

Supporting Information

Design and Discovery of an Orally Efficacious Spiroindolinone-Based Tankyrase Inhibitor for the Treatment of Colon Cancer

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1) Experimental procedures and analytical data for synthesized compounds.

Methyl 2-(4,6-difluoro-1'-(8-methyl-4-oxo-3,4,5,6,7,8-hexahydropyrido[2,3-d]pyrimidin-2-yl)-2-oxospiro[indoline-3,4'-piperidin]-1-yl)acetate (11g-Me).

To a solution of **24** (1 equiv, 60.6 mg, 0.163 mmol) in DMF (2 mL) were added methyl bromoacetate (11 equiv, 166 μ L, 1.79 mmol) and potassium carbonate (20 equiv, 495 mg, 3.58 mmol) at ambient temperature. After stirring for 1 hour, the reaction mixture was quenched with saturated ammonium chloride, extracted with ethyl acetate and washed with brine. The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was column chromatographed on silica gel (ethyl acetate/n-hexane = 50:50) to give 73.6 mg (quant., 0.179 mmol) of crude benzyl 4,6-difluoro-1-(2-methoxy-2-oxoethyl)-2-oxospiro[indoline-3,4'-piperidine]-1'-carboxylate (**25g**).

Crude **25g** (1 equiv, 73.6 mg, 0.163 mmol) was dissolved in methanol (1 mL) and 10% Pd-C (50% wet; 0.1 equiv, 34.0 mg, 0.016 mmol) was added. After stirring under a hydrogen atmosphere at ambient temperature for 1 hour, the mixture was filtered through a Celite pad and washed with methanol. The filtrate was evaporated under reduced pressure to give crude methyl 2-(4,6-difluoro-2-oxospiro[indoline-3,4'-piperidin]-1-yl)acetate (**21g**).

To a solution of crude compound **21g** in ethanol (1 mL) was added **28** (1.1 equiv, 35.7 mg, 0.18 mmol) and triethylamine (20 equiv, 499 μ L, 3.58 mmol) at ambient temperature. After stirring at 150 °C for 30 minutes with microwave irradiation, the reaction mixture was quenched with saturated sodium bicarbonate and extracted with chloroform. The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was column chromatographed on silica gel (methanol/chloroform = 10:90) to give 38.9 mg (46%, 0.082 mmol) of **11g-Me**.

2-Chloro-8-methylquinazolin-4(3H)-one (17).

To 2-Amino-3-methylbenzoic acid (1 equiv, 6.05 g, 40 mmol) in a round-bottom flask was added urea (5 equiv, 12.0 g, 200 mmol) at ambient temperature. After stirring at 180 °C for 1 h, the reaction mixture was cooled to ambient temperature, diluted with water (100 mL) and filtered to give crude 8-methylquinazoline-2,4(1H,3H)-dione. To this compound in a round-bottom flask was added phosphoryl chloride (10 equiv, 37 mL, 400 mmol) at ambient temperature. After stirring at 100 °C for 6 hours, the reaction mixture was cooled to ambient temperature and excess of phosphoryl chloride was removed under reduced pressure. The residue was diluted with chloroform, quenched with saturated sodium bicarbonate and extracted with chloroform. The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was column chromatographed on silica gel (ethyl acetate/n-hexane = 20:80) to give 6.86 g (81%, 32.2 mmol) of 2,4-dichloro-8-methylquinazoline (**16**).

To a solution of **16** (1 equiv, 2.13 g, 10 mmol) in acetonitrile (25 mL) was added 1N aq.

sodium hydroxide (1.5 equiv, 15 mL, 15 mmol) at ambient temperature. After stirring for 14 hours, the reaction mixture was quenched with saturated ammonium chloride and extracted with chloroform. The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was column chromatographed on silica gel (ethyl acetate/n-hexane = 50:50) to give 1.27 g (65%, 6.51 mmol) of **17**. ¹H NMR (400 MHz, DMSO-d₆) δ [ppm] 2.48 (s, 3H), 7.41-7.45 (m, 1H), 7.69-7.72 (m, 1H), 7.92-7.95 (m, 1H), 13.23 (br s, 1H).

2-Chloropyrido[2,3-*d*]pyrimidin-4(3H)-one (18).

To 2,4-dichloropyrido[2,3-*d*]pyrimidine (1 equiv, 4.0 g, 20 mmol) in a round-bottom flask was added 1N aq. sodium hydroxide (3 equiv, 60 mL, 60 mmol) at ambient temperature. After stirring for 1 hour, the reaction mixture was quenched with 1N aq. hydrochloric acid and filtered to give 3.49 g (96%, 19.2 mmol) of **18**.

2-Chloro-7-methyl-3,7-dihydro-4H-pyrrolo[2,3-*d*]pyrimidin-4-one (19).

To a solution of 2,4-dichloro-7H-pyrrolo[2,3-*d*]pyrimidine (1 equiv, 564 mg, 3.0 mmol) in THF (15 mL) were added iodomethane (1.5 equiv, 280 μL, 4.5 mmol) and sodium hydride (60% dispersion in paraffin liquid; 1.5 equiv, 180 mg, 4.5 mmol) at 0 °C. After stirring at ambient temperature, the reaction mixture was quenched with saturated ammonium chloride and extracted with chloroform. The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was column chromatographed on silica gel (ethyl acetate/n-hexane = 20:80) to give 487 mg (80%, 2.41 mmol) of 2,4-dichloro-7-methyl-7H-pyrrolo[2,3-*d*]pyrimidine.

To this compound (1 equiv, 206 mg, 1.02 mmol) in a round-bottom flask was added 2N aq. potassium hydroxide (5.4 equiv, 2.75 mL, 5.5 mmol) at ambient temperature. After stirring under reflux condition for 14 hours, the reaction mixture was quenched with saturated ammonium chloride and extracted with chloroform. The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was column chromatographed on silica gel (ethyl acetate/n-hexane = 50:50) to give 124 mg (61%, 0.67 mmol) of **19**. ¹H NMR (400 MHz, CDCl₃) δ [ppm] 3.77 (s, 3H), 6.71 (d, *J*=3.4 Hz, 1H), 6.83 (d, *J*=3.4 Hz, 1H), 12.83 (br s, 1H).

Benzyl 4,6-difluoro-2-oxospiro[indoline-3,4'-piperidine]-1'-carboxylate (24).

Synthetic procedure of **24** was modified according to the procedure of our previous paper²¹.

To a solution of 1-benzyloxycarbonyl-4-formylpiperidine (1 equiv, 3.81 g, 15.4 mmol) in chloroform (75 mL) were added 3,5-difluorophenylhydrazine hydrochloride (1.2 equiv, 3.34 g, 18.5 mmol) and trifluoroacetic acid (3 equiv, 3.5 mL, 46.3 mmol) at ambient temperature. The mixture was heated at reflux for 3 hours and cooled down to ambient temperature. To the reaction mixture was added *m*CPBA (2.4 equiv, 6.39 g, 37.0 mmol). After stirring for 1 hour, the reaction mixture was quenched with saturated sodium bicarbonate, extracted with chloroform and washed

with brine. The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was column chromatographed on silica gel (ethyl acetate/n-hexane = 50:50) to give 1.88 g (33%, 5.1 mmol) of **24**.

2-Chloro-8-methyl-5,6,7,8-tetrahydropyrido[2,3-d]pyrimidin-4(3H)-one (28).

To a solution of **18** (1 equiv, 6.36 g, 35 mmol) in THF-water (4:1, 200 mL) was added platinum (IV) oxide (0.1 equiv, 800 mg, 3.5 mmol). After stirring under a hydrogen atmosphere at ambient temperature for 14 hours, the mixture was filtered through a Celite pad and washed with tetrahydrofuran-water (5:1). The filtrate was evaporated under reduced pressure to give 6.00 g (92%, 32.3 mmol) of 2-chloro-5,6,7,8-tetrahydropyrido[2,3-d]pyrimidin-4(3H)-one. ¹H NMR (400 MHz, DMSO-d₆) δ [ppm] 1.65-1.80 (m, 2H), 2.32-2.40 (m, 2H), 3.17-3.25 (m, 2H), 7.37 (s, 1H), 11.80 (br s, 1H).

To a solution of 2-chloro-5,6,7,8-tetrahydropyrido[2,3-d]pyrimidin-4(3H)-one (1 equiv, 5.75 g, 31 mmol) in THF (100 mL) were added chlorotriisopropylsilane (1.5 equiv, 9.96 mL, 46.5 mmol) and imidazole (2 equiv, 4.21 g, 62 mmol) at ambient temperature. After stirring for 1 hour, the reaction mixture was quenched with water, extracted with ethyl acetate and washed with brine. The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was column chromatographed on silica gel (ethyl acetate/n-hexane=5:95) to give 11.2 g (quant., 32.7 mmol) of 2-chloro-4-((triisopropylsilyl)oxy)-5,6,7,8-tetrahydropyrido[2,3-d]pyrimidine (**27**).

To a solution of **27** (1 equiv, 9.69 g, 28.3 mmol) in DMF (150 mL) were added iodomethane (1.1 equiv, 1.95 mL, 31.3 mmol) and sodium hydride (60% dispersion in paraffin liquid; 1.5 equiv, 1.71g, 42.8 mmol) at 0 °C. After stirring at ambient temperature for 1 hour, the reaction mixture was quenched with saturated ammonium chloride, extracted with ethyl acetate and washed with brine. The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was dissolved in THF-acetic acid (10:1, 100 mL) and 1M tetrabutylammonium fluoride in THF (1 equiv, 28 mL, 28 mmol) was added at ambient temperature. After stirring for 30 minutes, the reaction mixture was quenched with water and extracted with chloroform. The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was column chromatographed on silica gel (methanol/chloroform = 3:97) to give 2.33 g (41%, 11.7 mmol) of **28**. ¹H NMR (400 MHz, CDCl₃) δ [ppm] 1.85-1.93 (m, 2H), 2.55-2.60 (m, 2H), 3.13 (s, 3H), 3.30-3.38 (m, 2H).

4-Fluoro-6-methoxy-1-methyl-2-oxospiro[indoline-3,4'-piperidine]-1'-carboxylate (33).

Synthetic procedure of **33** was modified according to the procedure of our previous paper²¹.

To a solution of 2-bromo-1,3-difluoro-5-methoxybenzene (1.12 g, 5.0 mmol) in 1,4-dioxane (25 mL) were added *tert*-butyl cyanoacetate (1.2 equiv, 856 μL, 6.0 mmol), sodium *tert*-butoxide (2.5 equiv, 1.20 g, 12.5 mmol), tris(dibenzylideneacetone) dipalladium(0) (0.01 equiv, 45.8 mg,

0.05 mmol) and tri-*tert*-butylphosphonium tetrafluoroborate (0.02 equiv, 29.0 mg, 0.10 mmol) at ambient temperature. After stirring at 180 °C for 30 minutes with microwave irradiation, the mixture was quenched with water and filtered through a Celite pad. The filtrate was extracted with ethyl acetate and washed with brine. The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was column chromatographed on silica gel (ethyl acetate/n-hexane = 20:80) to give 1.28 g (90%, 4.52 mmol) of *tert*-butyl 2-cyano-2-(2,6-difluoro-4-methoxyphenyl)acetate (**29**). ¹H-NMR (400 MHz, CDCl₃) δ: 1.50 (s, 9H), 3.81 (s, 3H), 4.92 (br s, 1H), 6.50-6.55 (m, 2H).

Compound **29** (1 equiv, 1.28 g, 4.52 mmol) was dissolved in toluene (20 mL) and added *p*-toluenesulfonic acid monohydrate (0.11 equiv, 85.9 mg, 0.5 mmol) at ambient temperature. After stirring at reflux for 1 hour, the reaction mixture was quenched with saturated sodium bicarbonate and extracted with ethyl acetate. The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was column chromatographed on silica gel (ethyl acetate/n-hexane = 20:80) to give 698.8 mg (85%, 3.82 mmol) of 2-(2,6-difluoro-4-methoxyphenyl)acetonitrile (**30**). ¹H-NMR (400 MHz, CDCl₃) δ: 3.64 (s, 2H), 3.80 (s, 3H), 6.48-6.54 (m, 2H).

Compound **30** (1 equiv, 699 mg, 3.82 mmol) was dissolved in DMF (20 mL) and *tert*-butyl bis(2-chloroethyl)carbamate (1.2 equiv, 1.11 g, 4.58 mmol) and sodium hydride (60% dispersion in paraffin liquid; 3 equiv, 458 mg, 11.45 mmol) were added at 0 °C. After stirring at 80 °C for 3 hours, the reaction mixture was quenched with saturated ammonium chloride and extracted with ethyl acetate. The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was column chromatographed on silica gel (ethyl acetate/n-hexane = 20:80) to give 881 mg (66%, 2.50 mmol) of *tert*-butyl 4-cyano-4-(2,6-difluoro-4-methoxyphenyl)piperidine-1-carboxylate (**31**). ¹H-NMR (400 MHz, CDCl₃) δ: 1.48 (s, 9H), 2.11-2.20 (m, 2H), 2.30-2.36 (m, 2H), 3.17-3.29 (m, 2H), 3.79 (s, 3H), 4.10-4.27 (m, 2H), 6.46-6.51 (m, 2H).

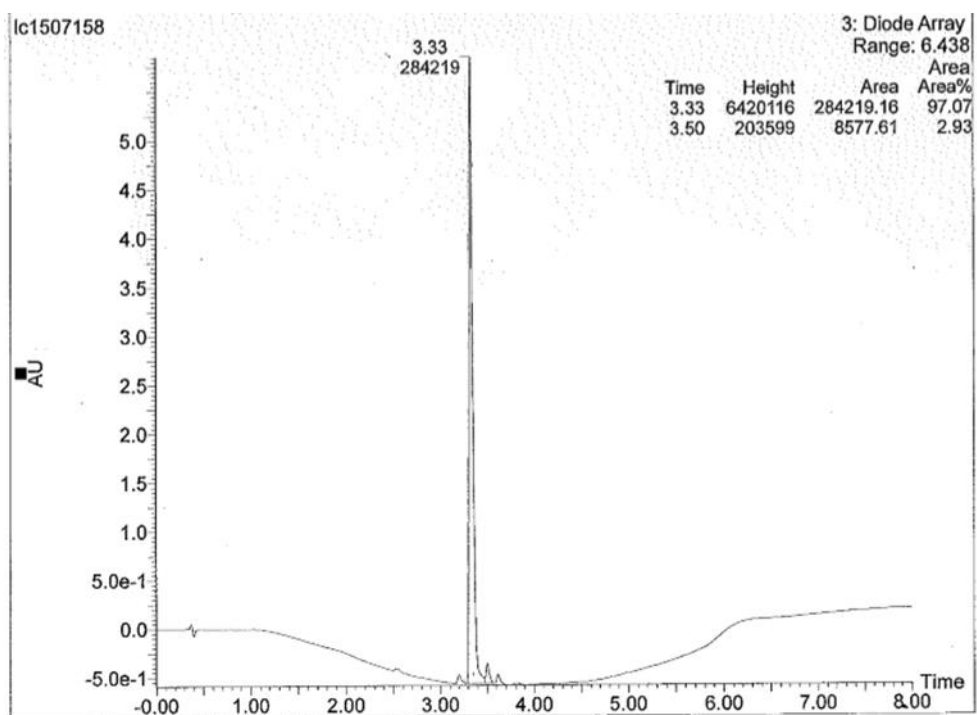
Compound **31** (1 equiv, 2.09 g, 5.92 mmol) was dissolved in DMSO (35 mL) and 5N aq. sodium hydroxide (2.11 equiv, 2.5 mL, 12.5 mmol) and 30% H₂O₂ aq. (3.7 equiv, 2.5 mL, 22.0 mmol) were added at 0 °C. After stirring at 80 °C for 14 hours, the reaction mixture was quenched with saturated ammonium chloride and extracted with ethyl acetate. The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was column chromatographed on silica gel (ethyl acetate/n-hexane = 20:80) to give 2.17 g (99%, 5.87 mmol) of *tert*-butyl 4-carbamoyl-4-(2,6-difluoro-4-methoxyphenyl) piperidine-1-carboxylate (**32**).

Compound **32** (1 equiv, 2.17 g, 5.87 mmol) was dissolved in DMF (30 mL) and lithium hydride (3 equiv, 140 mg, 17.6 mmol) was added at 0 °C. After stirring at 120 °C for 14 hours, the

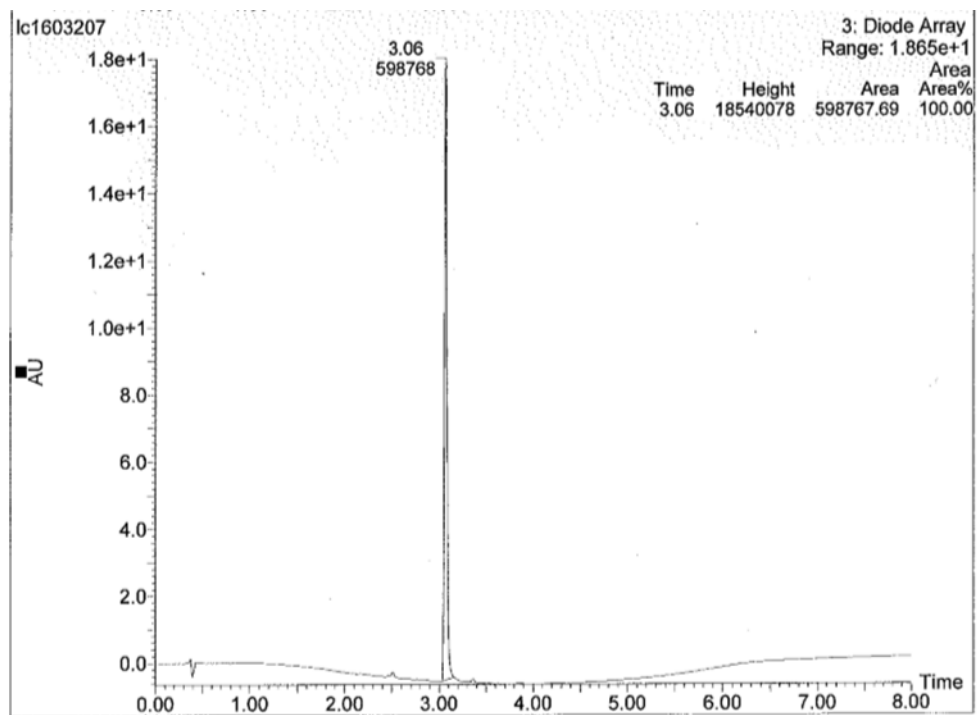
reaction mixture was cooled down to ambient temperature and iodomethane (3 equiv, 1.1 mL, 17.6 mmol) was added. After stirring at ambient temperature for additional 1 hour, the reaction mixture was quenched with saturated ammonium chloride and extracted with ethyl acetate. The organic layer was dried over magnesium sulfate, filtered and evaporated under reduced pressure. The residue was column chromatographed on silica gel (ethyl acetate/n-hexane = 50:50) to give 1.82 g (85%, 4.98 mmol) of **33**.

2) HPLC data of lead compounds 10, 11, 41d and 41g.

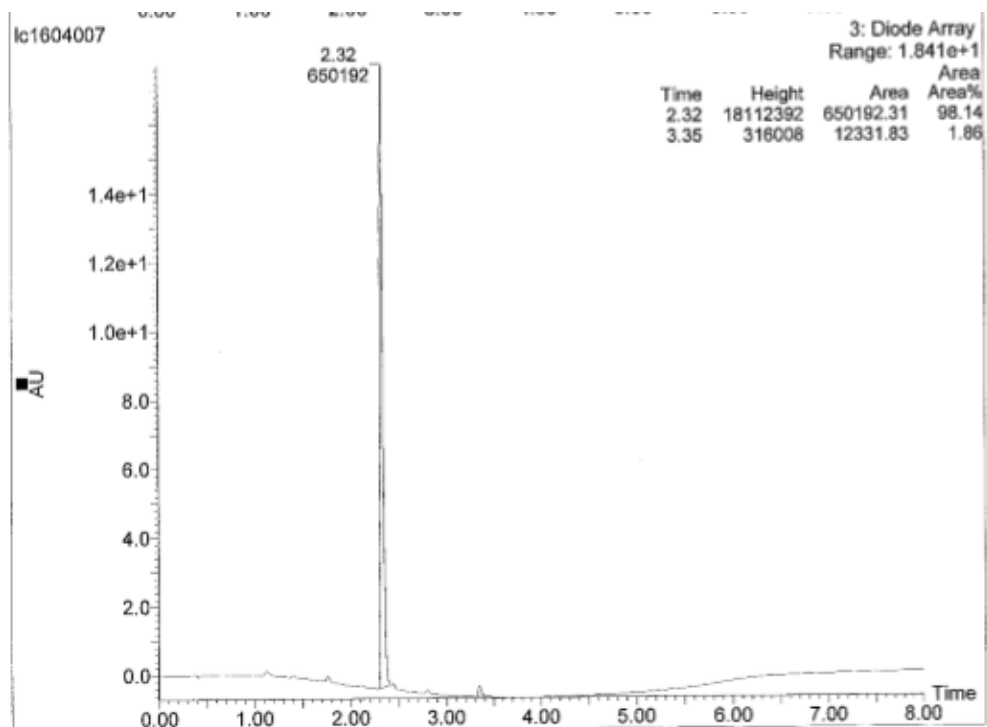
Compound 10.



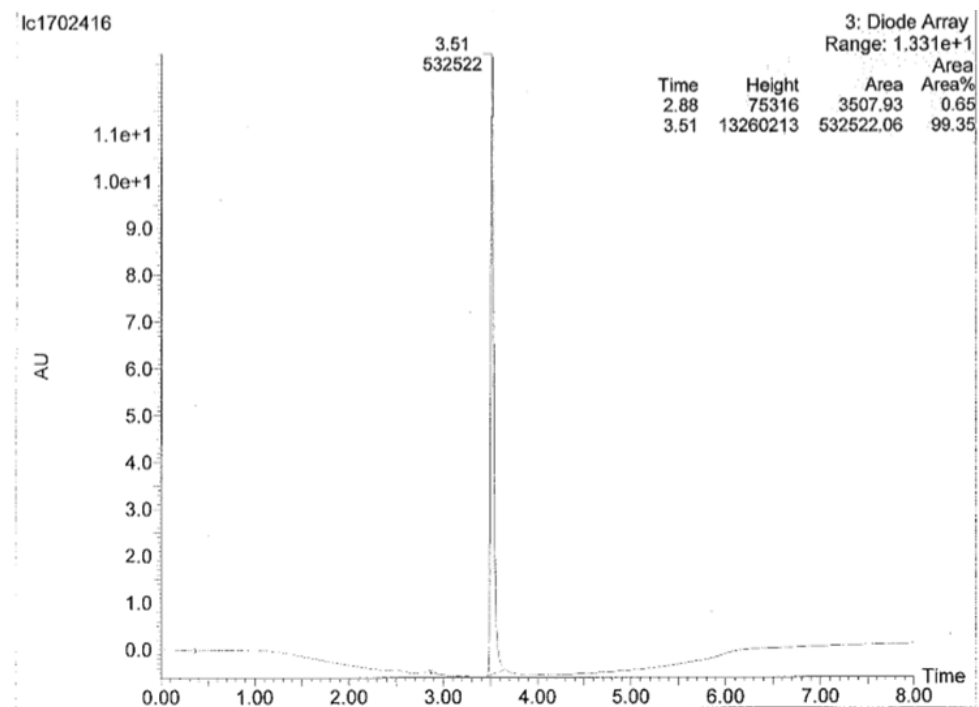
Compound 11.



Compound 41d.



Compound 41g.



3) *In vitro* biological data for synthesized compounds.

Table S1. *In vitro* data for analogs of tetrahydroquinazolinone moiety.

| No. | TNKS IC ₅₀ (nM) | SD | TNKS2 IC ₅₀ (nM) | SD | TCF reporter HEK293 (nM) | SD | TCF reporter DLD-1 (nM) | SD | COLO-320DM GI ₅₀ (μM) | SD |
|-----------|----------------------------|------|-----------------------------|------|--------------------------|------|-------------------------|------|----------------------------------|------|
| 1 | 14.3 | 1.1 | 10.6 | 2.4 | 12.1 | 4.5 | 77.6 | 26.8 | 1.63 | 0.40 |
| 7 | 14.9 | 5.2 | 20.6 | 6.6 | 2.1 | 1.8 | 71.2 | 23.3 | 2.27 | 1.12 |
| 8 | 5.5 | 2.1 | 8.5 | 1.1 | 3.1 | 2.6 | 13.7 | 4.9 | 0.72 | 0.28 |
| 9 | 5.3 | 2.4 | 3.4 | 2.3 | 0.2 | 0.1 | 4.8 | 6.1 | 2.49 | 0.92 |
| 10 | 13.6 | 6.9 | 21.4 | 0.5 | 2.7 | 3.2 | 7.0 | 4.1 | 0.25 | 0.05 |
| 11 | 7.5 | 4.8 | 13.5 | 5.0 | 1.1 | 0.6 | 3.9 | 2.9 | 0.25 | 0.10 |
| 12 | 3.8 | 0.1 | 7.0 | 0.1 | 1.1 | 0.3 | 7.1 | 1.6 | 3.56 | 2.78 |
| 13 | 4.5 | 0.8 | 10.2 | 0.5 | 1.5 | 0.8 | 6.3 | 0.1 | >20 | - |
| 14 | 53.5 | 29.2 | 73.6 | 36.2 | 36.5 | 14.4 | 140.6 | 34.3 | 5.01 | 0.51 |
| 15 | 14.1 | 5.4 | 27.6 | 4.0 | 4.4 | 0.9 | 32.9 | 22.0 | 2.37 | 1.43 |

Table S2. *In vitro* data for 1-substituted spiroindolinone derivatives.

| No. | TNKS IC ₅₀ (nM) | SD | TNKS2 IC ₅₀ (nM) | SD | TCF reporter HEK293 (nM) | SD | TCF reporter DLD-1 (nM) | SD | COLO-320DM GI ₅₀ (μM) | SD |
|------------|----------------------------|------|-----------------------------|------|--------------------------|------|-------------------------|-------|----------------------------------|------|
| 11 | 7.5 | 4.8 | 13.5 | 5.0 | 1.1 | 0.6 | 3.9 | 2.9 | 0.25 | 0.10 |
| 11a | 7.9 | 2.3 | 10.7 | 1.6 | 3.9 | 2.1 | 14.4 | 17.2 | 0.22 | 0.21 |
| 11b | 10.3 | 1.3 | 11.7 | 4.0 | 0.3 | 0.1 | 3.0 | 0.7 | 0.05 | 0.03 |
| 11c | 16.2 | 5.0 | 32.4 | 27.4 | 0.2 | 0.0 | 2.4 | 0.1 | 0.07 | 0.05 |
| 11d | 3.7 | 0.2 | 6.1 | 1.9 | 0.2 | 0.1 | 1.6 | 0.8 | 0.44 | 0.32 |
| 11e | 4.3 | 1.6 | 5.5 | 1.5 | 0.1 | 0.0 | 5.7 | 0.1 | 0.53 | 0.29 |
| 11f | 7.8 | 2.4 | 12.2 | 0.8 | 0.3 | 0.1 | 7.2 | 1.6 | 0.20 | 0.11 |
| 11g | 28.5 | 17.5 | 29.8 | 1.3 | 220.5 | 75.5 | 1185.3 | 146.4 | 12.59 | 3.27 |
| 11h | 8.9 | 2.8 | 12.5 | 1.7 | 0.7 | 0.0 | 14.5 | 6.6 | 0.71 | 0.38 |

Table S3. *In vitro* data for 6-alkoxy substituted spiroindolinone derivatives.

| No. | TNKS IC ₅₀ (nM) | SD | TNKS2 IC ₅₀ (nM) | SD | TCF reporter HEK293 (nM) | SD | TCF reporter DLD-1 (nM) | SD | COLO-320DM GI ₅₀ (μM) | SD |
|------------|-------------------------------|-----|--------------------------------|------|-----------------------------------|-------|----------------------------------|-------|-------------------------------------|------|
| 37a | 4.4 | 1.3 | 6.2 | 1.3 | 0.2 | 0.1 | 4.5 | 3.8 | 0.13 | 0.12 |
| 37b | 3.5 | 0.2 | 4.7 | 0.1 | 0.8 | 0.4 | 2.3 | 0.8 | 0.16 | 0.11 |
| 37c | 4.8 | 0.1 | 6.4 | 0.7 | 0.1 | 0.0 | 1.3 | 1.2 | 0.05 | 0.04 |
| 37d | 4.9 | 0.1 | 11.1 | 2.5 | 0.3 | 0.0 | 6.1 | 3.3 | 0.81 | 0.23 |
| 37e | 11.1 | 2.1 | 12.4 | 6.2 | 0.4 | 0.1 | 6.6 | 1.7 | 1.08 | 0.28 |
| 37f | 12.0 | 1.0 | 33.3 | 3.1 | 2.5 | 1.8 | 5.0 | 2.5 | 1.09 | 0.83 |
| 37g | 9.2 | 3.7 | 17.1 | 10.9 | 0.3 | 0.2 | 2.8 | 1.3 | 0.33 | 0.24 |
| 37h | 7.9 | 1.5 | 18.7 | 0.2 | 1.2 | 1.0 | 6.3 | 3.5 | 0.74 | 0.30 |
| 37i | 6.1 | 2.2 | 21.2 | 6.0 | 0.1 | 0.1 | 2.7 | 0.9 | 0.66 | 0.24 |
| 37j | 4.2 | 0.4 | 9.9 | 3.5 | 506.9 | 215.5 | 1877.2 | 616.6 | 0.30 | 0.19 |

Table S4. *In vitro* data for 6-amino substituted spiroindolinone derivatives.

| No. | TNKS IC ₅₀ (nM) | SD | TNKS2 IC ₅₀ (nM) | SD | TCF reporter HEK293 (nM) | SD | TCF reporter DLD-1 (nM) | SD | COLO-320DM GI ₅₀ (μM) | SD |
|------------|-------------------------------|------|--------------------------------|------|-----------------------------------|-----|----------------------------------|------|-------------------------------------|------|
| 41a | 6.3 | 1.7 | 13.0 | 10.5 | 4.6 | 1.8 | 20.7 | 8.8 | 1.17 | 0.11 |
| 41b | 21.3 | 2.2 | 49.1 | 18.7 | 0.7 | 0.4 | 6.4 | 1.1 | 0.30 | 0.36 |
| 41c | 12.8 | 2.1 | 23.4 | 3.3 | 3.2 | 2.1 | 29.1 | 12.0 | 1.12 | 0.62 |
| 41d | 7.7 | 3.0 | 12.3 | 7.1 | 0.8 | 1.5 | 8.4 | 10.6 | 0.19 | 0.17 |
| 41e | 11.1 | 6.5 | 20.2 | 10.6 | 0.1 | 0.1 | 4.3 | 1.3 | 0.85 | 0.66 |
| 41f | 14.9 | 11.5 | 10.5 | 0.2 | 0.1 | 0.1 | 2.0 | 2.1 | 0.10 | 0.08 |
| 41g | 36.1 | 5.6 | 39.2 | 2.2 | 0.3 | 0.2 | 3.1 | 1.1 | 0.23 | 0.03 |

4) AXIN2 and β -catenin protein expression analysis of compound **37j**.

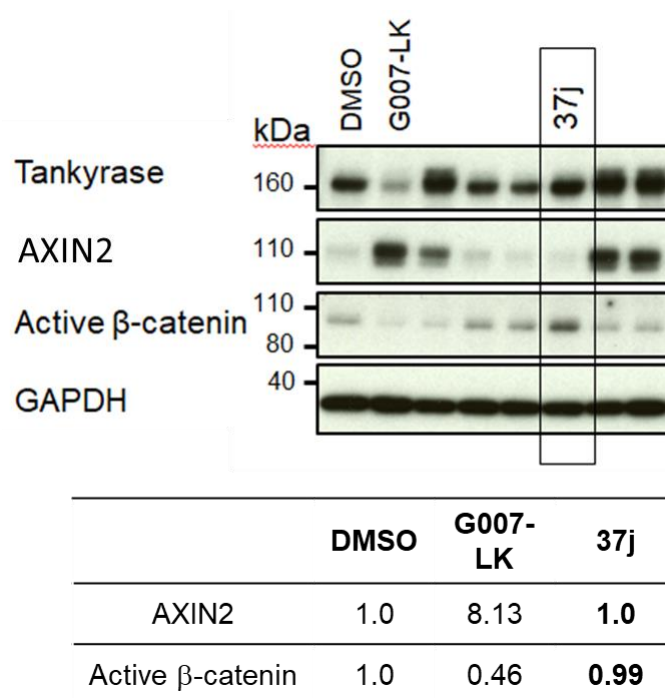


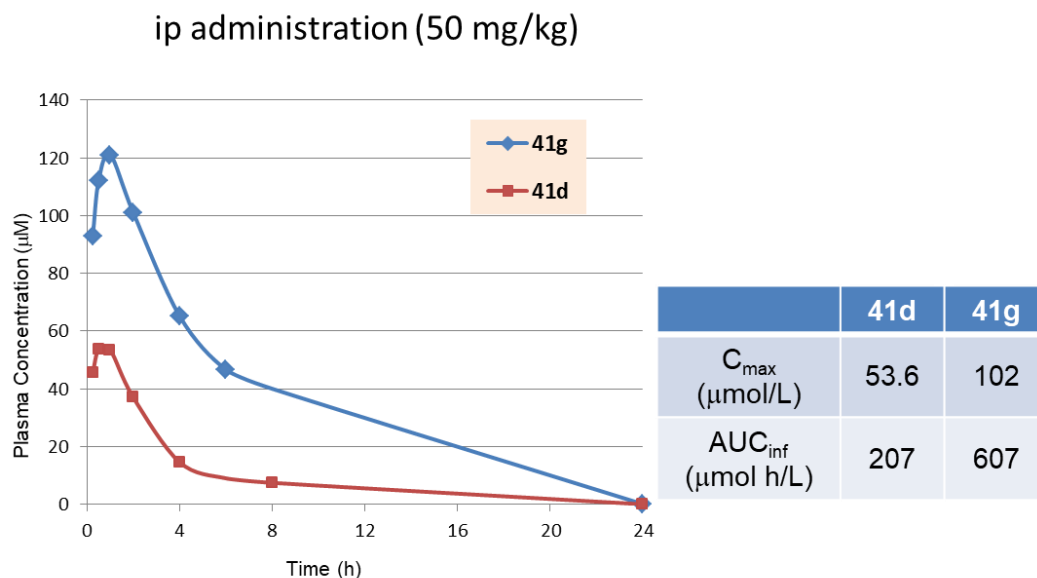
Figure S1. Western blot analysis of compound **37j** and G007-LK in cultured cells.

COLO-320DM cells were treated with 0.33 μ M for **37j** and G007-LK for 16 hours. Relative intensity value was determined by comparing with DMSO control.

Compound **37j** showed no accumulation of AXIN2 and attenuation of active β -catenin in protein expression level. These results indicated that cell growth inhibition of **37j** was not regulated by WNT/ β -catenin signaling pathway.

5) Mouse pharmacokinetics of 41d and 41g.

A



B

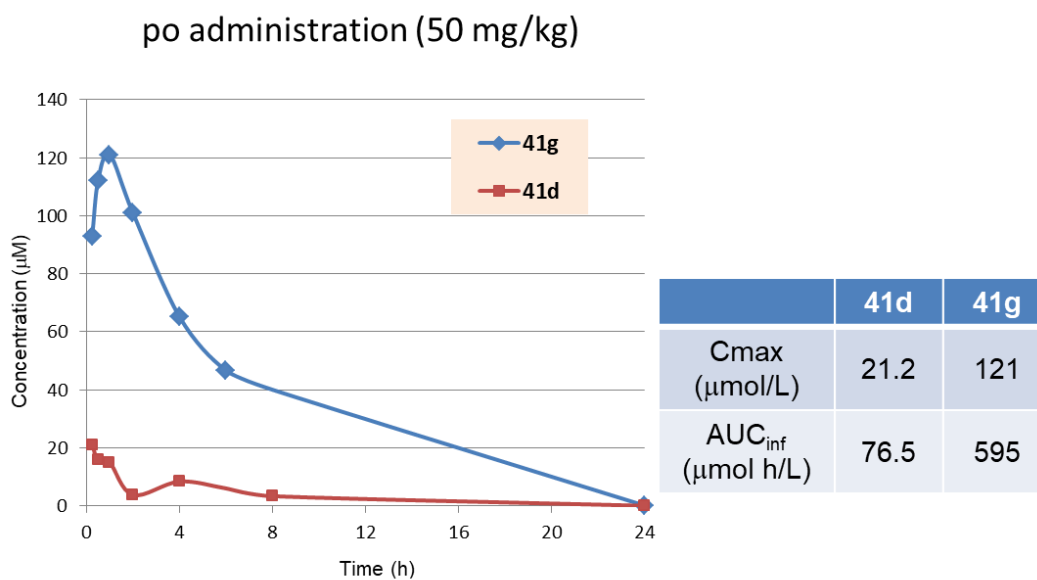
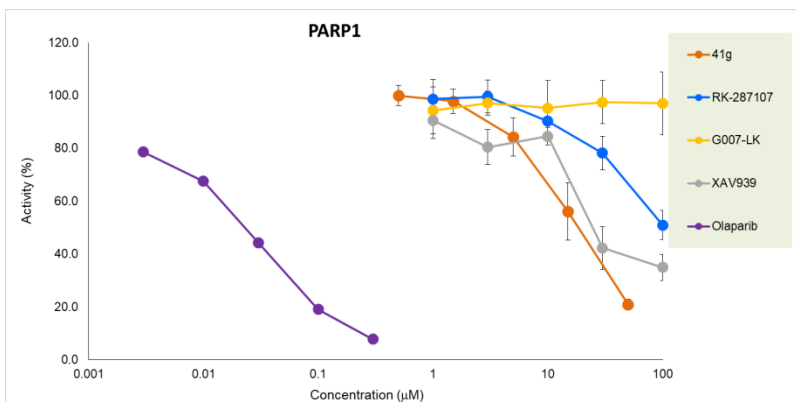


Figure S2. Mouse single dose pharmacokinetics of **41d** and **41g**.

A: 50 mg/kg intraperitoneal (ip) and **B:** 50 mg/kg per oral (po) administration, ICR mice; C_{max} and AUC_{inf} of **41g** were higher than those of **41d**, respectively. Pharmacokinetic parameters were calculated using the program, Phoenix WinNonlin ver. 8.1.0.¹

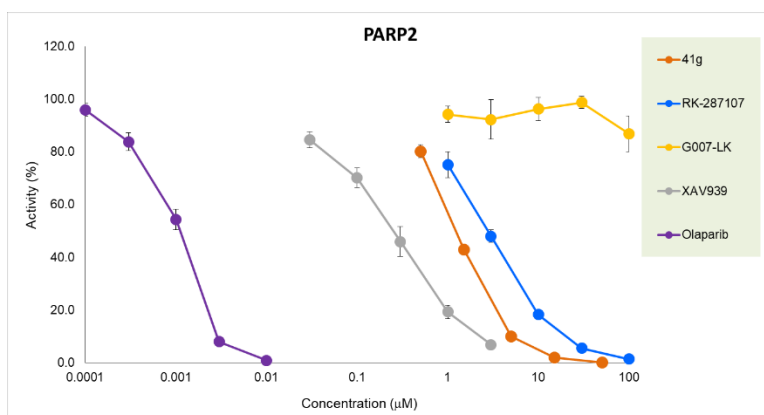
6) Enzyme selectivity of 41g.

A: PARP1



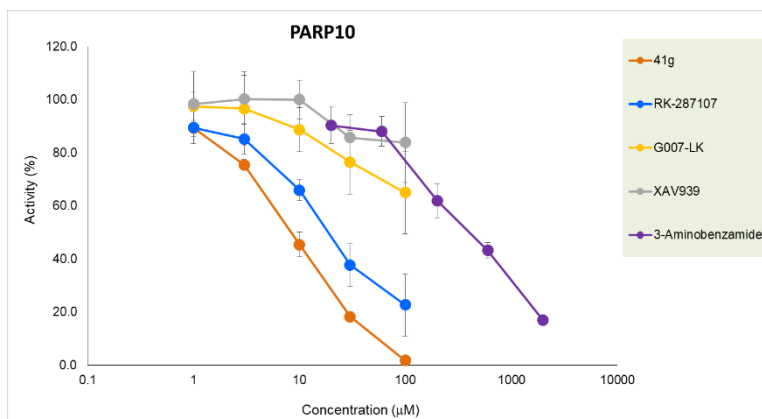
| Compound | PARP1 (μM) |
|--------------|-------------------------|
| 41g (RK-582) | 18.19 |
| RK-287107 | >100 |
| G007-LK | >100 |
| XAV939 | 34.91 |
| Olaparib | 0.021 |

B: PARP2



| Compound | PARP2 (μM) |
|--------------|-------------------------|
| 41g (RK-582) | 1.24 |
| RK-287107 | 2.72 |
| G007-LK | >100 |
| XAV939 | 0.24 |
| Olaparib | 0.001 |

C: PARP10



| Compound | PARP10 (μM) |
|------------------|--------------------------|
| 41g (RK-582) | 8.05 |
| RK-287107 | 19.81 |
| G007-LK | >100 |
| XAV939 | >100 |
| 3-Aminobenzamide | 391.79 |

Figure S3. PARP family enzyme inhibitory activity. Concentration-response curves for in vitro inhibition of A: PARP1, B: PARP2, and C: PARP10 enzymes by **41g** (RK-582), RK-287107, G007-LK, XAV-939, and Olaparib or 3-Aminobenzamide.

7) Structural analysis of the MTTP derivatives in PARP2.

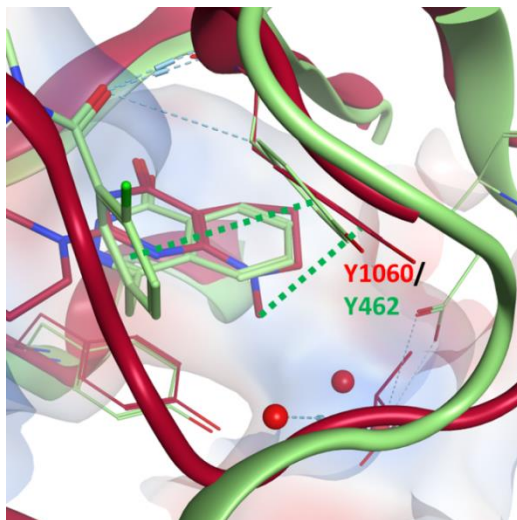


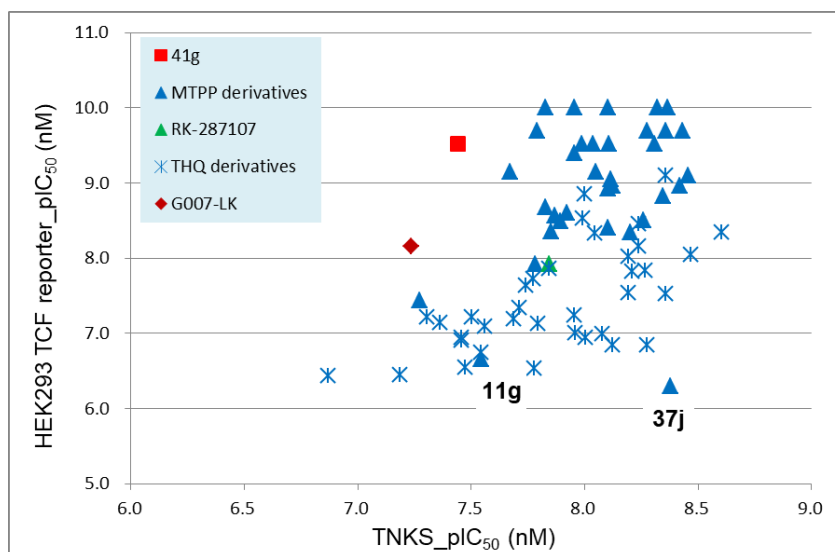
Figure S4. Superimposed complex structure of **41g** in TNKS2 and Olaparib in PARP2.

Compound **41g** in the TNKS2 (Red) and Olaparib in the PARP2 (Green) complex structures were superimposed. PARP2-Olaparib complex structure² was adopted from PDB database: 4TVJ.

Methyl substituent at 8-position of tetrahydropyridopyrimidinone (MTTP) moiety has CH- π interaction with Y1060 of TNKS2. On the other hand, the 8-methyl substituent has probably CH- π interaction with Y462 of PARP2, which is interacting with aromatic ring of Olaparib. Those data suggested that 8-methyl substituent might increase the inhibitory activity of TNKS2 and PARP2 similarly.

8) HEK293 and DLD-1 TCF reporter assay.

A



B

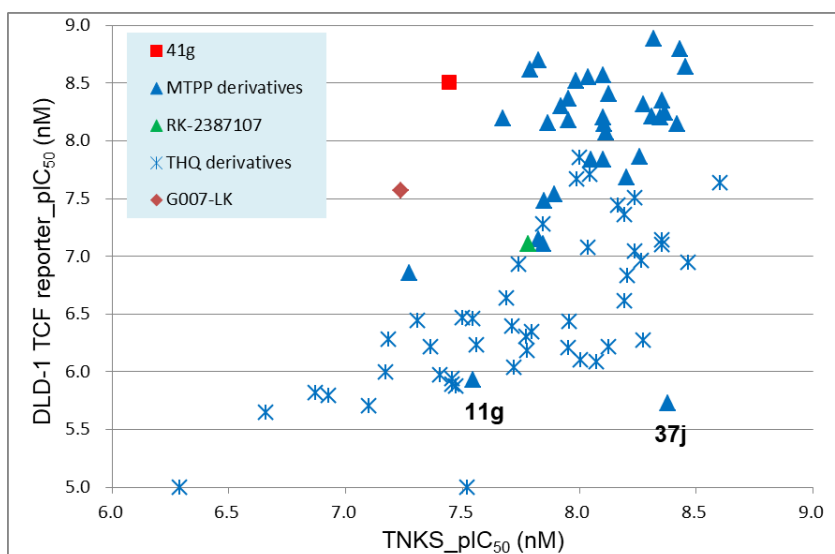


Figure S5. Correlation of pIC_{50} values between TNKS and HEK293 /DLD-1 TCF-reporter assay.

A: pIC_{50} of TNKS enzyme vs. HEK293 TCF reporter assay. **B:** pIC_{50} of TNKS enzyme vs. DLD-1 TCF reporter assay. (**41g**: Red rectangle; G007-LK: Brown rhombus; RK-287107: Green triangle; MTPP derivatives: Blue triangle; THQ derivatives of previous work³: Blue asterisk)

TCF reporter inhibitory activity is much stronger by introduction of MTPP moiety at the same IC_{50} value, and the enzyme inhibitory activities are correlated with the functional inhibition of Wnt/ β -catenin signaling without the compound **37j**, which has a carboxylic acid moiety. (Pearson's correlation $r = 0.54$ for MTPP and THQ series)

11g and **37g** show different behavior of correlation from other MTPP derivatives.

9) Cell growth inhibitory specificity by MTT cell assay.

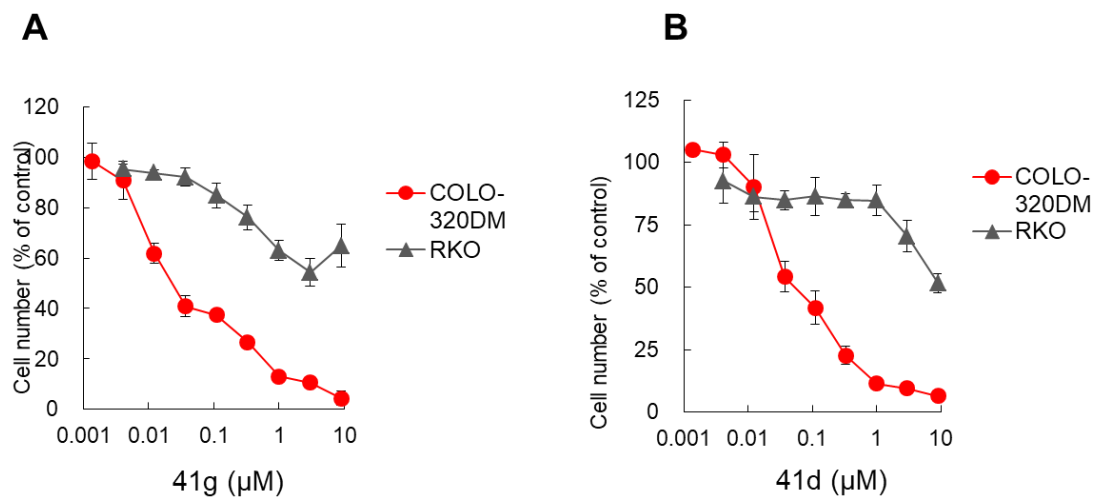


Figure S6. Concentration - COLO-320DM and RKO cell number curve using MTT in colorimetric colony assay. (A) COLO-320 DM and RKO cell proliferation assays for compound **41g**. (B) COLO-320 DM and RKO cell proliferation assays for compound **41d**.

10) Rat pharmacokinetic profile of 41g.

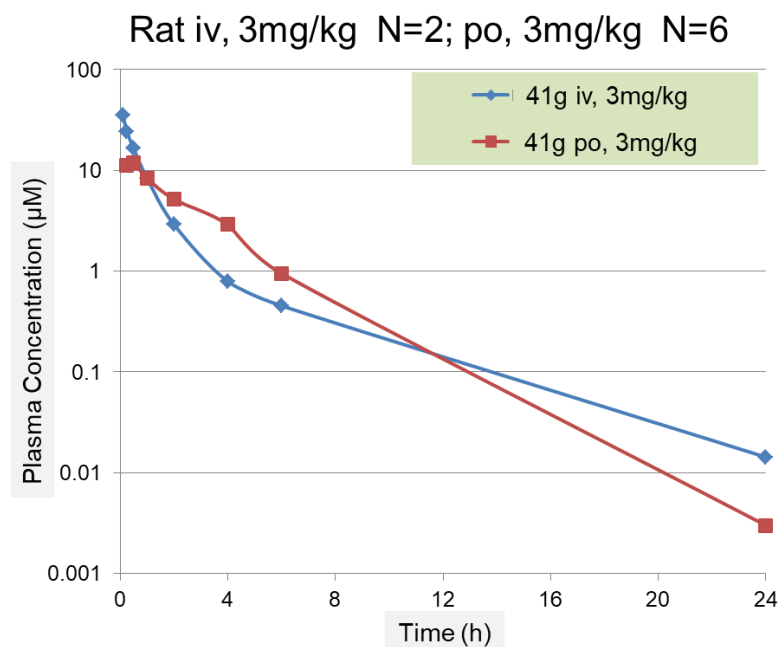


Figure S7. Rat pharmacokinetics of **41g** intravenous and oral administration. As the F value of **41g** exceeded 100% at a dose of 1 mg/kg iv administration, we investigated at doses of 3 mg/kg iv and po administration to confirm the good bioavailability.

Table S5. Rat pharmacokinetics parameters of **41g**.

| iv | $t_{1/2}$ (h) | C_0 (µmol/L) | AUC_{inf} (µmol h/L) | CL_{tot} (L/h/kg) | V_{dssh} (L/kg) |
|---------|---------------|--------------------|------------------------|---------------------|-------------------|
| 3mg/kg | 3.61 | 45.78 | 31.48 | 0.19 | 1.02 |
| po | t_{max} (h) | C_{max} (µmol/L) | AUC_{inf} (µmol h/L) | F (%) | |
| 3 mg/kg | 0.33 | 12.67 | 33.01 | ~100 | |

Pharmacokinetic parameters were calculated by the program, Phoenix WinNonlin ver. 8.1.0.¹ using the non-compartmental analysis. Data is presented as estimated average.

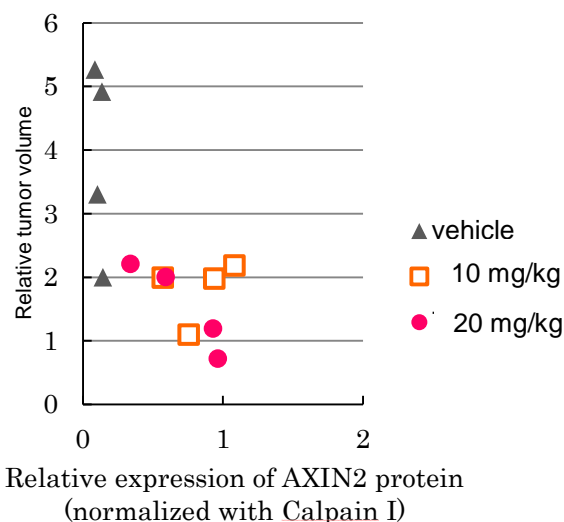
11) *In vitro* cytochrome P450 inhibition study.

Table S6. *In vitro* CYP inhibition data of **41g**.

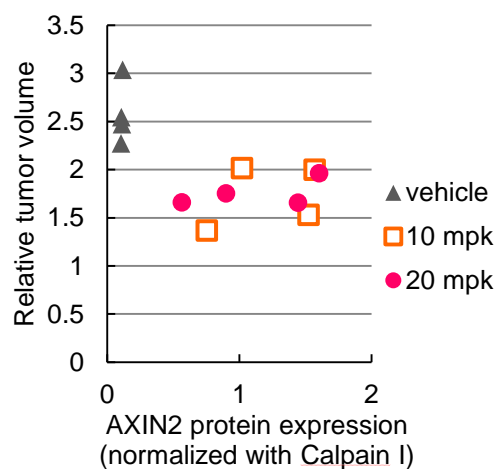
| <i>In vitro</i> CYP Inhibition | | | |
|--------------------------------|-----------------|---|-----------------------|
| Enzyme | Substrate | Control Inhibitor [IC ₅₀ (μM)] | IC ₅₀ (μM) |
| CYP1A2 | Phenacetin | α-Naphtoflavone [0.0022] | >50 |
| CYP2B6 | Bupropion | - | 10 |
| CYP2C8 | Amodiaquine | Quercetin [5.0] | 14 |
| CYP2C9 | Diclofenac | Sulfaphenazole [0.79] | 19 |
| CYP2C19 | (S)-Mephenytoin | (S)-(+)-N-3-benzyl nirvanol [0.53] | 22 |
| CYP2D6 | Bufuralol | Quinidine [0.16] | >50 |
| CYP3A4 | Midazolam | Ketoconazole [0.037] | >50 |

12) Mouse COLO-320DM tumor xenograft model study of 41g.

A



B



C

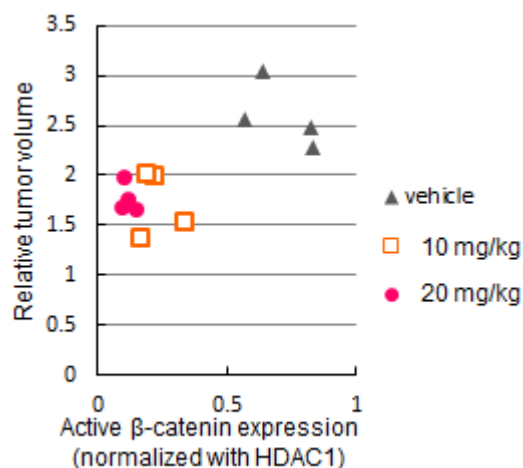


Figure S8 Relationship between the marker proteins of WNT/ β -catenin signaling and tumor volume. **A:** Intraperitoneal injection of **41g**; Relative tumor volumes showed good inverse correlation with the expression levels of AXIN2 protein (Pearson's correlation $r = -0.72$).

B, C: Oral administration of **41g**; Relative tumor volumes showed (**B**) good inverse correlation with the expression levels of AXIN2 protein (Pearson's correlation $r = -0.64$) and (**C**) good correlation with the expression levels of Active β -catenin protein (Pearson's correlation $r = 0.74$).

13) Co-crystal structure of TNKS2 and 41g.

Table S7. Crystallographic data collection and refinement statistics.

| PDB ID | 6KRO |
|------------------------------------|--|
| Data Collection | |
| Beamline | SPring-8 BL26B2 |
| Wavelength (Å) | 1.000 |
| Space group | <i>P</i> 4 ₁ 2 ₁ 2 |
| Unit cell dimensions | |
| <i>a</i> , <i>b</i> , <i>c</i> (Å) | 66.1, 66.1, 114.8 |
| <i>α</i> , <i>β</i> , <i>γ</i> (°) | 90, 90, 90 |
| Resolution (Å) | 46.8–1.90 (1.94–1.90) |
| Overall completeness (%) | 100.0 (100.0) |
| Multiplicity | 14.3 (14.5) |
| Mean <i>I</i> / <i>σ</i> | 16.3 (1.6) |
| <i>R</i> _{sym} (%) | 11.0 (197.5) |
| <i>R</i> _{pim} (%) | 3.0 (53.4) |
| CC _{1/2} | 0.999 (0.615) |
| Refinement | |
| <i>R</i> _{work} (%) | 17.2 |
| <i>R</i> _{free} (%) | 20.3 |
| R.m.s.d. bond lengths (Å) | 0.011 |
| R.m.s.d. bond angles (°) | 1.028 |
| No. of reflections (total) | 20773 |
| No. of reflections (test set) | 1058 |
| No. of atoms | |
| Protein | 1659 |
| Inhibitor | 37 |
| Ion/glycerol | 18 |
| Water | 113 |
| Mean B-factor (Å ²) | |
| Protein | 41.7 |
| Inhibitor | 39.7 |
| Ion/glycerol | 55.3 |
| Water | 45.7 |
| Ramachandran plot (%) | |
| Favored | 98.0 |
| Allowed | 2.0 |

14) References.

1. Phoenix WinNonlin ver. 8.1.0, 2018; Certara, L.P.; 210 North Tucker Boulevard Suite 350, St. Louis, MO 63101 USA.
2. Thorsell, A.G.; Ekblad, T.; Karlberg, T.; Low, M.; Pinto, A.F.; Tresaugues, L.; Moche, M.; Cohen, M.S.; Schuler, H. Structural Basis for Potency and Promiscuity in Poly(ADP-ribose) Polymerase (PARP) and Tankyrase Inhibitors. *J. Med. Chem.* **2017**, *60*, 1262-1271.
3. Shirai, F.; Tsumura, T.; Yashiroda, Y.; Yuki, H.; Niwa, H.; Sato, S., Chikada, T.; Koda, Y.; Washizuka, K.; Yoshimoto, N.; Abe, M.; Onuki, T.; Mazaki, Y.; Hirama, C.; Fukami, T.; Watanabe, H.; Honma, T.; Umehara, T.; Shirouzu, M.; Okue, M.; Kano, Y.; Watanabe, T.; Kitamura, K.; Shitara, E.; Muramatsu, Y.; Yoshida, H.; Mizutani, A.; Seimiya, H.; Yoshida, M.; Koyama, H. Discovery of Novel Spiroindoline Derivatives as Selective Tankyrase Inhibitors. *J. Med. Chem.* **2019**, *62*, 3407-3427.