Supplemental Materials

Investigation of 3-aryl-pyrimido[5,4-*e*][1,2,4]triazine-5,7-diones as small molecule antagonists of β-catenin/TCF transcription

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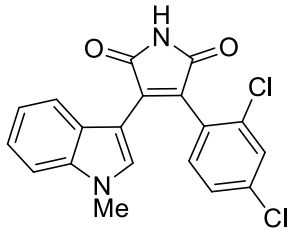
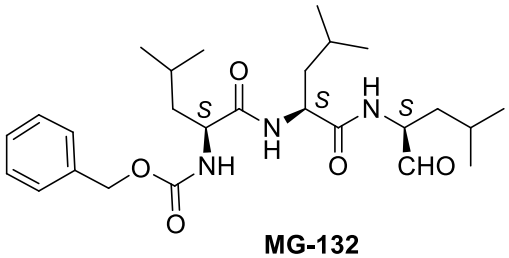
Chemistry Experimental Section

General Chemical Methods. All starting materials were obtained from commercial suppliers and were used without further purification. XAV939 was purchased from Selleck Chemicals, LLC. Glassware was oven-dried before use for reactions run under anhydrous conditions. Reactions were run under a blanket of nitrogen unless specified otherwise. Reaction extractions were dried over magnesium sulfate or sodium sulfate prior to concentration. Melting points were determined in open capillary tubes on a Laboratory Devices Mel-Temp apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian 300, Varian 400, or a Bruker

500 instrument. ^{13}C spectra were recorded on the Bruker instrument at 125 MHz. Mass spectra were recorded on a Micromass LCT time-of-flight instrument utilizing the electrospray ionization mode. Calphostin C (**1**) was purchased from Enzo Life Sciences. XAV939 (**2**) was purchased from Selleck Chemicals, LLC. SB216763 and MG-132 were purchased from Cayman Chemical.

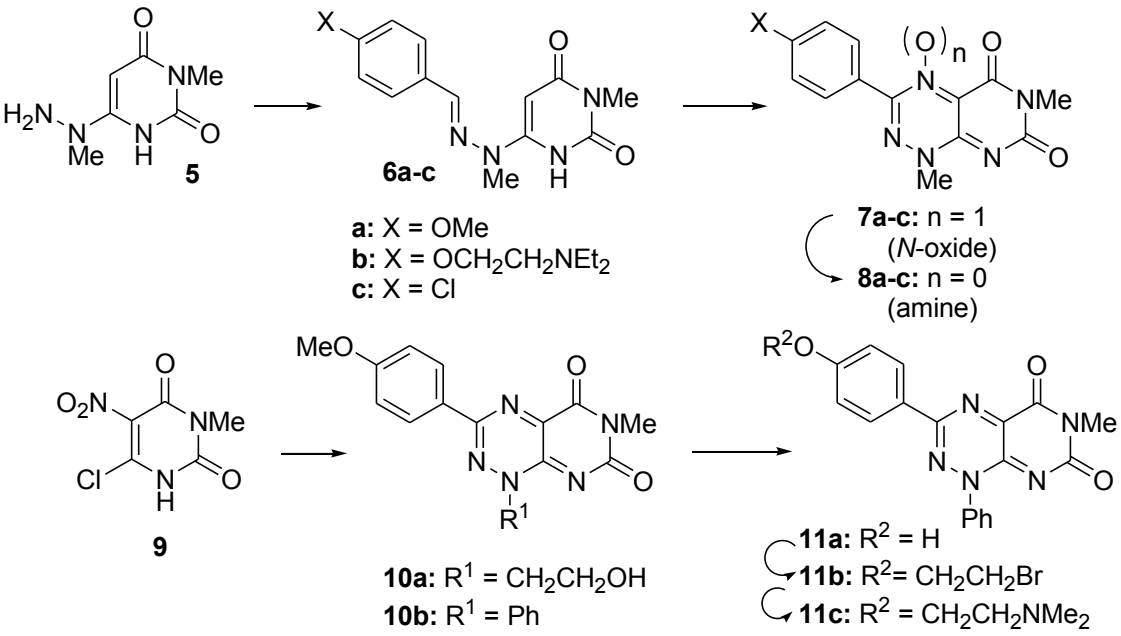


SB216763



MG-132

Scheme S1



3-Methyl-6-(1-methylhydrazinyl)pyrimidine-2,4(1*H*,3*H*)-dione (5). A solution of 6-chloro-3-methyluracil (5.0 g, 0.031 mol), methylhydrazine (8.3 mL, 0.16 mol) and absolute ethanol (45 mL) was heated at reflux for 1 h wherein TLC (20% MeOH/CH_2Cl_2) indicated consumption of the starting uracil. The mixture was cooled to room temperature and a precipitated white solid was collected, washed with ethanol, and dried to give **5** (3.492 g, 66%): mp 212-215 °C (lit[1] 207-

209 °C); ^{1}H NMR (DMSO-d_6) δ 4.66 (s, 1H), 3.02 (s, 3H), 2.97 (s, 3H). ^{13}C NMR (DMSO-d_6) δ 163.38, 154.30, 150.85, 72.74, 26.48.

6-(2-(4-Methoxybenzylidene)-1-methylhydrazinyl)-3-methylpyrimidine-2,4(1*H*,3*H*)-dione (6a). A suspension of 6-(hydrazinyl)pyrimidinedione **5** (2.97 g, 17.5 mmol), 4-anisaldehyde (2.34 mL, 19.2 mmol), and absolute ethanol (40 mL) was heated at reflux for 14 h wherein TLC (5% MeOH/CH_2Cl_2) indicated complete consumption of starting uracil. The mixture was cooled to room temperature, and a precipitated peach solid was collected, rinsed with ethanol, and dried to give **6a** (4.404 g, 87%): mp 228-230˚C (lit[2] 223 ˚C); ^{1}H NMR (DMSO-d_6) δ 10.65 (s, 1H), 7.93 (d, J = 6.2 Hz, 2H), 6.99 (d, J = 8.65 Hz, 2H), 5.21 (s, 1H), 3.81 (s, 3H), 3.12 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 163.59, 160.93, 151.08, 150.96, 140.88, 127.52, 129.84, 114.42, 76.93, 55.71, 32.14, 26.71; MS *m/z* 289.1 $(M+H)^+$.

Made in similar fashion were the following compounds:

6-(2-(4-(2-(Diethylamino)ethoxy)benzylidene)-1-methylhydrazinyl)-3-methylpyrimidine-2,4(1*H*,3*H*)-dione (6b). From pyrimidinone **5** and 4-[2-(diethylamino)ethoxy]benzaldehyde[3] for 2h at reflux (134 mg; 31%): mp 175-176 ˚C; ^{1}H NMR (DMSO-d_6) δ 7.90 (d, J = 8.2 Hz, 2H), 6.98 (d, J = 8.4 Hz, 2H), 5.20 (s, 1H), 4.06 (t, J = 7.0 Hz, 2H), 3.32 (s, 3H), 3.11 (s, 3H), 2.79 (t, J = 7.0 Hz, 2H), 2.56 (q, J = 7.1 Hz, 4H), 0.97 (t, J = 7.0, 6H); ^{13}C NMR (DMSO-d_6) δ 163.64, 160.19, 151.17, 140.73, 129.80, 127.48, 114.92, 76.88, 66.84, 51.74, 47.44, 32.06, 26.71, 12.27.

6-(2-(4-Chlorobenzylidene)-1-methylhydrazinyl)-3-methylpyrimidine-2,4(1*H*,3*H*)-dione (6c). From pyrimidinone **5** and 4-chlorobenzaldehyde for 2h at reflux (119 mg, 69%): mp 233-235 ˚C (lit[2] 194 ˚C); ^{1}H NMR ($CDCl_3$) δ 9.15 (s, 1H), 7.66 (s, 1H), 7.62 (d, J = 6.7 Hz, 2H), 7.42 (d, J = 6.1 Hz, 2H), 5.16 (s, 1H), 3.33 (s, 6H).

3-(4-Methoxyphenyl)-1,6-dimethyl-5,7-dioxo-1,5,6,7-tetrahydropyrimido[5,4-*e*][1,2,4]triazine 4-oxide (7a) and **3-(4-methoxyphenyl)-1,6-dimethylpyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione (8a).** A stirring solution of hydrazone **6a** (5.81g, 20.2

mmol) in glacial acetic acid (38 mL) cooled to 0˚C was treated slowly with a solution of sodium nitrite (2.04 g, 30 mmol) in water (4 mL). The mixture was gradually brought to room temperature and kept there until TLC (2% MeOH/ CH_2Cl_2) indicated consumption of the starting hydrazine and conversion to ~1:1 ratio of two products, the *N*-oxide (**7a**) and the corresponding pyrimidotriazinedione (**8a**). Ether was added to precipitate the mixture, and the resulting bright orange crystals were collected and dried to leave 4.90 g (~79%): NMR (DMSO-d_6) δ 8.14 (s, 2H), 7.14 (s, 2H), 3.85 (s, 3H), 3.34 (s, 3H); MS *m/z* 300.1 and 316.1 $(M+H)^+$.

3-(4-Methoxyphenyl)-1,6-dimethylpyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione (8a). A suspension of **7a** and **8a** (4.3g, ~14 mmol), dithiothreitol (6.69g, 43.4 mmol) and absolute ethanol (100 mL) was stirred for 14 h at room temperature wherein TLC (2% MeOH/ CH_2Cl_2) indicated complete conversion of the *N*-oxide to the reduced pyrimidotriazinedione (**8a**). Ether was added to precipitate crude product as a dark orange solid, which was collected and dried to leave 4.13 g. Recrystallization from 2-propanol gave **8a** (3.06 g, 74%): mp 262-264˚C (lit[2] 244˚C, dec); ^{1}H NMR (DMSO-d_6) δ 3.86 (s, 3H), 7.15 (d, J = 9.05 Hz, 2H), 8.15 (d, J = 9.05 Hz, 2H); ^{1}H NMR (*d*-TFA) 3.60 (s, 3H), 3.94 (s, 3H), 4.60 (s, 3H), 7.10 (d, J = 9.05 Hz, 2H), 8.34 (d, J = 9.05 Hz, 2H); ^{13}C DEPT NMR (DMSO-d_6) δ 129.62, 128.45, 114.60, 114.13, 55.98, 28.23, 27.22; MS *m/z* 300.1 $(M+H)^+$.

Made in similar fashion were the following compounds following dithiothreitol reduction on the mixture of **7** and **8**:

3-(4-(2-(Diethylamino)ethoxy)phenyl)-1,6-dimethylpyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione (8b). 15 mg (87%): mp > 240 ˚C, dec; ^{1}H NMR (DMSO-d_6) δ 8.18 (d, J = 7.9 Hz, 2H), 7.21 (d, J = 8.3 Hz, 2H), 4.41 (t, J = 7.1 Hz, 2H), 4.05 (s, 3H), 3.56 (t, J = 7.1 Hz, 2H), 3.29 (s, 3H), 3.25 (q, J = 7.0 Hz, 4H), 1.25 (t, J = 7.0 Hz, 6H); MS *m/z* 385.2 $(M+H)^+$.

3-(4-Chlorophenyl)-1,6-dimethylpyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione (8c). 12 mg (47%), mp 218-223 ˚C, dec (lit[2] 227˚C); ^{1}H NMR (DMSO-d_6) δ 7.75 (d, J =7.2 Hz, 2H), 7.52 (d, J = 7.2 Hz, 2H), 3.92 (s, 3H), 3.33 (s, 3H); MS *m/z* 304.0, 306.0 $(M+H)^+$.

1-(2-Hydroxyethyl)-3-(4-methoxyphenyl)-6-methylpyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione (10a). This compound was made from **9** by the procedure described in the literature.[4] Fluorescent orange solid, 95 mg (51%): mp 215-217˚C, dec; ^{1}H NMR (DMSO-d_6) δ 8.13 (d, J = 8.9 Hz, 2H), 7.14 (d, J = 8.9 Hz, 2H), 4.98 (t, J = 6.15 Hz, 1H), 4.53 (t, J = 5.7 Hz, 2H), 3.93 (q, J = 5.8 Hz, 2H), 3.86 (s, 3H), 3.28 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 162.25, 159.54, 154.58, 151.76, 149.76, 146.89, 128.89, 125.54, 115.12, 58.11, 57.10, 55.93, 28.75; MS *m/z* 330.1 $(M+H)^+$.

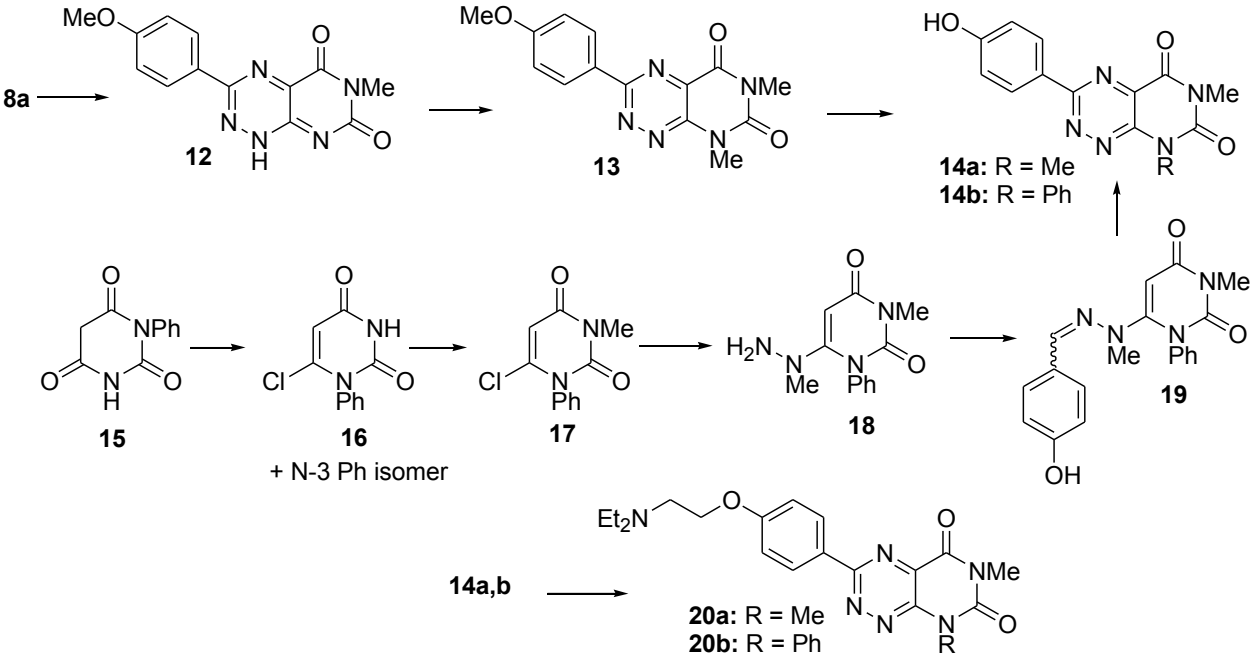
3-(4-Hydroxyphenyl)-6-methyl-1-phenyl-1*H*-pyrimido[5,4-*e*][1,2,4]triazine-5,7-dione (11a). A room temperature suspension of compound **10b**[4] (690 mg, 1.9 mmol) in 1,2-dichloroethane (6 mL) was treated drop-wise with BBr_3 (5.73 mL of 1M solution in CH_2Cl_2) and the mixture was heated at 70 ºC for 3 h while monitoring by TLC (5% MeOH/ethyl acetate). The flask was placed in an ice bath, and ethanol (25 mL) was added drop-wise to the stirring solution. The thick suspension was heated at reflux for 5 h, cooled, and filtered. The collected solids were washed well with ethanol and dried to leave **11a** (420 mg, 63%) as a deep red solid: mp >340 °C, dec; ^{1}H NMR (DMSO-d_6) δ 10.22 (s, 1H), 8.06 (d, J = 8.8, 2H), 7.75 (d, J = 7.5, 2H), 7.64 (m, 3H), 6.95 (d, J = 8.8, 2H), 3.17 (s, 3H); MS *m/z* 348.0 $(M+H)^+$.

3-[4-(2-Bromoethoxy)phenyl]-6-methyl-1-phenyl-1*H*-pyrimido[5,4-*e*][1,2,4]triazine-5,7-dione (11b). A suspension of phenol **11a** (347 mg, 1.0 mmol), Cs_2CO_3 (1.63 g, 5 mmol), 1,2-dibromoethane (5 mL) and DMF (5 mL) was stirred at room temperature for 20 h. The deep orange mixture was diluted with 0.5 N aqueous HCl and extracted with CH_2Cl_2 (4x). The combined extracts were washed with saturated brine, dried, and concentrated to an oil that was diluted with a small amount of ethyl acetate and refrigerated. After several hours, the precipitated solids were collected, washed with cold ethyl acetate, and dried to leave **11b** (213 mg, 47%) of an orange solid in two crops: mp 225 – 230 °C: ^{1}H NMR (DMSO-d_6) δ 8.11 (d, J = 8.9, 2H), 7.72 (d, J = 7.2, 2H), 7.60 (m, 3H), 7.13 (d, J = 8.9, 2H), 4.39 (t, J = 5.1, 2H), 3.82 (t, J = 5.1, 2H), 3.25 (s, 3H); MS *m/z* 453.9 $(M+H)^+$.

3-[4-(2-(Dimethylamino)ethoxy)phenyl]-6-methyl-1-phenyl-1*H*-pyrimido[5,4-*e*][1,2,4]triazine-5,7-dione (11c). A solution of compound **11b** (22.5 mg, 0.05 mmol),

dimethylamine (89 µL of 5.6 M solution in ethanol), and acetonitrile (2.8 mL) was heated in a sealed tube at 80 °C for 6 h. The cooled mixture was distributed between CH_2Cl_2 and 5% aqueous $NaHCO_3$. The combined organic extracts were dried, and concentrated to a solid that was triturated in 2-propanol, collected by filtration, and dried to leave **11c** (7 mg, 33%) as a bright orange solid: mp 234 – 235 °C; ^{1}H NMR (DMSO-d_6) δ 8.13 (d, J = 8.8, 2H), 7.70 (d, J = 6.6, 2H), 7.62 (m, 3H), 7.14 (d, J = 8.8, 2H), 4.14 (t, J = 5.7, 2H), 3.28 (s, 3H), 2.65 (t, J = 5.7, 2H), 2.22 (s, 6H); MS m/z 419.0 $(M+H)^+$.

Scheme S2



3-(4-Methoxyphenyl)-6-methylpyrimido[5,4-*e*][1,2,4]triazine-5,7(1*H*,6*H*)-dione (12).

Method A: from heating a mixture of **7a**/**8a**. A suspension of the mixture of **7a** and **8a** (4.9g, ~15.1 mmol) in anhydrous DMF (45 mL) was heated at 90˚C for 3 h, during which time it darkened and became homogeneous. After cooling to room temperature cold water (20 mL) was added and the mixture was refrigerated overnight. The resultant precipitate was collected by filtration and dried to give **12** (2.78g, 65%): mp 327-330 ˚C (lit[2] >300 ˚C); ^{1}H NMR (DMSO-

d$_6$) δ 12.77 (s, 1H), 8.34 (d, *J* = 7.2 Hz, 2H), 7.15 (d, *J* = 7.5 Hz, 2H), 3.87 (s, 3H), 3.25 (s, 3H); MS *m/z* 286.1 $(M+H)^+$.

Method B: from heating **8a**. Heating of a suspension of reduced pyrimidotriazinedione **8a** (1.87g, 6.2 mmol) in anhydrous DMF (30 mL) for 3 h followed by workup (addition of 20 mL of water) as described for Method A gave **12** (1.28 g, 73%): mp 331-340 ˚C, dec; ^{1}H NMR and MS data were the same as for Method A.

3-(4-Methoxyphenyl)-6,8-dimethylpyrimido[5,4-*e*][1,2,4]triazine-5,7(6*H*,8*H*)-dione (13). A mixture of pyrimidotriazinedione **12** (57 mg, 0.2 mmol), Cs_2CO_3 (98 mg, 0.3 mmol), dimethyl sulfate (21 μL, 0.22 mmol) and acetone (2 mL) was heated at 50 °C overnight. After cooling, the mixture was diluted with water and refrigerated for 3-4 h. The precipitate was collected, washed with water, and dried to give **13** (58 mg, 97%): mp 259-265˚C, dec (lit[5] 268˚C, dec); ^{1}H NMR (DMSO-*d*$_6$) δ 8.36 (d, *J* = 8.0 Hz, 2H), 7.18 (d, *J* = 8.0 Hz, 2H), 3.88 (s, 3H), 3.70 (s, 3H), 3.35 (s, 3H); MS *m/z* 300.1 $(M+H)^+$.

3-(4-Hydroxyphenyl)-6,8-dimethylpyrimido[5,4-*e*][1,2,4]triazine-5,7(6*H*,8*H*)-dione (14a). A solution of compound **13** (60 mg, 0.2 mmol) in CH_2Cl_2 (2 mL) was treated with BBr_3 (0.6 mL of 1M solution in CH_2Cl_2). The mixture was stirred at room temperature overnight and then ice-cooled and treated cautiously with excess methanol. After stirring for 30 min the solution was concentrated to a yellow solid that was triturated in methanol, collected, and dried to leave **14a** (37 mg, 65 %) as a yellow solid. The compound was carried directly to the next step without further characterization.

6-Chloro-1-phenylpyrimidine-2,4(1*H*,3*H*)-dione (16).[6] Phosphoryl chloride (8.44 mL, 90.6 mmol) was added drop-wise to a suspension of **15**[7] (1.53 g, 7.5 mmol) in 400 μL of water. The mixture was heated at reflux for 3 h and concentrated to a residue that was treated with ice chips while stirring rapidly. The formed precipitate was collected and suspended in a solution of saturated aqueous $NaHCO_3$. After stirring for 15 min, the precipitate was collected and crystallized from ethanol to yield **16** (800 mg, 48%): mp 257-260 °C (lit[6] 260-262 °C); ^{1}H NMR (*d*-DMSO) δ 6.09 (s, 1H), 7.45 (m, 2H), 7.50 (m, 3H), 11,72 (br s, 1H). The filtrate was

acidified with concentrated HCl to pH ~2, and a second precipitate formed, which was collected to yield isomeric compound 6-chloro-3-phenylpyrimidine-2,4(1*H*,3*H*)-dione (444 mg, 26%): mp 264-268 °C (lit[6] 268-270 °C); ^{1}H NMR (DMSO-d_6) δ 12.50 (br s, 1H), 7.50 (m, 3H), 7.45 (m, 2H), 6.09 (s, 1H).

6-Chloro-3-methyl-1-phenylpyrimidine-2,4(1*H*,3*H*)-dione (17). A suspension of **16** (1.0 g, 4.49 mmol), dimethyl sulfate (0.51 mL, 5.39 mmol), Cs_2CO_3 (2.19 g, 6.74 mmol) and acetone (15 mL) was stirred at room temperature for 16 h. The mixture was filtered with the collected solids washed excessively with water and dried to give **17** (890 mg, 84%): mp 270-275 °C, dec (lit[8] 273-275 °C); ^{1}H NMR (DMSO-d_6) δ 7.52 (m, 3H), 7.42 (m, 2H), 6.22 (s, 1H), 3.18 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 161.26, 151.09, 145.47, 137.48, 129.87, 129.73, 129.60, 101.52, 28.26.

3-Methyl-6-(1-methylhydrazinyl)-1-phenylpyrimidine-2,4(1*H*,3*H*)-dione (18). A suspension of chlorouracil **17** (0.89 g, 3.76 mmol), methylhydrazine (1.80 mL, 33.8 mmol) and absolute ethanol (5 mL) was heated at reflux for 2 h. The mixture was concentrated to a residue that was triturated in 2-propanol to provide a white solid that was collected and dried to give **18** (815 mg, 88%): mp 141-142 °C (lit[9] 146 °C); ^{1}H NMR (DMSO-d_6) δ 7.44 (m, 2H), 7.37 (d, J = 7.2 Hz, 1H), 7.29 (d, J = 7.2 Hz, 2H), 5.38 (s, 1H), 4.19 (br s, 2H), 3.13 (s, 3H), 2.59 (s, 3H).

6-(2-(4-Hydroxybenzylidene)-1-methylhydrazinyl)-3-methyl-1-phenylpyrimidine-2,4(1*H*,3*H*)-dione (19). A suspension of **18** (0.77 g, 3.11 mmol), 4-hydroxybenzaldehyde (0.418 g, 3.42 mmol), and absolute ethanol (10 mL) was heated at reflux for 1 h. The reaction mixture was cooled to room temperature and the formed precipitate was collected to yield **19** (693 mg, 64%): ^{1}H NMR (DMSO-d_6) δ 9.74 (s, 1H), 7.48 (s, 1H), 7.38 (m, 4H), 7.28 (d, J = 8.4 Hz, 3H), 6.73 (d, J = 8.5 Hz, 2H), 5.59 (s, 1H), 3.20 (s, 3H), 2.97 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 163.02, 158.75, 156.34, 152.16, 139.84, 138.51, 129.34, 128.81, 128.13, 126.19, 115.81, 88.49, 36.32, 28.04; MS *m/z* 351.1 $(M+H)^+$, 373.1 $(M+Na)^+$.

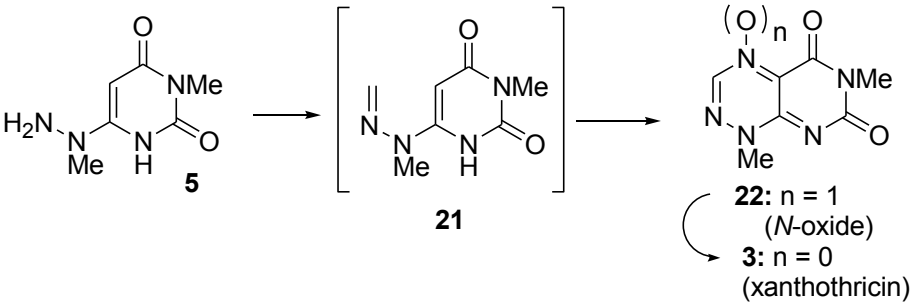
3-(4-Hydroxyphenyl)-6-methyl-8-phenylpyrimido[5,4-*e*][1,2,4]triazine-5,7(6*H*,8*H*)-dione (14b). Reaction of a mixture of hydrazone **19** (0.693 g, 1.98 mmol), sodium nitrite (0.205 g, 2.97 mmol), glacial acetic acid (8 mL) and H_20 (500 uL) was carried out similarly to the

formation of **8a** described above to give **14b** (300 mg, 44%): ^{1}H NMR (DMSO-d_6) δ 10.19 (s, 1H), 8.25 (d, J = 8.7 Hz, 2H), 7.62 (t, J = 7.3 Hz, 2H), 7.55 (t, J = 7.3 Hz, 1H), 7.44 (d, J = 7.3 Hz, 2H), 6.97 (d, J = 8.7 Hz, 2H), 3.40 (s, 3H); MS *m/z* 348.1 $(M+H)^+$.

3-(4-(2-(Diethylamino)ethoxy)phenyl)-6,8-dimethylpyrimido[5,4-*e*][1,2,4]triazine-5,7(6*H*,8*H*)-dione (20a). A mixture of **14a** (37 mg, 0.13 mmol), Cs_2CO_3 (93 mg, 0.29 mmol), 2-(diethylamino)ethyl chloride hydrochloride (25 mg, 0.15 mmol) and acetone (1.3 mL) in a sealed reaction vial was heated at 50 ˚C overnight. The reaction was cooled to room temperature and filtered. The filtrate was concentrated and triturated in ethanol. The precipitate was collected by filtration and dried to give **20a** (31 mg, 63%) as a yellow solid: mp 195˚C, dec; ^{1}H NMR (DMSO-d_6) δ 8.36 (d, J = 8.3 Hz, 2H), 7.17 (d, J = 8.4 Hz, 2H), 4.13 (t, J = 5.65 Hz, 2H), 3.71 (s, 3H), 2.82 (t, J = 5.6 Hz, 2H), 2.59 (q, J = 6.9 Hz, 4H), 2.55 (s, 3H), 1.00 (t, J = 6.9 Hz, 6H); MS *m/z* 385.2 $(M+H)^+$.

3-(4-(2-(Diethylamino)ethoxy)phenyl)-6-methyl-8-phenylpyrimido[5,4-*e*][1,2,4]triazine-5,7(6*H*,8*H*)-dione (20b). A mixture of pyrimidotriazine **14b** (0.070 g, 0.202 mmol), 2-(diethylamino)ethyl chloride hydrochloride (0.042 g, 0.242 mmol), Cs_2CO_3 (0.165 g, 0.505 mmol) and acetone (3 mL) was stirred at room temperature for 72 h and then concentrated. The solids were triturated in 2-propanol, collected, washed sequentially with water and then 2-propanol, and dried to give **20a** (40 mg, 44%): mp 185-195°C, dec; ^{1}H NMR (DMSO-d_6) δ 8.32 (d, J = 8.8 Hz, 2H), 7.59 (m, 3H), 7.43 (d, J = 6.9 Hz, 2H), 7.15 (d, J = 8.8 Hz, 2H), 4.12 (t, J = 6.1 Hz, 2H), 3.40 (s, 3H), 2.81 (t, J = 6.1 Hz, 2H), 2.56 (q, J = 7.1 Hz, 4H), 0.98 (t, J = 7.1 Hz, 6H); ^{13}C NMR (DMSO-d_6) δ 161.83, 160.74, 160.18, 151.09, 149.91, 135.74, 133.28, 129.96, 129.51, 129.44, 129.39, 126.65, 115.67, 67.13, 51.72, 47.44, 29.23, 12.36; MS *m/z* 447.2 $(M+H)^+$.

Scheme S3



Xanthothricin (3). A room temperature solution of hydrazinyl pyrimidinone **5** (1.47 g, 8.64 mmol) in glacial acetic acid (50 mL) was treated with 37% aqueous formaldehyde (707 uL, 9.5 mmol) to form the hydrazone **21**. After 30 min, a solution of sodium nitrite (894 mg, 13 mmol) in water (6 mL) was added and stirring was continued for 45 min. TLC (9:1 CH_2Cl_2 : MeOH) showed complete consumption of starting hydrazone and the appearance of two yellow spots. Diethyl ether was added to precipitate solids that were collected, washed successively with minimal water and then ethanol, and dried to leave a mixture of xanthothricin (**3**) and its corresponding N_4-oxide **22** (616 mg, ~35 %): MS *m/z* (xanthothricin) 216 $[M+Na]^+$, 248 $[M+Na+MeOH]^+$; (*N*-oxide) 232 $[M+Na]^+$, 264 $[M+Na+MeOH]^+$. A room temperature suspension of the mixture (235mg, 1.17mmol) in MeOH (15 mL) was treated with dithiothreitol (45 mg. 0.29 mmol). Two additional 45 mg charges of dithiothreitol were added to the stirring suspension after 20 and 30 min, respectively, while monitoring by TLC. After 40 min, the reaction mixture was diluted with 2-propanol and cooled in an ice bath. The resulting precipitate was collected, triturated in 2-propanol and dried to yield crude product which was purified by flash silica gel chromatography using a gradient elution of ~50 mL volumes of 0%, 5%, and 7% MeOH in DCM. Product fractions were pooled and concentrated to provide **3** (81 mg, 13% from **5**): mp 163 °C, dec (lit[1] 172-173 °C); ^{1}H NMR ($CDCl_3$) δ 8.82 (s, 1H), 4.17 (s, 3H), 3.50 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 158.45, 154.23, 150.39, 145.50, 145.23, 43.40, 29.18; MS *m/z* 216 $[M+Na]^+$, 248 $[M+Na+MeOH]^+$.

Biology Methods:

Cell culture: All cells were grown at 37 °C in 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. All cell lines were obtained from American

Type Culture Collection (ATCC). All cell lines have been authenticated in November 2011 by comparing short tandem repeats of cell lines used in these experiments with newly purchased cell lines from ATCC. Analysis of putative Wnt/β-catenin/TCF-regulated target gene expression in rat intestinal IEC-6 epithelial cells (Figure 2A): IEC-6 cells were plated at 50,000 cells per 6 cm dish and grown without insulin for 2 d. After 2 d, cells were treated for 12 h with 50 ng/mL recombinant mouse Wnt-3a (R&D Systems). RNA was extracted using Trizol (Invitrogen). 1-2 μg RNA was reverse transcribed into cDNA (High Capacity cDNA kit, Applied Biosystems). Gene expression levels relative to U6 gene expression levels were determined by quantitative PCR with gene specific primers using Power SYBR Green (Applied Biosystems). Each panel in Figure 2A represents the mean of three independent experiments.

Small molecule inhibition of Wnt target genes in IEC-6 cells: IEC-6 cells were grown as described above. Small molecule inhibitors were added and incubated for 6 h followed by incubation for 12 h with 50 ng/mL recombinant mouse Wnt-3a (R&D Systems). The base-line relative levels of *Lgr5* and *Axin2* expression (relative to U6 expression) after Wnt-3a stimulation was set to 100%. The relative target gene expression for each compound at various concentrations (log (M) = -6 corresponds to 1 μM) was determined. RNA was extracted 12 h after Wnt-3a treatment. cDNA was synthesized as described above. Gene expression levels were determined by quantitative PCR with gene specific primers using Power SYBR Green according to manufacturer instructions. Gene expression levels for *Lgr5* and *Axin2* were normalized to 50% of the cycle number of U6.

Colony formation assay: RKO and DLD-1 cells were plated at 500 and 1,000 cells in 12-well plates, respectively. Compounds and media were changed every 3 d. RKO and DLD-1 colonies were stained when clearly visible by eye. Colonies were stained with 2 mg/mL crystal violet in 10% buffered formalin phosphate and quantified after staining. Intragroup comparison was performed for each group using a one-way ANOVA followed by Dunnett's multiple comparison test to compare each value (compound treatment) with the control value (DMSO) (Figures 3C and 3E).

GST pull-down assays and Western blot analysis: HEK293T cells were plated at 400,000 cells per 6 cm dish and grown for 2 d. Cells were treated with 5 μM SB216763, 10 μM MG-132 and compound **8c** for 3 h before lysis with RIPA buffer containing protease inhibitor (Roche, Complete Mini). To test effects of compound **8c** in the presence of dnβTrCP (dominant negative

βTrCP) HEK293T cells were transiently transfected with dnβTrCP pcDNA plasmid using Mirus LT1 (Mirus) transfection reagent according to manufacturer's instructions. DLD-1 and SW480 cells were plated at 500,000 cells per 6 cm dish and grown for 2 d. Cells were treated with compound **8c** for 20 h before lysis with RIPA buffer containing protease inhibitor. Protein concentration was determined using a BCA kit (Pierce). To determine free β-catenin levels, 50 μg total protein were incubated for 1 h at 4 °C with 5 μL recombinant GST-E-cadherin[10] coupled to glutathione sepharose 4B beads (Bioworld). Beads were washed four times with RIPA buffer. Bound protein was eluted with loading buffer and analyzed by SDS-PAGE and Western blot. The following antibodies were used for detection: mouse monoclonal anti-β-catenin antibody (BD Biosciences), mouse monoclonal anti-β-actin antibody clone AC-74 (Sigma), polyclonal goat anti-GST-antibody (GE Healthcare), rabbit monoclonal anti-cyclinD1 (SP4) (Abcam), rabbit monoclonal anti-Axin2 clone 76G6 (Cell Signaling).

Data analysis: Data was analyzed using GraphPad Prism (GraphPad Software Incorporation)

Microsomal Stability. Pooled mouse liver microsomes, Xenotech lot# 1010423, were stored at -70 ºC prior to use. 10 μL of microsome (20 mg/mL) were diluted into 366 μL of 0.1M phosphate buffer containing 3.3 mM MgCl2; 4 μL of compound **11c** at 100 μM in PBS containing 16% methanol was added to phosphate buffer containing microsomes. The solution mixture was pre-incubated in a water bath at 37 ºC for 3 min. 15 μL of 20 mg/mL NADPH solution was added to the above mixture to initiate the reaction. The final concentration of test compound in the mixture was 1 μM. 30 μL of the reaction mixture was taken out into 90 μL (3 volumes) of cold acetonitrile to quench the reaction. Samples were taken at 0, 2, 10 and 60 minutes. The quenched sample mixtures were centrifuged at 14000 rpm for 10 min. 5 μL of the supernatant was used for LC/MS (liquid chromatography–mass spectrometry) analysis. The initial rate of hydrolysis was used to obtain the apparent first-order rate constant and to calculate the half-life. The apparent first-order degradation rate constant of **11c** at 37°C was determined by plotting the logarithm of this compound remaining as a function of time. The slope of this plot is related to the rate constant, k, and given by

k = 2.303 slope (log C vs. time)

The degradation half-life was then calculated by the equation: $t_{1/2} = 0.693/k$

Table S1. Oligos nucleotide sequences

Target gene	**Sequence (5' – 3')**
Axin2 forward	ACGCGCTGACCGACGATTCC
Axin2 reverse	GCGGTGGGTTCTCGGGAAGT
Bmp4 forward	CTTCCCGGTCTCAGGTATCA
Bmp4 reverse	TGAGCCTTTCCAGCAAGTTT
Edn1 forward	GGTCTTGATGCTGTTGCTGA
Edn1 reverse	ACCACAGACCAAGGGAACAG
Irs1 forward	CCAGAAGCAACCAGAGGA
Irs1 reverse	CCATGAGTTAAAAAGGAGGAT
Lgr5 forward	GCTGCCAAATTGTTGGTTTT
Lgr5 reverse	CAGGCTAGAAAGGGGAGCTT
Nkd1 forward	AGGACGACTTCCCCCTAGAA
Nkd1 reverse	TGCAGCAAGCTGGTAATGTC
U6 forward	GTGCTCGCTTCGGCAGCACATAT
U6 reverse	AAAAATATGGAACGCTTCACGAA

Table S2. Compound **11c** Metabolic Stability in Mouse Liver Microsomes

Time (min)	% **11c**
0	100.0
3	110.0
10	92.2
30	70.7
60	31.8
$t_{1/2}$ (min)	34.7

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