Supplementary Data: Experimental, characterization and results

HER2 inhibition efficiency of 6-amino-2-methyl-2-phenethyl-2H-benzopyran and feasibility of radioactive $^{64}$Cu-labeled benzopyran derivative in cancer diagnosis

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1. Synthesis of Compound 1

The 6-amino-2-methyl-2-phenethyl-2H-benzopyran (compound 4) was synthesized using 2-Hydroxy-5-nitroacetophenone as a starting compound and it (1.24 g, 6.8 mmol) was dissolved in 10 mL of toluene with the subsequent addition of 150 μL (1.8 mmol) pyrrrolidine. Then, 1 mL (6.6 mmol) of benzyl acetone was slowly added to the solution under constant stirring for 10 min and refluxed for 6 h. The resultant mixture was cooled to room temperature and extracted three times with 30 mL of distilled water and ethyl acetate (EtOAc). The separated organic layer was collected, washed with brine, dried over anhydrous MgSO$_4$ and distilled under reduced pressure. Furthermore, the mixture was purified by column chromatography (EtOAc: Hexane = 1: 5, v/v, Rf = 0.23) and obtained 0.94 g (3.0 mmol) to attain a pale-yellow oil, 2-methyl-6-nitro-2-phenethylbenzopyran-4-one (Compound 1) with the yield of 46%.

The characterization of the Compound 1 revealed, ρ 1.4 (density); LC-MS m/z 312, (M+H)$^+$; C$_{13}$H$_{15}$NO$_{6}$ 312.34 (calculated) and 312.2 (observed); $^1$H-NMR (500 MHz, DMSO-d$_6$): δ 8.48 (d, J = 2.9 Hz, 1H), 8.39 (dd, J = 9.1, 2.9 Hz, 1H), 7.27 (m, 3H), 7.19 (m, 3H), 3.11 (d, J = 16.7 Hz, 1H), 2.95 (d, J = 16.7 Hz, 1H), 2.75 (m, 2H), 2.05 (m, 2H), 1.48 (s, 3H); $^{13}$C NMR (125 MHz, DMSO-d$_6$): δ 191.24, 164.1, 141.7, 141.5, 131.1, 128.9, 128.7, 126.4, 122.4, 120.4, 119.9, 83.9, 46.5, 40.8, 29.6, 23.7.

2. Synthesis of Compound 2

Compound 1 (0.94 g, 3.0 mmol) was dissolved in 15 mL of tetrahydrofuran and 15 mL of methanol. NaBH$_4$ (0.20 g, 5.1 mmol) was slowly added with the subsequent intervals and stirred for 1 h at 10°C. Then, it was extracted 3 times with 30 mL of distilled water and EtOAc. The separated organic layer was collected, washed with brine, dried over anhydrous MgSO$_4$, filtered and distilled under reduced pressure. The mixture was purified by column chromatography (EtOAc: Hexane = 1: 5, v/v, Rf = 0.25) to yield 97% of 2-methyl-6-nitro-2-phenethylbenzopyran-4-ol (Compound 2) (0.93 g, 2.9 mmol).

The characterization showed, ρ 1.4(density); LC-MS m/z 314, (M+H)$^+$; C$_{14}$H$_{17}$NO$_{7}$ 314.36 (calculated) and 314.2 (observed); $^1$H-NMR (500 MHz, DMSO-d$_6$): δ 8.36 (m, 1H), 8.04 (m, 1H), 7.27 (m, 2H), 7.18 (m, 2H), 6.94 (dd, J = 10.3, 9.1 Hz, 1H), 5.83 (dd, J = 30.1, 6.0 Hz, 1H), 4.81 (m, 1H), 2.25 (m, 1H), 2.00 (m, 1H), 1.86 (m, 2H), 1.42 (s, 3H); $^{13}$C NMR (125 MHz, DMSO-d$_6$): δ 159.2, 142.3, 140.8, 128.8, 128.7, 127.8, 127.6, 126.3, 124.9, 124.8, 124.6, 118.0, 80.4, 61.2, 43.4, 29.9, 26.2, 24.2.

3. Synthesis of Compound 3

Compound 3 was synthesized by dissolving 0.94 g (2.9 mmol) of 2-methyl-6-nitro-2-phenethylbenzopyran-4-ol (compound 2) with 10 mL of CH$_2$Cl$_2$ and 1.0 mL (6.0 mmol) of N, N-diisopropylethylamine (DIPEA) and stirred for 5 min. Then, 465 μL (6.0 mmol) of methanesulfonyl chloride was slowly added and stirred under room temperature for 8 h. After this, the mixture was extracted three times with 100 mL of 1 N HCl and 30 mL of CH$_2$Cl$_2$. The
separated organic layers were combined, dried over anhydrous MgSO₄, filtered and distilled under reduced pressure. Then the obtained mixture was dissolved in 10 mL of toluene and 540 μL (3.6 mmol) of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was added slowly and refluxed for 16 h. The resultant mixture was cooled to room temperature and extracted three times with 30 mL of distilled water and 30 mL of EtOAc. The organic layer was collected, washed with brine, dried over anhydrous MgSO₄, filtered and distilled under reduced pressure. The mixture was purified by column chromatography (EtOAc: Hexane = 1: 4, v/v, Rf = 0.21) and obtained 69 % of 2-methyl-6-nitro-2-phenethyl-2H-benzopyran (compound 3).

Moreover, the characterization of compound 3 depicted ρ 1.3 (density); LC-MS m/z 296, (M+H)+; C₁₈H₁₈NO₃ 296.34 (calculated) and 296.2 (observed); ¹H-NMR (500 MHz, DMSO-d₆): δ 8.07 (m, 1H), 8.02 (d, J = 9.0 Hz, 1H), 7.26 (t, J = 7.4 Hz, 2H), 7.18 (dd, J = 15.0, 7.4 Hz, 3H), 6.94 (d, J = 8.9 Hz, 1H), 6.69 (d, J = 10.1 Hz, 1H), 6.00 (d, J = 10.1 Hz, 1H), 2.68 (t, J = 8.5 Hz, 2H), 2.01 (m, 2H), 1.47 (s, 3H); ¹³C NMR (125 MHz, DMSO-d₆): δ 159.1, 141.9, 141.3, 131.9, 128.8, 128.7, 126.3, 125.7, 122.5, 121.6, 121.8, 116.8, 81.4, 43.2, 30.2, 27.4.

4. Synthesis of Compound 4

Finally, compound 4 was synthesized by dissolving 0.60 g (2.0 mmol) of 2-methyl-6-nitro-2-phenethyl-2H-benzopyran in 5 mL of EtOAc, and 10 % Pd/C (0.1 g, 0.1 mmol) was slowly added to the solution. The reaction mixture was stirred at room temperature under of H₂ atmosphere for 20 h. After completion of the reaction, the reaction mixture was filtered and distilled under reduced pressure. The resultant mixture was purified by column chromatography (EtOAc: Hexane = 1: 4, v/v, Rf = 0.21) to yield 95% of 6-amino-2-methyl-2-phenethyl-2H-benzopyran (compound 4) (0.50 g, 1.9 mmol).

Here, the characterization showed ρ 1.6 (density); LC-MS m/z 266, (M+H)+; C₁₈H₂₀NO 266.36 (calculated) and 266.2 (observed); ¹H-NMR (500 MHz, DMSO-d₆): δ 7.26 (m, 3H), 7.17 (m, 4H), 6.51 (d, J = 8.4 Hz, 1H), 6.37-6.30 (m, 2H), 5.73 (d, J = 9.8 Hz, 1H), 2.66 (m, 2H), 1.87 (m, 2H), 1.35 (s, 3H); ¹³C NMR (125 MHz, DMSO-d₆): δ 143.9, 142.8, 142.6, 143.9, 130.9, 128.8, 128.6, 126.1, 123.6, 121.7, 116.4, 115.1, 112.4, 77.4, 42.5, 30.3, 25.9.


Compound 5 was synthesized by dissolving ~30 mg (0.11 mmol) of 6-amino-2-methyl-2-phenethylbenzopyran and 50 mg (0.09 mmol) of p-SCN-Bn-NOTA in 1 mL of CHCl₃ and 45 μL (0.27 mmol) of triethylamine was added into the mixture. After stirring for 24 h at room temperature, a semi-preparative HPLC column (C18, 5 μm, 250 × 10.0 mm, 0.1% TFA in water: 0.1% TFA in ACN = 55:45 , 254 nm, Rt = 37.8 min) to give 57 mg (0.08 g) of pale yellow solid precursor NOTA-Bn-SCN-2-methyl-2-phenethyl-2H-benzopyran was synthesized with the yield of 89%.

Characterization: 180-182 °C; LC-MS m/z 716, (M+H)+; C₃₂H₂₆N₄O₈S 716.87 (calculated) and 716.1 (observed); ¹H NMR (500 MHz, DMSO-d₆): δ 7.44 (d, J = 8.1 Hz, 2H), 7.40 (d, J = 8.4 Hz, 1H), 7.27 (m, 3H), 7.19 (m, 3H), 7.11 (d, J = 7.3 Hz, 2H), 6.75 (d, J = 9.1 Hz, 1H), 6.49 (d, J = 10.0 Hz, 1H), 5.82 (d, J = 9.9 Hz, 1H), 3.86-3.75 (br, 3H), 3.18-2.92 (br, 6H), 2.87-2.62 (br, 4H), 2.10-2.05 (m, 8H), 1.97-1.90 (m, 2H), 1.39 (s, 3H); ¹³C NMR (125 MHz, DMSO-d₆): δ 179.5, 177.0, 172.7, 149.8, 142.0, 137.4, 134.8, 131.0, 129.1, 128.9, 128.8, 128.7, 127.6, 125.0, 124.9, 123.6, 122.0, 121.2, 115.9, 77.5, 68.9, 63.5, 63.1, 62.8, 62.0, 58.3, 58.1, 55.7, 42.4, 40.4, 30.3, 25.9.


To synthesis Cu-6, ~11g (80 μmol) of CuCl₂ was dissolved in 10 mL of distilled water to prepare a stock solution. Then, 2.9 mg (4.0 μmol) of compound 5 and 500 μL (4.0 μmol, 1 eq) of CuCl₂ stock solution were added to the reaction vessel and the pH of the reaction mixture was adjusted to 5.1 using 5 % ammonium citrate buffer. After completion of the reaction, a semi-preparative HPLC column (C18, 5 μm, 250 × 10.0 mm, 0.1% TFA in water: 0.1% TFA in ACN = 55:45 (v/v) Rt = 20.0 min) to obtain 2.3 mg of the reference substance Cu-NOTA-Bn-SCN-2-methyl-2-phenethyl-2H-benzopyran (Cu-NOTA-Bn-SCN-Bzpyran) (3.0 μmol) with a yield of 75%.

Characterization: m.p. 193-195 °C; LC-MS m/z 777, (M+H)+; C₃₂H₂₆Bu₅N₄O₈S 777.39 (calculated) and 777.3 (observed); ¹H-NMR(500 MHz, DMSO-d₆): δ 7.46 (d, J = 8.4 Hz, 2H), 7.41 (d, J = 8.4 Hz, 1H), 7.27 (m, 3H), 7.19 (m,
ell proliferation was measured at 450 nm using a PerkinElmer VICTOR X4 Multimod. The proteins in the late was treated for 1 h incubation for 48 h in the incubator, the cells were collected in the supernatant was collected. 20 μL (50 μg) of each protein was treated with sodium dodecyl sulfate (SDS) gel and separated by SDS-

Western Blotting

For the Western blotting test, the SK-BR-3 cell line was sub-cultured in 100 mm culture plate at a concentration of 6 × 10^5 cells/plate and stabilized for a day. After stabilization, each plate was washed twice with 10 mL of PBS and the control group was treated with 1 mL of medium, and the comparative group was treated with 1 mL of 20 μM compound 4 at concentrations of 0 (DMSO 1: 1000), 1, 2, 5, 10, 20, 50, 80 and 100 μM. After incubation for 48 h, each well was washed twice with 100 μL of PBS and was treated with 100 μL of 10% EZ cytox and incubated for 4 h in the incubator. Then the cell proliferation was measured at 450 nm using a PerkinElmer VICTOR X4 Multimode Plate Reader instrument.
polyacrylamide gel electrophoresis (PAGE, at 160 V, 1 h). Gel proteins were transferred to polyvinylidene difluoride (PVDF) membrane by electrophoresis (90 V, 90 min). The membranes were treated with 10 mL of 5% skim milk (Skim milk 0.5 g in 10 mL TBS-T buffer) for blocking the non-specific binding of the antibody. 10 mL of 1% primary antibody was applied to the membrane and maintained over-night at 4 °C. After washing 3 times with 10 mL of TBS-T solution, 10 mL of 0.2% secondary antibody was treated and reacted at room temperature for 1 h. Then, the cells were reacted in 1 mL of electrochemiluminescence (ECL, Pierce Biotechnology) detection solution for 1 min and the proteins were identified on X-ray films.

11. Studies on Cellular uptake of $^{64}$Cu-6

The cellular uptake of $^{64}$Cu-6 was performed on SK-BR-3, BT-474 and MDA-MB-231 cell lines. The cells were placed in a 24 well plate with 1.0 x 10$^5$ cells per well and treated with $^{64}$Cu-6. After incubation at 37°C for 24 h, the supernatant was removed and the cells were washed by PBS to remove surface-bound radioactivity materials. Then, the cells were suspended in 0.1% SDS in PBS and the radioactivity of the supernatant and cells were determined by a gamma counter. The experiments of cell viability and cell uptake were carried out in triplicate.

12. Structural Stability of $^{64}$Cu-6

Structural stability of the labelled compounds was evaluated using the human serum at 0.5, 1, 2, 4, 16, and 24 h respectively. After incubation, the cells were incubated at 37 °C with a radio-TLC scanner. $^{64}$Cu-6 of 3.7 MBq/ml was added to 1 ml of serum, and the stability was over 94% after 24 h. Based on the stability evaluation results, it was proved that $^{64}$Cu labelled with the chelate reaction was released on the labelled compound was stable without being decomposed by another metabolism.

13. Lipophilicity of $^{64}$Cu-6

$$\log P = \log \frac{\text{concentration dissolved in octanol}}{\text{concentration dissolved in water}}$$

The result of fat-soluble evaluation, $^{64}$Cu-6 showed a partition coefficient value of 0.15. The distribution coefficient values are close to hydrophilicity when the value is negative with respect to 0, and lipophilic when the value is positive. Thus, it was found that the labeled compound synthesized in this study has considerable affinity.
Figure S1. Cu-64 labeled data (radio-TLC)

Radiochemical yield: 90%, Radiochemical purity: >98%, Specific activity: 3.7 GBq/μmol

Figure S2. Stability of $^{64}$Cu-6 in human serum
Figure S3. Cell viability analysis of compound 4 treated HER2-positive breast cancer cell, SK-BR-3 (IC₅₀ = 19 μM) (a) and HER2-negative breast cancer cell, MCF-7 (IC₅₀ = 62 μM) (b).

Figure S4. Cytotoxicity effect in A549 and KB, HER2(-) cell lines (compound 4 at 50 μM concentration)