Cells treated with various concentrations (0, 10, 20, 40, 80, and 100 μM) of HYK-16 or HYK-23 for 24, 48, and 72 hours. Results are expressed as a percentage of cell viability relative to untreated controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control. Data represent the mean ± SEM.

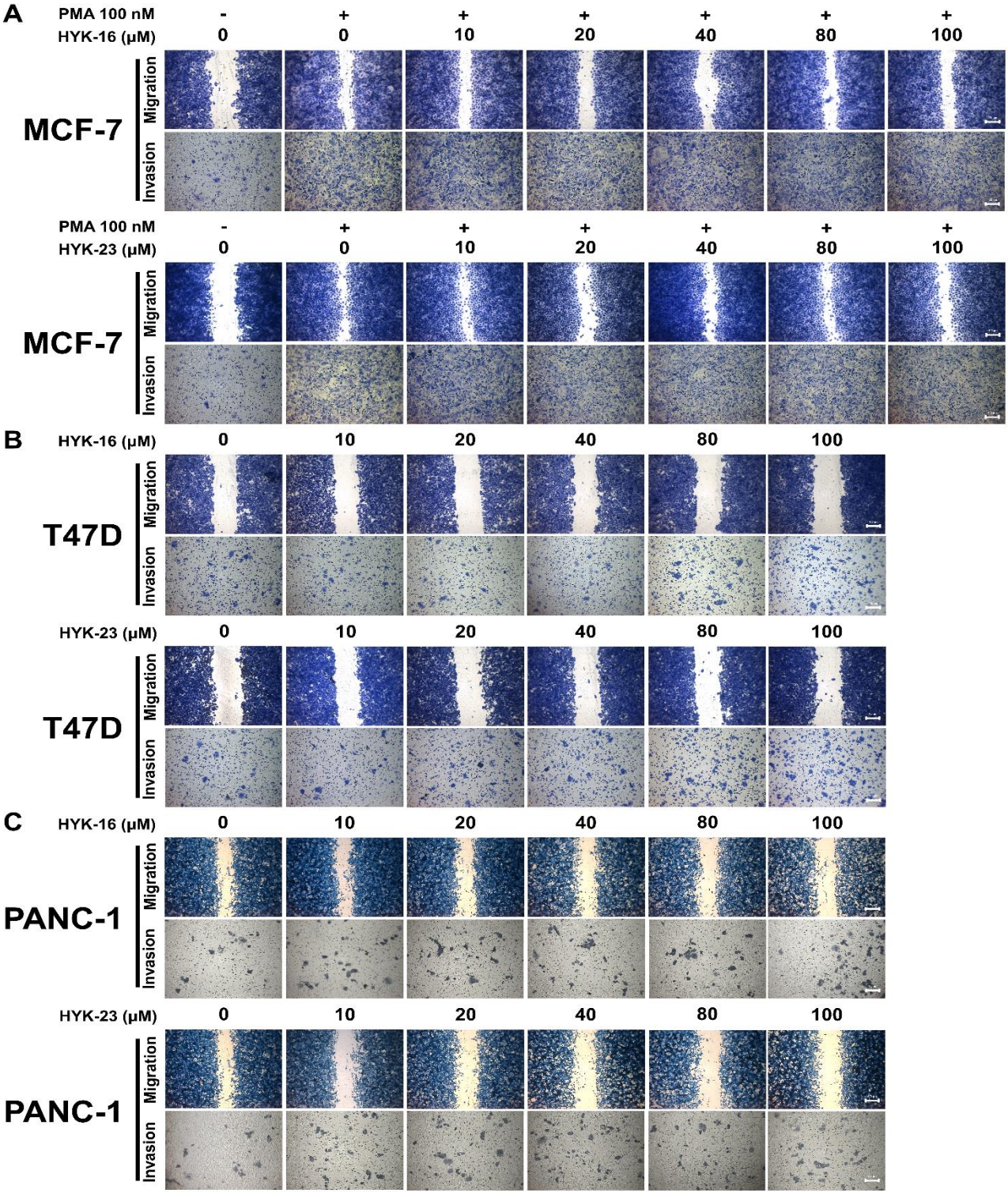


Figure S3. Effect of HYK-16 or HYK-23 on Wound Healing and Invasion Assay in Cancer Cells: (A) Effect of HYK-16 or HYK-23 on migration and invasion in MCF-7. (B) Effect of HYK-16 or HYK-23 on migration and invasion in T47D. (C) Effect of HYK-16 or HYK-23 on migration and invasion in PANC-1. Scale bar = 100 μm.