## Supporting Information

# Celastrol Analogs as Inducers of the Heat Shock Response. Design and Synthesis of Affinity Probes for the Identification of Protein Targets

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## 1. Chemistry

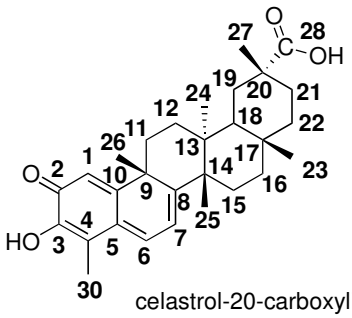
**General Methods.** Proton nuclear magnetic resonances ($^1$H NMR) were recorded in deuterated solvents on a Varian Inova 500 (500 MHz) or a Brucker (500 MHz) NMR spectrometer. Chemical shifts are reported in parts per million (ppm, δ). $^1$H NMR splitting patterns are designated as singlet (s), doublet (d), triplet (t), and quartet (q). Splitting patterns that could not be interpreted or easily visualized were recorded as multiplet (m) or broad (br). Coupling constants are reported in Hertz (Hz). Proton decoupled carbon ($^{13}$C NMR) spectra were recorded on a Varian Inova 500 (125 MHz) or a Brucker (125 MHz) NMR spectrometer and are reported in ppm using the solvent as an internal standard ($CDCl_3$, δ 77.23; MeOH, δ 3.34). High-resolution electrospray mass spectra (HR-ESMS) were obtained using an LCQ-Advantage with methanol as the solvent in the positive ion mode, unless otherwise stated. For new compounds, $^1$H and $^{13}$C NMR, HR-MS, and HPLC data are presented. Sorbent Technologies silica gel 60 (200- 400 mesh) was used for column chromatography. Thin-layer chromatography was carried out on E. Merck precoated silica gel 60 $F_{254}$ plates with visualization accomplished with phosphomolybdic acid, iodine, or with a UV-visible lamp.

Compounds **3-5**, **8, 9**, **14**, **15**, **19-25** were purified by HPLC using a semi-preparative column [Phenomenex Luna 5μ, 250 x 10.00 mm, C18(2)] with a precolumn [Phenomenex Luna 5μ, 50 x 10, C18(2)] at a flow rate of 4 mL/min. Sample elution was detected by absorbance at 254 nm. The mobile phase was isocratic (water + 0.01% TFA; $CH_3CN$). The HPLC was performed on a Beckman System Gold chromatograph (model 125P solvent module and model 166 detector). Fractions containing the pure product were concentrated *in vacuo*, and the residue was additionally dried on a high vacuum pump. The purity of compounds was determined by the same HPLC system using analytical columns Phenomenex Gemini-NX 5μ C18 110A, 250 x 4.60 mm, and Phenomenex Gemini 5μ C18 110A, 250 x 4.60 mm at a flow rate of 1 mL/min. Sample elution was detected by absorbance at 254 nm.

All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA) and were used without further purification. PEGylated reagents were from Quanta Biodesign (Powell, OH) and Peptides International (Louisville, CT). Celastrol was purchased from the GAIA Chemical Corporation (Gaylordsville, CT) and Shanghai Yingxuan Chempharm Co., Ltd. (Shanghai, China).

**General Procedure for Synthesis of 3-5**, **8**, **9**, **14**, **15**, **19**, **20**, **25.** To a suspension of HATU (0.022 mmol) in anhydrous DMF (0.5 mL) was added a solution of celastrol **(1)** (0.022 mmol) in anhydrous DMF (1 mL) at 0 $^o$C under an argon atmosphere, followed by the addition of DIEA (0.044 mmol). The reaction mixture was stirred for 15 min followed by addition of the appropriate amine (0.044 mmol), and stirring was continued for 20 h (0 $^o$C to room temperature). Saturated aqueous $NH_4Cl$ (20 mL) was added, and the mixture was extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with 0.1M HCl (2 x 10 mL), saturated $NaHCO_3$ (20 mL), water (2 x 10 mL), and dried over $Na_2SO_4$. Filtration and solvent removal afforded the crude amide (~ 80%), which was purified by reversed-phase HPLC.

Numbering convention:




celastrol-20-carboxylic acid

**Celastrol-20-carboxamide (3).** $^{1}$H NMR (500 MHz, $CDCl_3$) δ: 0.73 (s, 3H, $CH_3$), 1.02 (br d, 1H, *J* = 13.9 Hz, C(22)-H), 1.12 (s, 3H, $CH_3$) 1.21 (s, 3H, $CH_3$), 1.27 (s, 3H, $CH_3$), 1.44 (s, 3H, $CH_3$), 1.47-1.70 (m, 8H), 1.85-2.18 (m, 5H), 2.22 (s, 3H, $CH_3$), 2.42 (d, 1H, *J* = 15.6 Hz, C(19)-H), 5.54 (br s, 1H, NH), 5.70 (br s, 1H, NH), 6.33 (d, 1H, *J* = 7.1 Hz, C(7)-H), 6.52 (s, 1H, C(1)-H), 7.01 (d, 1H, *J* = 6.96 Hz, C(6)-H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ: 10.49, 19.00, 22.04, 28.90, 29.66, 30.64, 31.01, 31.31, 31.78, 33.61, 33.95, 35.08, 36.53, 38.44, 39.52, 40.63, 43.20, 44.48, 45.23, 117.29, 118.24, 119.70, 127.63, 134.16, 146.22, 164.96, 170.44, 178.56, 181.12. HPLC (Gemini-NX C18 5μ; 20% $H_2O$ (0.01% TFA) - 80% $CH_3CN$) $R_f$ = 6.18 min; Gemini 5μ C18; 10% $H_2O$ (0.1% TFA) - 90% MeOH) $R_f$ = 5.98 min. HRMS (ESI, MeOH) calcd for $C_{29}H_{39}NO_3$: 449.2936; found: 449.2930.

***N*-Methyl celastrol-20-carboxamide (4).** $^{1}$H NMR (500 MHz, $CDCl_3$) δ: 0.62 (s, 3H, $CH_3$), 1.03 (br d, 1H, *J* = 13.4 Hz, C(22)-H), 1.13 (s, 3H, $CH_3$) 1.16 (s, 3H, $CH_3$), 1.27 (s, 3H, $CH_3$), 1.45 (s, 3H, $CH_3$), 1.48-1.75 (m, 8H), 1.83-2.18 (m, 5H), 2.21 (s, 3H, $CH_3$), 2.46 (d, 1H, *J* = 15.3 Hz, C(19)-H), 2.68 (d, 3H, *J* = 4.6, NH-$CH_3$), 5.73 (d, 1H, *J* = 4.0 Hz, NH), 6.34 (d, 1H, *J* = 7.0 Hz, C(7)-H), 6.54 (s, 1H, C(1)-H), 6.98 (br s, 1H, OH), 7.02 (d, 1H, *J* = 7.5 Hz, C(6)-H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ: 10.27, 18.07, 21.71, 26.50, 28.63, 29.42, 30.12, 30.86, 31.25, 31.56, 33.45, 33.63, 34.97, 36.34, 38.20, 39.33, 40.26, 43.00, 44.33, 45.06, 117.08, 117.99, 119.52, 127.36, 134.09, 145.97, 164.78, 170.39, 178.33, 178.46. HPLC (Gemini-NX C18 5μ; 20% $H_2O$ (0.01% TFA) - 80% $CH_3CN$) $R_f$ = 7.00 min; Gemini 5μ C18; 10% $H_2O$ (0.1% TFA) - 90% MeOH) $R_f$ = 6.28 min. HRMS (ESI, MeOH) calcd for $C_{30}H_{41}NO_3$: 463.3073; found: 463.3086.

***N*,*N*-Dimethyl celastrol-20-carboxamide (5).** $^{1}$H NMR (500 MHz, $CDCl_3$) δ: 0.53 (s, 3H, $CH_3$), 0.98 (br d, 1H, *J* = 14 Hz, C(22)-H), 1.14 (s, 3H, $CH_3$) 1.27 (s, 3H, $CH_3$), 1.28 (s, 3H, $CH_3$), 1.46 (s, 3H, $CH_3$), 1.48-1.88 (m, 14H), 2.05-2.20 (m, 2H), 2.22 (s, 3H, $CH_3$), 2.31-2.48 (m, 2H), 2.80 (br s, 3H), 3.18 (br s, 3H), 6.36 (d, 1H, *J* = 7.5 Hz, C(7)-H), 6.53 (s, 1H, C(1)-H), 6.96 (br s, 1H), 7.03 (d, 1H, *J* = 7 Hz, C(6)-H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ: 10.29, 18.44, 22.11, 28.80, 30.04, 30.41, 30.79, 30.96, 31.97, 33.45, 34.35, 36.01, 36.36, 38.30, 39.56, 40.20, 42.93, 44.94, 45.07, 117.30, 118.31, 119.42, 127.33, 134.38, 145.99, 164.75, 170.22, 176.83, 178.30. HPLC (Gemini-NX C18 5μ; 20% $H_2O$ (0.01% TFA) - 80% $CH_3CN$) $R_f$ = 10.20 min; Gemini 5μ C18; 10% $H_2O$ (0.1%

TFA) - 90% MeOH) $R_f$ = 7.18 min. HRMS (ESI, MeOH) calcd for $C_{31}H_{41}NO_3$: 477.3243; found: 477.3236.

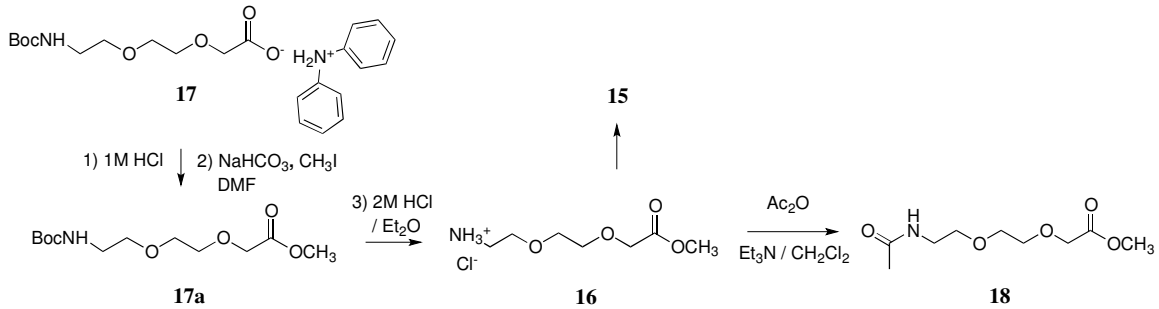
***N*-Hydroxycelastrol-20-carboxamide (8).** $^1$H NMR (500 MHz, CDCl3) δ: 0.41 (s, 3H, $CH_3$), 0.94-1.03 (m, 1H), 1.09 (s, 3H, $CH_3$) 1.18 (s, 6H, $CH_3$), 1.35 (s, 3H, $CH_3$), 1.39-1.92 (m**,** 11H), 2.01-2.12 (m, 1H), 2.21 (s, 3H, $CH_3$), 2.51 (d, 1H, *J* = 15.0 Hz, C(19)-H), 6.05 (d, 1H, *J* = 10 Hz), 6.58 (s, 1H, C(1)-H), 6.92-7.10 (m, 2H), 8.10 (br s, 1H), 9.00 (br s, 1H); $^{13}$C NMR (125 MHz, CDCl3) δ: 10.29, 18.34, 21.56, 28.38, 29.20, 30.62, 30.77, 31.37, 33.19, 33.56,36.10, 38.10, 38.98, 39.07, 43.12, 44.02, 45.02, 117.14, 117.67, 127.21, 134.23, 146.08, 164.99, 170.98, 178.40. HPLC (Gemini-NX C18 5μ; 20% $H_2O$ (0.01% TFA) - 80% $CH_3CN$) $R_f$ = 10.55 min; Gemini 5μ C18; 10% $H_2O$ (0.1% TFA) - 90% $CH_3OH$) $R_f$ = 5.38 min. HRMS (ESI, $CH_3OH$) calcd for $C_{29}H_{39}NO_4$: 465.2879; found: 465.2883.

***N*-(2-Hydroxyethyl)celastrol-20-carboxamide (9).** $^1$H NMR (500 MHz, $CDCl_3$) δ: 0.65 (s, 3H, $CH_3$), 1.02 (br d, 1H, *J* = 12.8 Hz, C(22)-H), 1.12 (s, 3H, $CH_3$) 1.17 (s, 3H, $CH_3$), 1.26 (s, 3H, $CH_3$), 1.44 (s, 3H, $CH_3$), 1.48-1.72 (m, 8H), 1.80-2.18 (m, 6H), 2.21 (s, 3H, $CH_3$), 2.45 (d, 1H, *J* = 15.6 Hz, C(19)-H), 3.30 (br s, 2H), 3.64 (br s, 2H), 6.25-6.38 (m, 2H), 6.52 (s, 1H, C(1)-H), 6.94-7.06 (m, 2H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ: 10.28, 18.33, 21.72, 28.64, 29.47, 30.12, 30.83, 31.08, 31.59, 33.47, 33.91, 34.93, 36.32, 38.19, 39.33, 40.36, 42.52, 42.97, 44.31, 45.01, 62.30, 117.03, 118.05, 119.57, 127.41, 134.03, 145.98, 164.71, 170.12, 178.34, 179.17. HPLC (Gemini-NX C18 5μ; 20% $H_2O$ (0.01% TFA) - 80% $CH_3CN$) $R_f$ = 5.20 min; Gemini 5μ C18; 10% $H_2O$ (0.1% TFA) - 90% $CH_3OH$) $R_f$ = 5.65 min. HRMS (ESI, $CH_3OH$) calcd for $C_{31}H_{43}NO_4$: 493.3192; found: 493.3202.

***N*-Dodecyl-*N*-methyl celastrol-20-carboxamide (14).** $^1$H NMR (500 MHz, $CDCl_3$) δ: 0.58 (s, 3H, $CH_3$), 0.88 (br s, 3H), 0.98 (d, 1H, *J* = 14 Hz), 1.42 (s, 3H), 0.90-1.40 (m, 25 H), 1.46 (s, 3H), 1.49-1.85 (m, 13H), 2.05-2.20 (m, 2H), 2.21 (s, 3H, $CH_3$), 2.33 (d, 1H, *J* = 13.5 Hz), 2.40 (d, 1H, *J* = 16 Hz), 3.14 (br s, 2H), 6.35 (d, 1H, *J* = 7.5 Hz), 6.53 (d, 1H, *J* = 1.5 Hz), 6.97 (br s, 1H, OH), 7.03 (dd, 1H, $J_1$ =1.5 Hz, $J_2$ = 7.0 Hz). HPLC (Gemini-NX C18 5μ; 1% $H_2O$ (0.1% TFA) - 99% $CH_3CN$) $R_f$ = 31.07 min; Gemini-NX 5μ C18; 1% $H_2O$ (0.1% TFA) - 99% $CH_3OH$) $R_f$ = 8.02 min. HRMS (ESI, $CH_3OH$) calcd for $C_{42}H_{65}NO_3$ $[M+1]^+$: 632.5037; found: 632.5026.

**Celastrol-20-carboxamido(diethyleneglycol)methyl acetate (15).**

**Scheme S-1.** Synthesis of **18** and **25**



Boc-8-amino-3,6-dioxaoctanoic acid, DCHA **(17)** (57 mg, 0.128 mmol) was dissolved in water (3 mL), and 1 M HCl was added until a white precipitate formed, which was subsequently dissolved in EtOAc (70 mL). The water solution was additionally extracted with EtOAc (3 x 20 mL), and the combined organic layers were dried over $Na_2SO_4$. Filtration and solvent removal afforded the acid as a white solid (26 mg, 77%), which was immediately used directly in the next reaction. To a solution of the acid (26 mg, 0.098 mmol) in DMF (2 mL) was added $NaHCO_3$ (16.5 mg, 0.196 mmol) and iodomethane (69.5 mg, 0.49 mmol). The reaction solution was stirred under an argon atmosphere at room temperature for 48 h. DMF was removed under reduced pressure, and the resulting yellow residue was dissolved in EtOAc (70 mL) and washed with water (2 x 15 mL). The organic layer was dried over $Na_2SO_4$. Filtration and evaporation afforded a pale yellow oily product. The product was purified by column chromatography on silica-gel (hexanes / EtOAc, 1:1) to afford methyl ester **17a** (19.7 mg, 89%) as a colorless oil. $^1$H NMR (500 MHz, $CDCl_3$) δ: 1.39 (s, 9H), 3.29-3.35 (m, 2H), 3.52-3.57 (m, 2H), 3.64-3.68 (m, 2H), 3.70-3.74 (m, 2H), 3.76 (s, 3H, $OCH_3$), 4.17 (s, 2H), 5.02 (s, 1H, NH); $^{13}$C NMR (125 MHz, $CDCl_3$) δ: 28.64, 40.57, 52.06, 68.79, 70.50, 70.54, 71.12, 79.45, 155.90, 170.94.

Methyl ester **17a** was dissolved in 2M HCl in dioxane (2 mL) and stirred for 4 h at room temperature during which time the formation of a white solid was observed. The solvent was evaporated under reduced pressure to afford a white oily salt **16** (11.4 mg, 78%), which was used in the next step without further purification. $^1$H NMR (500 MHz, $CD_3OD$) δ: 3.04-3.19 (m, 2H), 3.63-3.84 (m, 9H), 4.20 (s, 2H).

Celastrol **(1)** was coupled to **16** using the General Procedure above to give **15**. $^1$H NMR (500 MHz, $CDCl_3$) δ: 0.65 (s, 3H, $CH_3$), 1.00 (br d, 1H, *J* = 13.5 Hz, C(22)-H), 1.13 (s, 3H, $CH_3$) 1.16 (s, 3H, $CH_3$), 1.26 (s, 3H, $CH_3$), 1.45 (s, 3H, $CH_3$), 1.47 - 1.76 (m, 8H), 1.82-2.18 (m, 6H), 2.22 (s, 3H, $CH_3$), 2.45 (d, 1H, *J* = 15.6, C(19)-H), 3.31 -3.39 (m, 2H), 3.59-3.66 (m, 2H), 3.68-3.74 (m, 2H), 3.77 (s, 3H, $OCH_3$), 4.15 (s, 2H), 6.27 (br t, 1H, *J* = 5.0 Hz, NH), 6.34 (d, 1H, *J* = 7.1 Hz, C(7)-H), 6.52 (s, 1H, C(1)-H), 7.02 (d, 1H, *J* = 7.1 Hz, C(6)-H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ: 10.40, 18.51, 21.96, 28.89, 29.59, 30.28, 30.28, 31.03, 31.33, 31.86, 33.74, 33.95, 35.17, 36.61, 38.41, 39.37, 39.57, 40.48, 43.23, 44.62, 45.28, 52.10, 68.69, 69.73, 70.29, 71.10, 117.22, 118.22, 119.74, 127.60, 134.27, 146.21, 165.01, 170.54, 177.99, 178.57. HPLC (Gemini-NX C18 5μ; 20% $H_2O$ (0.01% TFA) - 80 % $CH_3CN$) $R_f$ = 6.65 min; Gemini 5μ C18; 10% $H_2O$ (0.1% TFA) - 90 % MeOH) $R_f$ = 6.00 min. HRMS (ESI, MeOH) calcd for $C_{36}H_{51}NO_7$: 609.3688; found: 609.3666.

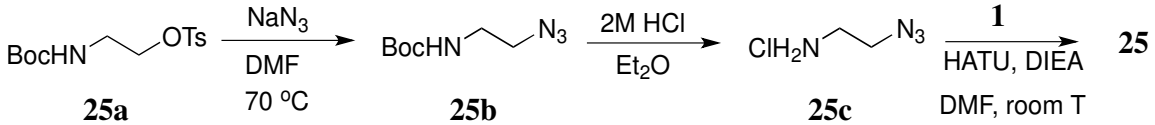
**Methyl 2-(2-(2-acetamidoethoxy)ethoxy)acetate (18**, **Scheme S-1).** To a suspension of **16** (103 mg, 0.48 mmol, Scheme S-1) in anhydrous $CH_2Cl_2$ (3 mL) was added $Ac_2O$ (0.06 ml, 0.58 mmol) at 0 °C followed by the addition of $Et_3N$ (0.08 ml, 0.58 mmol). The reaction was stirred for 20 h at room temperature, after which 0.1M HCl (10 mL) was added, and the solution was extracted with EtOAc (3 x 10 mL), washed with water (20 mL), and brine (20 mL). The combined organic extracts were dried over $Na_2SO_4$. Filtration and solvent removal afforded the crude product, which was purified by column chromatography (silica gel, ethyl acetate - hexanes) to yield **18** (30 mg, 28%) as a colorless viscous liquid. $^1$H NMR (500 MHz, $CDCl_3$) δ: 2.01 (s, 3H, $CH_3$), 3.44-3.48 (m,

2H), 3.56-3.61 (m, 2H), 3.65-3.68 (m, 2H), 3.71-3.74 (m, 2H), 3.77 (s, 3H, $OCH_3$), 4.17 (s, 2H), 6.28 (s, 1H, NH); $^{13}C$ NMR (125 MHz, $CDCl_3$) δ: 23.21, 39.25, 51.93, 68.44, 69.93, 70.08, 71.00, 170.33, 170.97. HRMS (ESI, $CH_3OH$) calcd for $C_9H_{17}NO_5$ $[M+Na]^+$: 242.0999; found: 242.1004.

**3-(Celastrol-20-carboxamido(diethyleneglycol)) *tert*-butyl propionate (19).** $^1H$ NMR (500 MHz, $CDCl_3$) δ: 0.64 (s, 3H, $CH_3$), 1.01 (br d, 1H, *J* = 14.0 Hz, C(22)-H), 1.12 (s, 3H, $CH_3$) 1.15 (s, 3H, $CH_3$), 1.26 (s, 3H, $CH_3$), 1.44 (s, 3H, $CH_3$), 1.45 (s, 9H, $3CH_3$), 1.48-2.16 (m, 16H), 2.21 (s, 3H, $CH_3$), 2.46 (d, 1H, *J* = 15.5, C(19)-H), 2.51 (t, 2H, *J* = 7.0 Hz, $CH_2COOtBu$), 3.32 (br s, 2H), 3.44-3.50 (m, 2H), 3.53-3.57 (m, 2H), 3.58-3.61 (m, 2H), 3.72 (t, 2H, *J* = 6.5 Hz), 6.28 (br t, 1H, *J* = 5.0 Hz, NH), 6.34 (d, 1H, *J* = 7.5 Hz, C(7)-H), 6.52 (s, 1H, C(1)-H), 6.98 (br s, 1H, OH), 7.01 (d, 1H, *J* = 7.5 Hz, C(6)-H); $^{13}C$ NMR (500 MHz, $CDCl_3$) δ: 10.28, 18.29, 21.73, 28.10 ($3CH_3$), 28.65, 29.37, 30.05, 30.81, 31.09, 31.62, 33.49, 33.74, 34.94, 36.22, 36.37, 38.19, 39.11, 39.32, 40.26, 43.01, 44.36, 45.05, 66.85, 69.52, 70.11, 70.23, 80.69, 117.03, 117.99, 119.50, 127.35, 134.09, 145.96, 164.79, 170.37, 170.87, 177.77, 178.33. HPLC (Gemini-NX C18 5μ; 20% $H_2O$ (0.01% TFA) - 80 % $CH_3CN$) $R_f$ = 12.83 min; Gemini 5μ C18; 10% $H_2O$ (0.1% TFA) - 90 % MeOH) $R_f$ = 8.13 min. HRMS (ESI, MeOH) calcd for $C_{40}H_{59}NO_7$: 665.4321; found: 665.4292.

**2-(Celastrol-20-carboxamido(triethyleneglycol)) ethanol (20).** $^1H$ NMR (500 MHz, $CDCl_3$) δ: 0.64 (s, 3H, $CH_3$), 0.90 (br d, 1H, *J* = 14.0 Hz, C(22)-H), 1.12 (s, 3H, $CH_3$) 1.16 (s, 3H, $CH_3$), 1.26 (s, 3H, $CH_3$), 1.44 (s, 3H, $CH_3$), 1.45 (s, 9H, $3CH_3$), 1.47-2.17 (m, 16H), 2.21 (s, 3H, $CH_3$), 2.46 (d, 1H, *J* = 16.0, C(19)-H), 3.38-3.39 (m, 2H), 3.41-3.49 (m, 3H), 3.54-3.58 (m, 3H), 3.63-3.67 (m, 6H), 3.69-3.70 (m, 4H overlap with OH), 6.34 (d, 1H, *J* = 7.0 Hz, C(7)-H), 6.52 (s, 1H, C(1)-H), 6.91 (br t, 1H, *J* = 5.0 Hz, NH), 6.99 (br s, 1H, OH), 7.02 (d, 1H, *J* = 6.5 Hz, C(6)-H); $^{13}C$ NMR (125 MHz, $CDCl_3$) δ: 10.29, 18.27, 21.72, 28.68, 29.35, 29.94, 30.72, 31.08, 31.67, 33.63, 33.67, 35.04, 36.42, 38.19, 39.32, 39.42, 40.13, 43.03, 44.48, 45.09, 61.36, 69.83, 70.02, 70.17, 70.41, 70.44, 72.49, 117.12, 118.00, 119.43, 127.30, 134.27, 145.97, 164.89, 170.59, 178.06, 178.33. HPLC (Gemini-NX C18 5μ; 20% $H_2O$ (0.01% TFA) - 80% $CH_3CN$) $R_f$ = 4.85 min; Gemini 5μ C18; 10% $H_2O$ (0.1% TFA) - 90% MeOH) $R_f$ = 5.73 min. HRMS (ESI, MeOH) calcd for $C_{37}H_{55}NO_7$: 625.3979; found: 625.3988.

**Celastrol-(2-azidoethyl)-20-carboxamide (25, Scheme S-2).**
Scheme S-2. Synthesis of **25**.




To a solution of 2-(*tert*-butoxycarbonylamino)ethyl 4-methylbenzenesulfonate (**25a**, 65 mg, 0.2 mmol) in anhydrous DMF was added sodium azide (15 mg, 0.23 mmol), and the mixture was heated at 65 °C for 72 h. The solvent was removed *in vacuo*, and the residue was dissolved in EtOAc (70 mL) then washed with water (2 x 30 mL) and brine (30 mL). Filtration and solvent removal afforded **25b** as a white solid (35 mg, 95%). The crude

product was used in the next reaction without further purification. $^1$H NMR (500 MHz, $CDCl_3$) δ: 1.29 (s, 9H), 3.14 (m, 2H), 3.24 (m, 2H), 5.35 (br s. 1H).

A solution of **25b** (22.3 mg, 0.12 mmol) in 2 M HCl in ether (2 mL) was stirred for 24 h at room temperature during which time the formation of a white solid was observed. The solvent was evaporated under reduced pressure to afford a pale yellow salt (**25c**, 11.4 mg, 78%), which was used in the next step without further purification.
$^1$H NMR (500 MHz, $CD_3OD$) δ: 3.09 (m, 2H), 3.70 (m, 2H).

Celastrol **(1)** was coupled to **25c** using the above General Procedure to give **25**. $^1$H NMR (500 MHz, $CDCl_3$) δ: 0.64 (s, 3H, $CH_3$), 1.05 (br d, 1H, *J* = 13.4 Hz, C(22)-H), 1.14 (s, 3H, $CH_3$) 1.17 (s, 3H, $CH_3$), 1.27 (s, 3H, $CH_3$), 1.45 (s, 3H, $CH_3$), 1.50-1.78 (m, 8H), 1.80-2.18 (m, 6H), 2.21 (s, 3H, $CH_3$), 2.48 (d, 1H, *J* = 15.0 Hz, C(19)-H), 3.20-3.50 (m, 4H), 6.05 (br s, 1H, NH), 6.35 (d, 1H, *J* = 6.7 Hz, C(7)-H), 6.53 (s, 1H, C(1)-H), 6.98 (br s, 1H, OH), 7.01 (d, 1H, *J* = 6.7 Hz, C(6)-H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ: 9.26, 17.39, 20.69, 27.60, 28.44, 29.06, 29.79, 29.94, 30.58, 32.42, 32.68, 33.89, 35.28, 37.16, 38.02, 38.30, 39.43, 41.95, 43.25, 43.98, 49.81, 116.09, 117.06, 118.51, 126.36, 133.09, 144.95, 163.73, 169.10, 177.13, 177.31. HPLC (Gemini-NX C18 5μ; 20% $H_2O$ (0.01% TFA) - 80% $CH_3CN$) $R_f$ = 8.68 min; Gemini 5μ C18; 10% $H_2O$ (0.1% TFA) - 90 % MeOH) $R_f$ = 6.78 min. HRMS (ESI, MeOH) calcd for $C_{31}H_{42}N_4O_3$ $[M+1]^+$: 519.3330; found: 519.3326.

***N*-(2-(2-((4-(2-Oxohexahydro-1H-thieno[3,4]-dimidazol-4-yl)butanamido)methoxy)ethoxy)ethyl)celastrol-20-carboxamide (21).** Celastrol **(1)** was coupled to EZ-link amine-$PEG_2$-biotin using the General Procedure. $^1$H NMR (500 MHz, MeOD) δ: 7.24 (d, 1H, *J* = 6.76 Hz), 6.49 (m, 2H), 4.50 (m, 1H, CH-bridge), 4.31 (m, 1H, CH-bridge), 3.47-3.59 (m, 8H, $OCH_2$), 3.33-3.38 (m, 4H, $NHCH_2$), 3.29 (m, 1H, CHS), 3.20 (m, 1H, CH'HS), 2.93 (dd, 1H, $J_1$ = 5.44 Hz, $J_2$ = 7.45 Hz, CH'HS), 2.72 (m, 1H, CH'H-4), 2.51 (d, 1H, *J* = 15.75 Hz, CH'H-4), 2.23 (s, 3H, $CH_3$), 2.12 (t, 2H, *J* = 11.55 Hz, $CH_2$), 1.83-2.02 (m, 2H, $CH_2$), 1.70-1.80 (m, 2H, $CH_2$), 1.59-1.70 (m, 4H, 2 x $CH_2$), 1.52-1.58 (m, 2H, $CH_2$), 1.41-1.51 (m, 4H, 2 x $CH_2$), 1.37 (m, 1H), 1.34 (s, 3H, $CH_3$), 1.31 (s, 3H, $CH_3$), 1.17 (s, 3H, $CH_3$), 1.16 (s, 3H, $CH_3$), 0.99-1.05 (m, 2H, $CH_2$), 0.90-0.99 (m, 2H, $CH_2$), 0.69 (s, 3H, $CH_3$). $^{13}$C NMR (125 MHz, MeOD) δ: 10.42, 19.45, 22.19, 26.89, 29.53, 29.81, 30.51, 30.81, 31.80, 31.85, 32.09, 34.23, 34.35, 34.84, 36.16, 36.77, 37.64, 38.97, 40.31, 40.69, 41.10, 41.46, 41.52, 44.28, 45.88, 46.35, 57.06, 61.64, 63.39, 70.33, 70.61, 71.21, 71.25, 71.31, 118.73, 119.77, 120.11, 120.61, 136.43, 159.85, 166.13, 166.48, 176.14, 181.01, 181.07. $^{13}$C NMR (125 MHz, MeOD) δ: 0.71, 9.3, 10.6, 20.1, 20.9, 21.2, 23.9, 24.9, 25,2, 27.8, 28.1, 28.4, 28.4, 28.9, 30.5, 32.1, 32.6, 35.3, 35.5, 35.8, 37.4, 38.4, 39.2, 39.8, 40.3, 40.7, 41.2, 41.4, 44.3, 48.5, 59.9, 61.8, 111.5, 118.2, 120.0, 120.7, 128.6, 135.2, 136.7, 145.5, 164.9, 168.5, 176.1, 181.0. HPLC: Gemini 5μ C-18 (10% $H_2O$ (0.1% TFA) - 90 % $CH_3CN$): Rf = 5.08 min; Gemini-NX 5μ C-18 (10% $H_2O$ (0.1% TFA) - 90% $CH_3OH$): $R_f$ = 4.82 min. HRMS (ESI, $CH_3OH$) calcd for $C_{45}H_{66}N_4O_7S$ $[M+1]^+$: 807.4725; found: 807.4731.

***N*-(7-(4-(2-Oxohexahydro-1H-thieno[3,4]-dimidazol-4-yl)butanamido)heptyl)celastrol-20- carboxamide (22).** Celastrol **(1)** was coupled to EZ-link pentylamine-biotin using the General Procedure. $^1$H NMR (500 MHz, MeOD) δ: 0.68 (s, 3H, $CH_3$), 1.16 (s, 3H, $CH_3$), 1.17 (s, 3H, $CH_3$), 1.33 (s, 3H, $CH_3$), 1.22-1.35 (m,

3H), 1.39-1.57 (m, 4H), 1.49 (s, 3H, $CH_3$), 1.57-1.78 (m, 10H, 5 x $CH_2$), 1.79-2.00 (m, 2H, $CH_2$), 2.24 (s, 3H, $CH_3$), 2.50 (d, 1H, $J$ = 14.92 Hz, CHH'-3), 2.68 (d, 1H, $J$ = 13.78 Hz, CHH'-3), 2.91 (dd, 1H, $J_1$ = 5.02 Hz, $J_2$ = 7.72 Hz, CH-S), 3.01-3.11 (m, 2H, $CH_2$), 3.12-3.16 (m, 5H, 2x $CH_2$ + CH'HS), 3.16-3.22 (m, 5H, 2x $CH_2$ + CH'HS), 3.38-3.57 (m, 2H, $CH_2$), 4.30 (dd, 1H, $J_1$ = 4.39 Hz, $J_2$ = 7.82 Hz, CH-bridge) [minor = 4.25], 4.49 (dd, 1H, $J_1$ = 4.77 Hz, $J_2$ = 7.82 Hz, CH-bridge) [minor = 4.45], 6.49 (d, 2H, $J$ = 5.31 Hz), 7.23 (d, 1H, $J$ = 7.11 Hz). $^{13}$C NMR (125 MHz, MeOD) δ: 0.71, 9.3, 10.6, 21.2, 24.9, 20.1, 20.9, 23.9, 24.9, 25.2, 27.8, 28.1, 28.4, 28.9, 30.5, 32.1, 32.6, 35.3, 35.5, 35.8, 37.4, 38.4, 39.2, 39.8, 40.3, 40.8, 41.1, 41.4, 44.3, 48.5, 59.9, 61.8, 111.5, 118.2, 120.0, 120.7, 128.6, 135.2, 136.7, 145.5, 164.9, 168.5, 176.1, 181.0. HPLC: Gemini 5µ C-18 (80% $H_2O$ (0.1% TFA) - 50% $CH_3CN$): $R_f$ = 5.42 min; Gemini 5µ C-18 (80% $H_2O$ (0.1% TFA) - 20% MeOH): $R_f$ = 9.13 min. HRMS (ESI, MeOH) calcd for $C_{44}H_{64}N_4O_5S$ $[M+1]^+$: 761.4670; found: 761.4668.

***N*-(2-(4-(2-Oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)butanamido)ethyl)celastrol-20- carboxamide (23).** Celastrol **(1)** was coupled to *N*-(2-aminoethyl)biotinamide hydrobromide using the General Procedure.
$^1$H NMR (500 MHz, MeOD) δ: 7.21 (d, 1H, $J$ = 7.33), 6.44 (2H, m), 4.46 (m, 1H, CH-bridge), 4.46 (m, 1H, CH-bridge), 4.27 (dd, 1H, $J_1$ = 4.62 Hz, $J_2$ = 3.54 Hz, CH-bridge), 3.26 (m, 1H, CHS), 3.21 (m, 1H, CH-HS), 3.11-3.19 (m, 4H, $NHCH_2CH_2NH$), 2.89 (dd, 1H, $J_1$ = 5.08 Hz, $J_2$ = 8.13 Hz, CH-HS), 2.68 (m, 1H), 2.47 (m, 1H), 2.20 (s, 3H, $CH_3$), 2.16 (m, 2H, $CH_2$), 2.03 (m, 2H, $CH_2$), 1.47-1.66 (m, 13H), 1.36-1.42 (m, 2H), 1.45 (s, 3H, $CH_3$), 1.29 (s, 3H, $CH_3$), 1.13 (s, 3H, $CH_3$), 1.12 (s, 3H, $CH_3$, 0.60 (s, 3H, $CH_3$). $^{13}$C NMR (125 MHz, MeOD) δ: 10.40, 19.39, 22.21, 26.86, 29.51, 29.86, 30.45, 30.67, 31.82, 32.03, 34.21,34.69, 36.10, 36.86, 37.52, 38.91, 39.61, 40.61, 41.07, 41.44, 41.54, 44.25, 45.76, 46.35, 57.02, 61.66, 63.38, 109.33, 118.73, 120.13, 120.52, 128.61, 136.46, 142.36, 145.13, 146.28, 147.91, 166.45, 176.79, 180.09, 181.38 HPLC: Gemini 5µ C-18 (20% $H_2O$ (0.1% TFA) - 80% $CH_3CN$): $R_f$ = 4.72 min; Gemini-NX 5µ C-18 (20% $H_2O$ (0.1% TFA) - 80% $CH_3OH$): $R_f$ = 11.42 min. HRMS (ESI, $CH_3OH$) calcd for $C_{41}H_{58}N_4O_5S$: 718.4128; found: 718.4152.

***N*-(4-(2-Oxohexahydro-1H-thieno[3,4]-dimidazol-4-yl)butyl)celastrol-20-carboxamide (24).** Celastrol **(1)** was coupled to norbiotinamine hydrochloride using the General Procedure.
$^1$H NMR (500 MHz, MeOD) δ: 7.24 (d, 1H, $J$ = 7.10 Hz, 6.48 (m, 2H), 4.42 (m, 1H, CH-bridge), 4.21 (m, 1H, CH-bridge), 3.04 (m, 1H), 3.17 (m, 1H), 2.88-3.08 (m, 4H, $CH_2$), 2.53 (d, 1H, $J$ = 12.5 Hz), 2.46 (d, 1H, $J$ = 15 Hz), 2.11 (s, 3H), 1.99 (m, 2H), 1.56 (m, 4H), 1.46-1.87 (m, 8H), 1.37 (s, 3H), 1.25 (m, 2H), 1.19 (s, 3H), 1.04 (s, 3H), 1.03 (s, 3H), 0.86 (m, 2H), 0.58 (s, 3H).$^{13}$C NMR (125 MHz, MeOD) δ: 11.3, 12.4, 14.2, 26.1, 28.6, 28.8, 30.2, 30.8, 33.0, 36.0, 36.5, 37.4, 37.4, 38.87, 39.3, 39.6, 40.55, 40.67, 41.06, 41.51, 41,45, 44.0, 46.02, 46.31, 45.86, 45.36, 47.8, 54.9, 61.5, 61.8, 117.3, 118.2, 119.4, 135.2, 166.05, 172.25, 180.81. HPLC: Gemini-NX 5µ C-18 (80% $H_2O$ (0.1% TFA) - 20% $CH_3CN$): $R_f$ = 4.45 min; Gemini 100 5µ C-18 (70% $H_2O$ (0.1% TFA) - 30% $CH_3OH$): $R_f$ = 5.55 min. HRMS (ESI, $CH_3OH$) calcd for $C_{38}H_{53}N_4O_5S$ $[M+1]^+$: 648.3830; found: 648.3835.

## 2. Biology

### 2.1. RNA isolation and reverse transcription - PCR

Human HeLa cells, grown to approximately 80% confluence were treated with varying concentrations of indicated compounds for 8 h. Total RNA was isolated from cells using an RNeasy Extraction kit (Qiagen, Valencia, CA) with on-column DNase I treatment according to the manufacturer's directions. RNA (1.0 µg) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). PCR was performed using PCR primers specific for Hsp70 and 18S. The human Hsp70 primers were:

5′-GCCCAAGGTGCGCGTATGCTA-3′ (forward) and
5′-CGCCTCGGCCGTCTCCTTCA-3′ (reverse);

the human 18S primers were:

5′- GCCCGAGCCGCCTGGATACC-3′ (forward) and
5′-TCACCTCTAGCGGCGCAATACGAA-3′ (reverse).

PCR products were amplified with Taq polymerase (Promega, Madison, WI) using the standard cycling conditions.

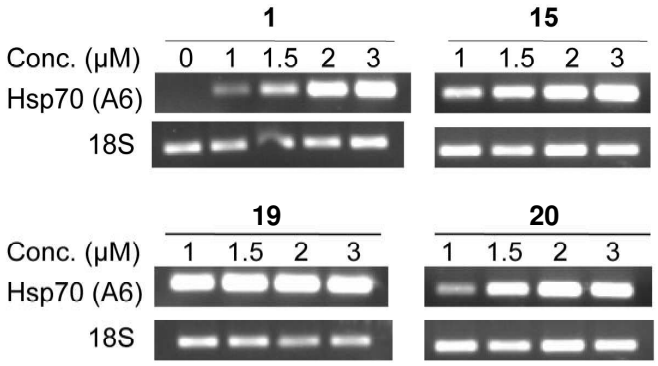



**Figure S-1.** RT-PCR of Hsp70 mRNA levels by long chain analogs **15**, **19**, and **20** compared to celastrol **(1)**

## 3. Mass Spectrometric Analysis

### 3.1 Sample Preparation

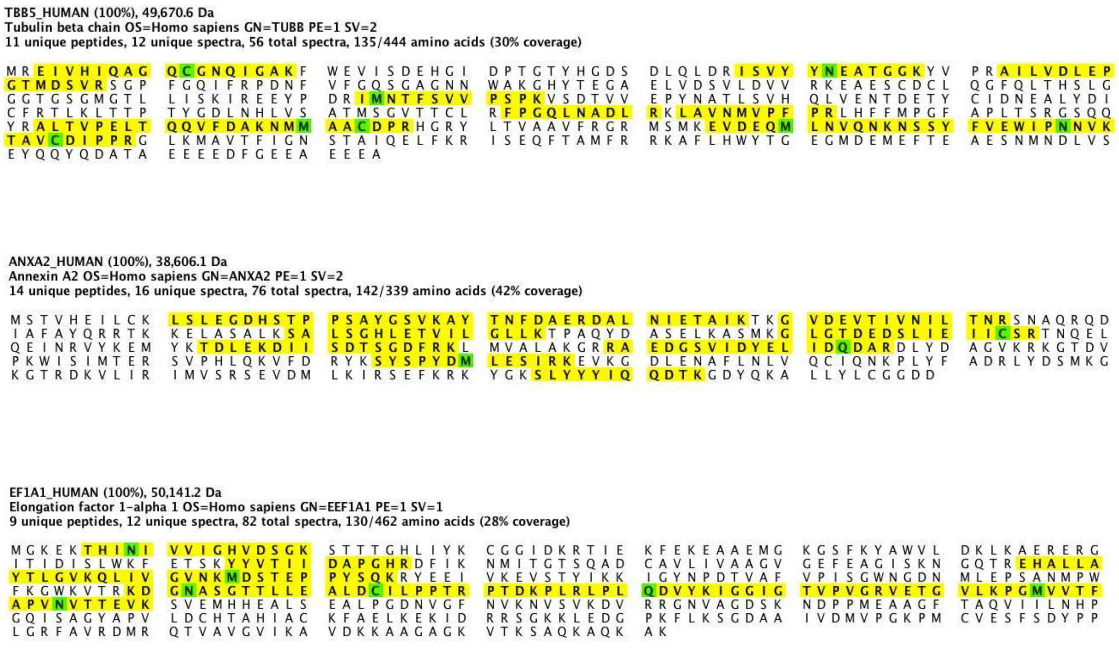
Mass spectrometric analysis was performed by the Chicago Biomedical Consortium/University of Illinois at Chicago Research Resources Center Proteomics and Informatics Services Facility. The in-gel tryptic digestion was carried out by following the protocol described by Kinter and Sherman.[1] Briefly, the gel bands were cut into 1-$mm^3$ pieces, rinsed, and dehydrated, and the protein was reduced with DTT and alkylated with iodoacetamide in the dark prior to overnight digestion with trypsin at 37 °C in 50 mM ammonium bicarbonate. The peptides were concentrated and analyzed using a Thermo LTQ-FT Ultra mass spectrometer equipped with a Dionex 3000 nanoflow HPLC system controlling a reverse-phase column (Agilent Zorbax 300SB-C18, 3.5 µm, 75 µm x 150mm) at a flow rate of 250 nL/min. The peptides were separated and eluted with a linear gradient of 10–60% solution B (95% acetonitrile, 0.1% formic acid) in 60 min. The RAW data file was converted to mzXML (version 2.1) using the Institute for Systems Biology's readw.exe conversion tool (version 4.0.2), converted to the Mascot generic

format (MGF) using MzXML2Search and then submitted to a Mascot search engine (version 2.2.04).

### 3.2 Database Searching

Tandem mass spectra were extracted by using readw.exe version 4.0.2 (Institute for Systems Biology). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.2.04). Mascot was set up to search the SwissProt_57.15 database (selected for Homo sapiens, 20266 entries) assuming the digestion enzyme was trypsin with two missed cleavages allowed. Mascot was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 ppm. Deamidation of asparagine, oxidation of methionine, acetylation of lysine, and the iodoacetamide derivative of cysteine werespecified in Mascotas variable modifications. Criteria for Protein Identification Scaffold (version Scaffold_3_00_02, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability as specified by the Peptide Prophet algorithm.[2] Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.[2,3] Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

### 3.3 Peptide coverage for significant proteins:



**4**. **Prediction of pharmacokinetic properties of 21-24**

Computational approach (QikProp v3.2 software) was used to evaluate the physicochemical properties, such as the octanol - water partition (log P), water solubility (log S), and ADME properties, such as the apparent cell permeability based on *Caco-2* and *MDCK* cell lines of biotinylated analogues **21-24** in comparison to celastrol **(1)** and primary amide **3** (Table S-1).

**Table S-1.** Prediction of pharmacokinetic properties of biotinylated derivatives **21**- **24** and comparison to **1** and **3**. Predictions were calculated using QikProp v3.2 software.

| ***Compound*** | | ***Properties*** | | ***Permeability*** *(nm/sec)* | |
|---|---|---|---|---|---|
| ***#*** | ***MW*** | ***Log P*** | ***Log S*** | ***Caco-2*** | ***MDCK*** |
| **1** | 450.61 | 4.99 | - 6.50 | 69 | 35 |
| **3** | 449.63 | 3.47 | - 5.30 | 151 | 111 |
| **21** | 807.10 | 5.26 | - 10.11 | 6 | 8 |
| **22** | 761.07 | 5.86 | - 10.14 | 7 | 10 |
| **23** | 718.99 | 3.86 | - 5.81 | 14 | 15 |
| **24** | 647.91 | 5.48 | - 8.91 | 32 | 31 |

***Expected values:*** *Log P for octanol/water (8 – 35.0);*
*Log S for aqueous solubility (- 6.5 - 0.5);*
*Permeability Caco-2 cells (<25 poor, >500 great);*
*Permeability MDCK cells (<25 poor, >500 great)*

**5. Western blot analysis**

HEK293 cells were grown to ~80% confluence and treated with indicated concentrations of celastrol or DMSO control for 3 h. The whole cell lysate was analyzed for the level of alpha-, beta-, and gamma-tubulin as described previously (see Methods). The following antibodies were used: anti-α-tubulin antibody (T5168; Sigma, St. Louis, MO) in 1:5,000 dilution; anti-β-tubulin antibody (T-4026; Sigma, St. Louis, MO) in 1:200 dilution and anti-γ-tubulin antibody (T-6557; Sigma, St. Louis, MO) in 1:5,000 dilution. Equal loading was verified by constitutive non-inducible Hsc70 (SPA-815, Stressgen) in 1:1,500 dilution.

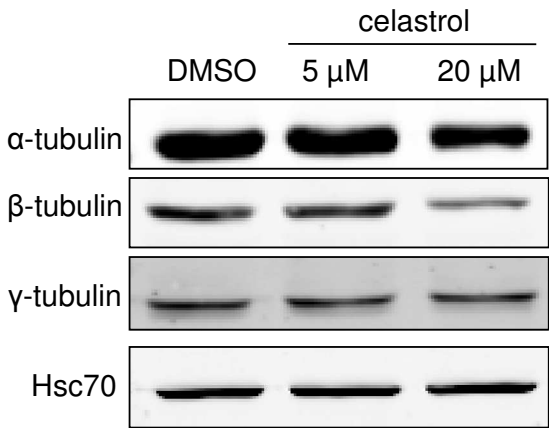


Figure S-2. Western blot analysis of intracellular tubulin levels following celastrol treatment.

**References:**

1. Kinter, M., Sherman, N. E. : Protein Sequencing and Identification Using Tandem Mass Spectrometry. *Wiley-Interscience, Inc., New York* **2000.**
2. Keller, A.; Nesvizhskii, A. I.; Kolker, E.; Aebersold, R. (2002) Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem. 74,* 5383-92.
3. Nesvizhskii, A. I., Keller, A., Kolker, E., and Aebersold, R. (2003) A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem. 75*, 4646– 4658.