Supporting Information

Targeting YAP degradation by a novel 1,2,4-oxadiazole derivative via restoration of the function of the Hippo pathway

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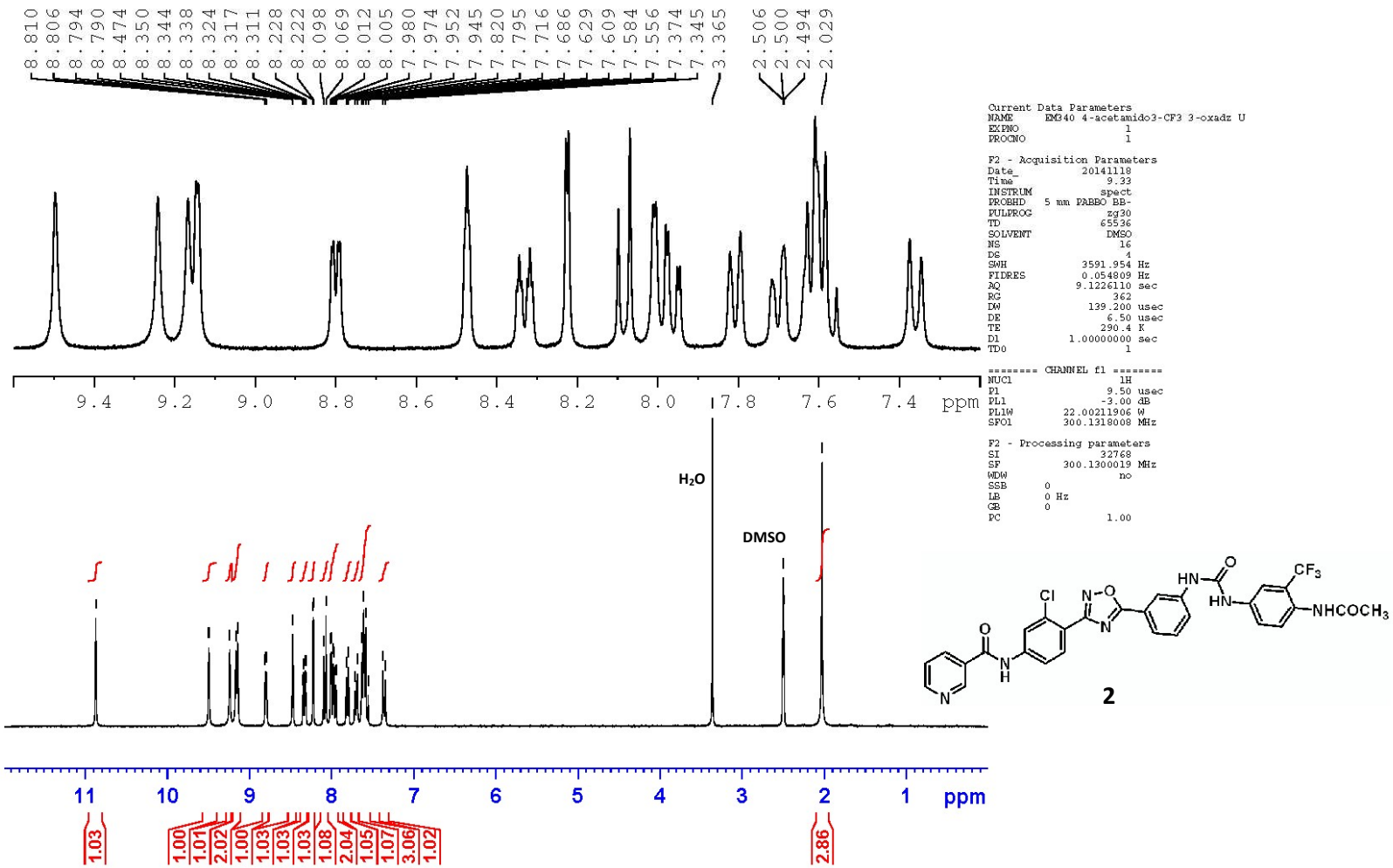
Chemistry, spectroscopic and purity data for compound 2

All commercially available reagents were used without further purification unless otherwise stated. Routine ^{1}H and ^{13}C nuclear magnetic resonance spectra were recorded on Bruker AV300 or Bruker Ascend 400. Samples were dissolved in deuterated chloroform ($CDCl_3$) or dimethyl sulfoxide (DMSO-d_6), and tetramethylsilane (TMS) was used as a reference. Electrospray ionization mass spectrometry analyses were performed with a Micromass Q-T of II high resolution electrospray mass spectrometer. All compounds for bioassay were identified with ^{1}H NMR, ^{13}C NMR, and HRMS, and in purity higher than 95%. The purity was confirmed by a Hitachi Elite LaChrom HPLC system (comprised of a Versa Grad Prep 36 pump, an L-2400 UV detector, an L-2200 auto sampler and a 150 × 4.6 mm Agilent ZORBAX Eclipse XDB-C18 5μ column; detection, 254 nm). A linear solvent gradient with a mobile phase of 30% water in methanol to 100% methanol in 20 min was used. Compound **2** was synthesized according to a previously reported procedure,[1] as depicted in **Scheme 1**. R_f = 0.25 (EtOAc/MeOH 4:0.25). Light-pink solid, overall yield 26.8%. **^{1}H NMR** (300 MHz, DMSO-d_6) δ 10.87 (s, 1H), 9.50 (s, 1H), 9.24 (s, 1H), 9.20 – 9.11 (m, 2H), 8.80 (dd, *J* = 4.8, 1.7 Hz, 1H), 8.47 (d, *J* = 1.9 Hz, 1H), 8.33 (dt, *J* = 8.1, 2.0 Hz, 1H), 8.23 (d, *J* = 2.0 Hz, 1H), 8.13 – 7.91 (m, 3H), 7.81 (dt, *J* = 7.6, 1.4 Hz, 1H), 7.75 – 7.52 (m, 4H), 7.36 (d, *J* = 8.7 Hz, 1H), 2.03 (s, 3H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 174.72, 169.27, 166.80, 164.66, 152.50, 148.79, 142.24, 140.52, 137.95, 135.60, 132.34, 132.21, 131.21, 130.15, 130.01, 129.26, 124.79, 123.67, 123.54, 123.06, 122.36, 122.07, 121.46, 121.32, 120.22, 118.69, 117.21, 115.54, 115.48, 22.78. **HRMS** exact mass of $C_{30}H_{21}ClF_3N_7O_4$ $(M+Na)^+$: 658.1189 amu; found: 658.1203 amu. **HPLC purity**: 98.37%, **HPLC t_R**: 8.47 min.

[1] Dokla, E. M. E., Fang, C.-S., Abouzid, K. A. M. & Chen, C. S. 1,2,4-Oxadiazole derivatives targeting EGFR and c-Met degradation in TKI resistant NSCLC. *Eur. J. Med. Chem.* **182**, 111607 (2019).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7.6
7.4
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DMSO
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10
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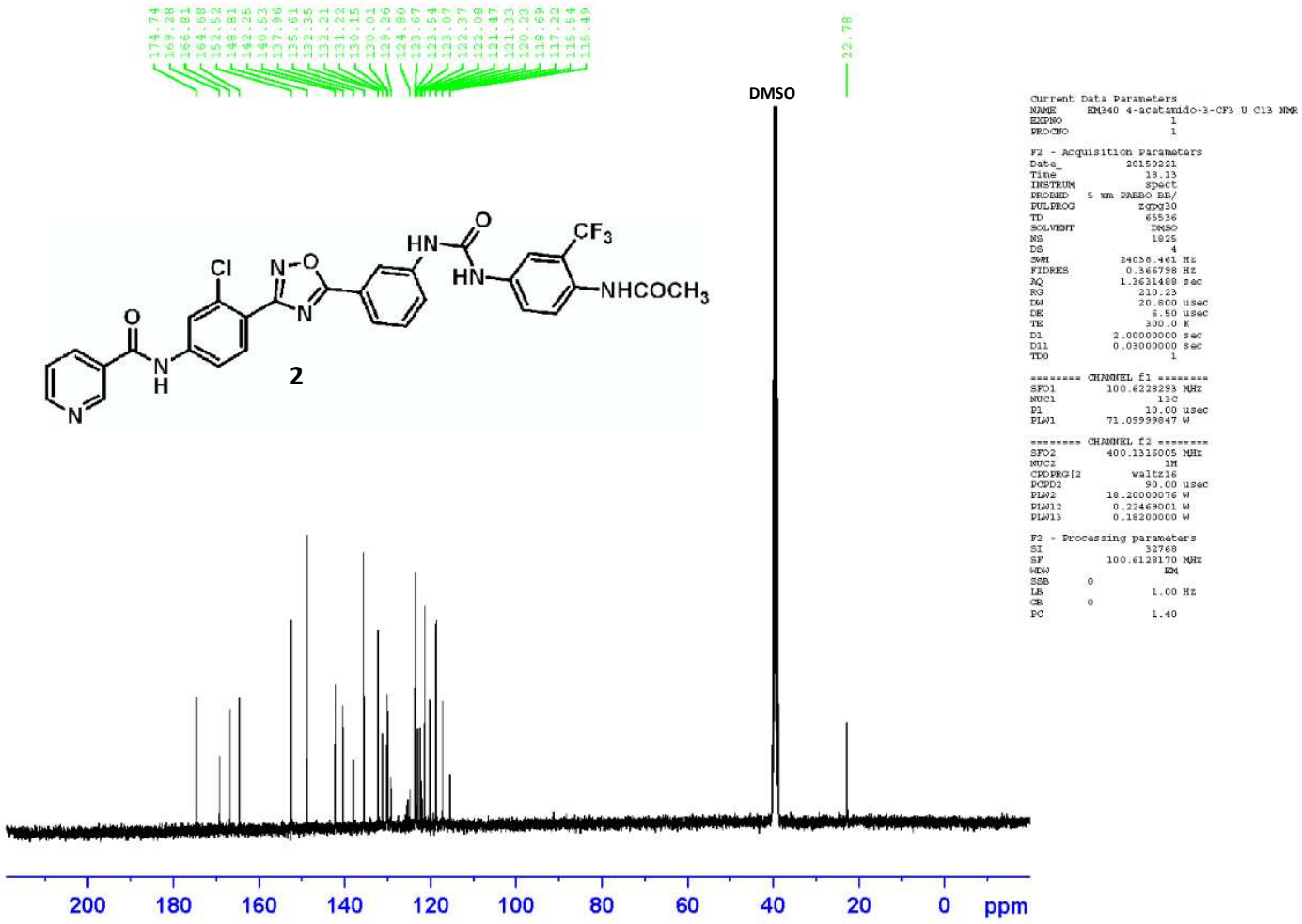


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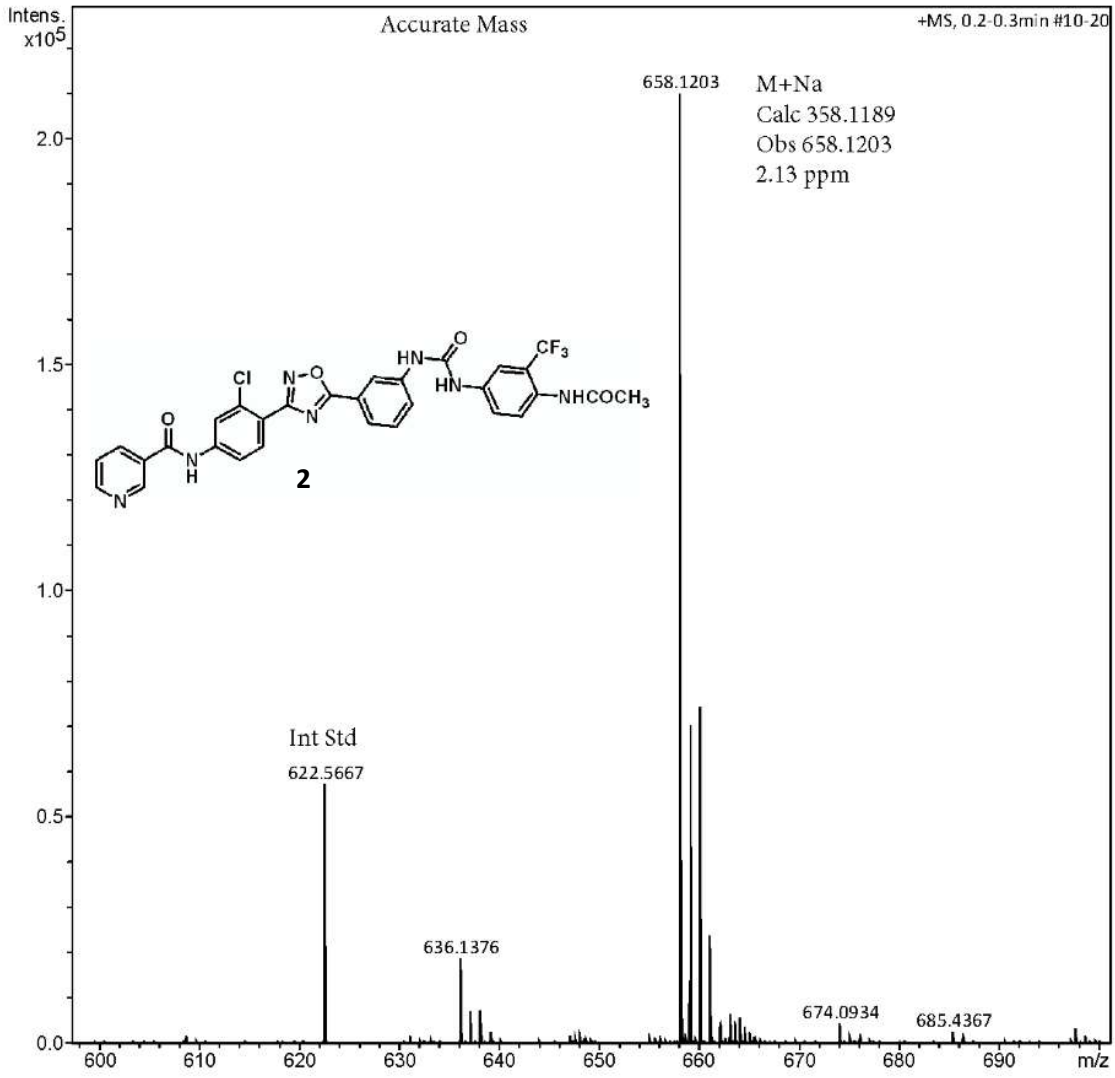

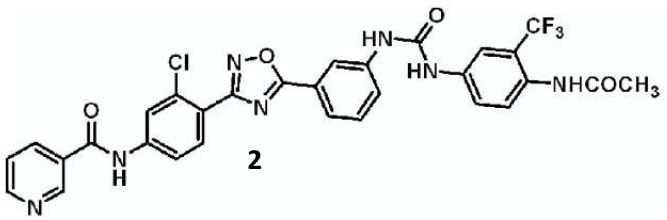

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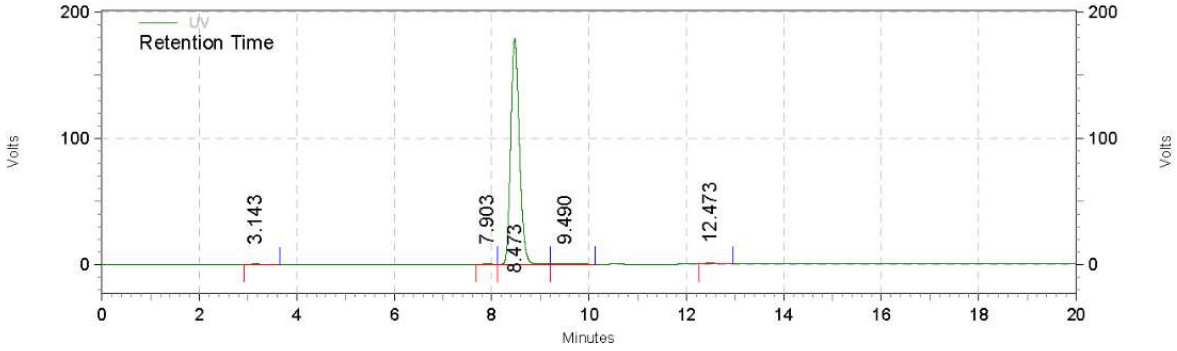

Modified UV Results

Retention Time	Area	Area %	Height	Height %
10.383	46130	100.00	3751	100.00

Totals	46130	100.00	3751	100.00

UV Results

Retention Time	Area	Area %	Height	Height %
3.143	36316	0.40	2184	0.30
7.903	17787	0.20	1393	0.19
8.473	8841768	98.37	716085	98.71
9.490	33019	0.37	744	0.10
12.473	58986	0.66	5020	0.69

Totals	8987876	100.00	725426	100.00

Materials and methods for biological evaluation of compound 2

Cancer cell lines, cell culture, reagents, and antibodies

Cancer cell lines, reagents, inhibitors and plasmids

SUM159 cells were purchased from Asterand Biosciences (Detroit, MI), MCF-7, MDA-MB-231, and PC3 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA), and PC9 cells were kindly provided by Professor Pan-Chyr Yang (Institute of Biomedical Science, Academia Sinica, Taiwan)[2]. Individual cell lines were cultured in the following media (all purchased from Life Technologies, Grand Island, NY): SUM159, Ham's F-12 with 5 μg/ml insulin and 1 μg/ml hydrocortisone; MDA-MB-231, DMEM; PC3 and PC9, RPMI 1640; MCF-7, 1:1 DMEM/F-12 with 2.5 μg/ml insulin and 0.5 μg/ml hydrocortisone. All media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies). Cells were incubated at 37°C in a humidified incubator containing 5% CO_2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), a cocktail consisting of protease and phosphatase inhibitors, insulin, hydrocortisone, cycloheximide, and MG132 were obtained from Sigma-Aldrich (St. Louis, MO). BCA Protein Assay kit was obtained from Thermo Fisher Scientific (Waltham, MA). Western Lighting Chemiluminescence Plus-ECL was purchased from Perkin-Elmer (Waltham, MA). Lipofectamine 2000 reagents were purchased from Life Technologies. 8xGTIIC-Luc plasmid was a gift from Stefano Piccolo (Addgene plasmid # 34615) and has been described previously[3] and pCMV-Luc was a gift from Ralf Kuehn (Addgene plasmid # 45968).

Reporter activity assay

Luciferase reporter assays were performed in SUM159 cell line using 8xGTIIC-Luc plasmid with the YAP/TAZ-TEAD responsive reporter, which contains TEAD-binding sites 8xGTIIC. The reporter plasmid (2 μg) was first transiently transfected into SUM159 cells using Lipofectamine 2000 reagents according to the manufacturer's instructions. After 24-h incubation, cells were treated with indicated concentrations of compound **2** for 12 h. An internal control pCMV-Luc plasmid was used as control. Cell lysates were analyzed using the Dual-Luciferase Reporter Assay Kit (Promega) and the luminescence was detected with the aid of an infinite M1000 microplate reader (TECAN; Mänedorf, Switzerland).

Cell viability assay

Cell viability was evaluated using the MTT assay in six replicates. Cancer cells (5000 cells/well) were incubated in a 96-well plate (Corning Inc; Union City, CA) with DMSO or test

[2] Yen, H.-Y. *et al.* Effect of sialylation on EGFR phosphorylation and resistance to tyrosine kinase inhibition. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 6955–60 (2015).
[3] Dupont, S. *et al.* Role of YAP/TAZ in mechanotransduction. *Nature* **474**, 179–183 (2011).

compound at indicated concentrations for 12, 24, 48 and 72 h. A stock solution of MTT (0.5 mg/ml) was then added to each well containing the treated cells, followed by incubation at 37 °C for 1 h. After removal of the medium, the reduced MTT dye was solubilized with dimethyl sulfoxide (DMSO) and the absorbance was determined by an iMarker Microplate spectrophotometer (Bio-Rad; Hercules, CA) at 570 nm. The inhibitory (IC_{50}) value is defined as the concentration of the compound required to inhibit cell growth by 50% compared to untreated controls and is determined by interpolation based on the absorbance values obtained directly above and directly below the midpoint absorbance (the absorbance value halfway between positive and negative controls).

Transient transfection

Wild type YAP-Flag plasmid was obtained from Addgene (plasmid #66853). Transfection was performed using Lipofectamine 2000 according to the manufacture's protocol. After 24 h of transfection, cells (5000 cells/well) were seeded into 96-well plate and cell viability was assessed using MTT assay as described above.

RT-PCR analysis

Total RNA was isolated from cells using TRIzol reagent (Thermo Fisher Scientific; Waltham, MA) by following the manufacturer's protocol. One μg RNA from each sample was reverse-transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad; Hercules, CA). Diluted cDNA samples were used to perform polymerase chain reaction (PCR) and the resulting PCR products were resolved by electrophoresis in 2% agarose gels, and the signals were detected by ethidium bromide staining. The sequences of primers used for RT-PCR were as follows. YAP, forward primer: 5′- CCT TCT TCA AGC CGC CGG AG -3′; reverse primer: 5′- CAG TGT CCC AGG AGA AAC AGC -3′ (146 bp).[4] GAPDH, forward primer: 5′- AAG CCC ATC ACC ATC TTC CAG-3′; reverse primer: 5′- AGG GGC CAT CCA CAG TCT TCT -3′ (361 bp).[5]

Immunoblotting and antibodies

Total cell lysates were collected by scraping and then lysed in SDS lysis buffer (1% SDS, 50 mM Tris-HCl pH 8.0, 10 mM EDTA) containing a commercial cocktail of protease and phosphatase inhibitors. Samples were then sonicated using a VCX500 sonicator (Sonics; Newtown, CT). The protein concentrations were determined using the BCA Protein Assay kit. Equal amounts of proteins were analyzed by SDS-PAGE, transferred to a nitrocellulose membrane (Millipore; Billerica, MA), and then immunoblotted with primary antibodies at 4 °C overnight. Immunoblots were incubated with specific HRP-conjugated secondary antibodies and signals were

[4] Zhang, J. *et al.* YAP-dependent induction of amphiregulin identifies a non-cell-autonomous component of the Hippo pathway. *Nat. Cell Biol.* **11**, 1444–1450 (2009).
[5] Chou, C.-W., Wu, M.-S., Huang, W.-C. & Chen, C.-C. HDAC Inhibition Decreases the Expression of EGFR in Colorectal Cancer Cells. *PLoS One* **6**, e18087 (2011).

visualized by the Western Lighting Chemiluminescence Reagent Plus-ECL. Antibodies used in this study were obtained from the following sources: GAPDH, HRP-conjugated anti-mouse and rabbit secondary antibodies, Santa Cruz Biotechnology (Santa Cruz, CA); YAP, S397-YAP, TAZ, MST1, MST2, p-T183/T180-MST1/2, MOB, p-T35-MOB, LATS1, p-T1079-LATS1, p-S909-LATS1, c-Myc and Cyclin D1, Cell Signaling (Beverly, MA).

In vivo efficacy of compound 2

All experimental procedures were carried out in accordance with guidelines approved by the experimental animal facilities of IBMC (Institute of Biomedical Sciences) and the Animal Care Ethics Commission Academia Sinica (Taipei). Female Balb/c nude mice (Athymic Nude-Foxn1^{nu}; 5 weeks of age; National Animal Research Laboratory, Taipei, Taiwan) were group-housed under constant photoperiod (12-h light/12-h dark) with *ad libitum* access to sterilized food and water. To assess the role of compound **2** *in vivo,* MDA-MB-231 cells were harvested and subcutaneously injected (10^6 cells/mouse) in the right flank of mice. When tumor volumes reached approximately 50 mm^3, mice were randomized to different groups (n = 7/each group) and treated once daily by oral gavage with (1) control group: vehicle solution (10% DMSO/ 0.1% Tween 80/ 0.5% methylcellulose [v/v] in sterile water), and (2) treatment group: compound **2** at 200 mg/kg. Body weight and tumor volume ($0.52 \times \text{Width}^2 \times \text{Length}$) were monitored twice a week by direct measurement with scales and calipers, respectively.[6] At the end of the study (40 days after inoculation), mice were sacrificed by CO_2 asphyxiation and tumor samples were analyzed by immunoblotting.

Maximum tolerated dose (MTD) test and serum chemistry analysis

To assess the MTD of compound **2** *in vivo*, mice were randomly assigned to different groups (n = 3/each group) and treated once daily for 14 days with (1) control group: vehicle solution (10% DMSO/ 0.1% Tween 80/0.5% methylcellulose [v/v] in sterile water) and (2) treatment groups: compound **2** at 200 mg/kg. Body weight was monitored twice a week by direct measurement. The weight variation of the animals in the treatment group did not exceed 10% of the mean weight. At the end of the study, mice were sacrificed by CO_2 asphyxiation and blood was collected through cardiac puncture. Serum chemistry values were determined by using a Hitachi 7080 chemistry automatic analyzer. Aspartate transaminase (AST), alanine transaminase (ALT), albumin (Alb), total bilirubin (T-Bil), blood urea nitrogen (BUN), creatinine (Crea), and total cholesterol (T-Cho) were determined.

[6] Tomayko, M. M. & Reynolds, C. P. Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother. Pharmacol.* **24**, 148–54 (1989).

Statistical analysis

In vitro experiments were performed at least three times and data are presented as means ± SD. Group means were compared using one-way ANOVA (two-tailed) followed by Student's *t* tests. For the *in vivo* experiments, differences in tumor volume were analyzed by Student's *t* test. Differences were considered significant at $P < 0.05$.