Supporting Information

Zingerone nanotetramer strengthened the polypharmacological efficacy of zingerone on human hepatoma cell lines

Mei-Lang Kung[#,1], Pei-Ying Lin[#,1], Shih-Tsung Huang[2,3], Ming-Hong Tai [2,3,4,5], Shu-Ling Hsieh [6], Chih-Chung Wu[7], Bi-Wen Yeh [8,9], Wen-Jeng Wu [8,9,10] and Shuchen Hsieh*[,1,11]

[1]Department of Chemistry, National Sun Yat-sen University, Kaohsiung, 80424, Taiwan

[2]Doctoral Degree Program in Marine Biotechnology, National Sun Yat-Sen University, Kaohsiung, 80424, Taiwan

[3]Doctoral Degree Program in Marine Biotechnology, Academia Sinica, Taipei, 11529, Taiwan

[4]Institute of Biomedical Sciences, National Sun Yat-Sen University, Kaohsiung, 80424, Taiwan

[5]Center for Neuroscience, National Sun Yat-Sen University, Kaohsiung, 80424, Taiwan

[6] Department of Seafood Science, National Kaohsiung University of Science and Technology, Kaohsiung, 81157, Taiwan

[7]Department of Food and Nutrition, Providence University, Taichung, 43301, Taiwan

[8]Department of Urology, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, 80708, Taiwan

[9]Department of Urology, School of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, 80708, Taiwan

[10]Department of Urology, Kaohsiung Municipal Ta-Tung Hospital, Kaohsiung Medical University, Kaohsiung, 80708, Taiwan

[11]School of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, 80708, Taiwan

[#]These authors contributed equally to this work.

***Corresponding Author:**

Prof. Shuchen Hsieh

Department of Chemistry and Center for Nanoscience and Nanotechnology

National Sun Yat-Sen University, Kaohsiung, Taiwan 80424

Phone: +886 7 525-2000 ext. 3931

Fax: +886 7 525-3908

Email: shsieh@faculty.nsysu.edu.tw

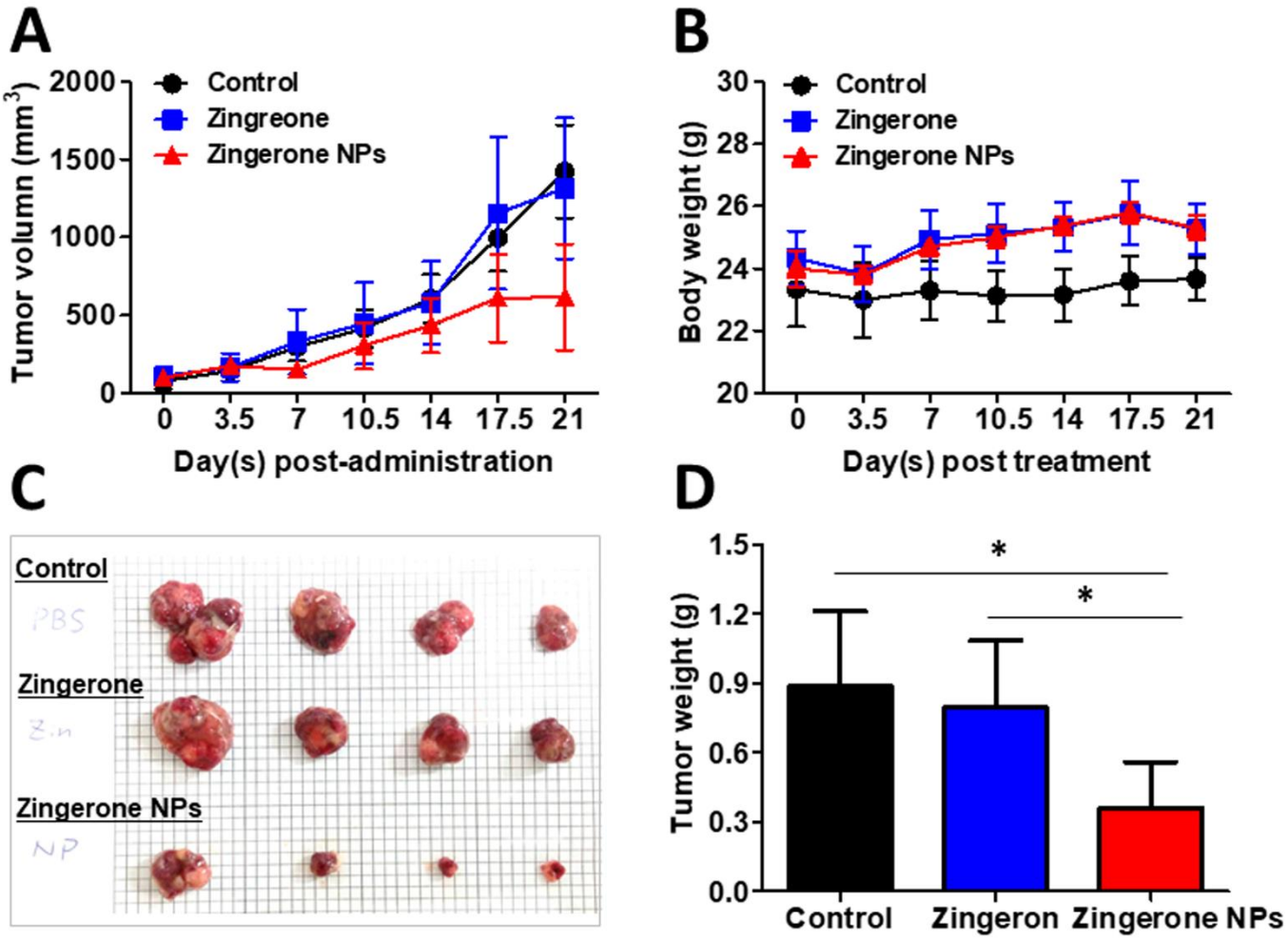

Figure S1. Zingerone NPs suppress tumor growth in Huh7 tumor xenograft model of NOD/SCID mice. After tumor development, mice were randomly divided into three groups (n= 4 per group) and treated with 100 μM of zingerone, zingerone NPs or PBS every 3.5 day by intratumor administration for 21 days. (A) Tumor volume, (B) body weight, (C) tumors and (D) tumor weight were measured and recorded. Tumor images and tumor weight of xenografts were obtained after the mice were euthanized at the end of the experiment. The data are shown as the mean ± SEM from 4 individual tumor nodes (n= 4) in each group from one experiment. *$P < 0.05$, compared with the controls.

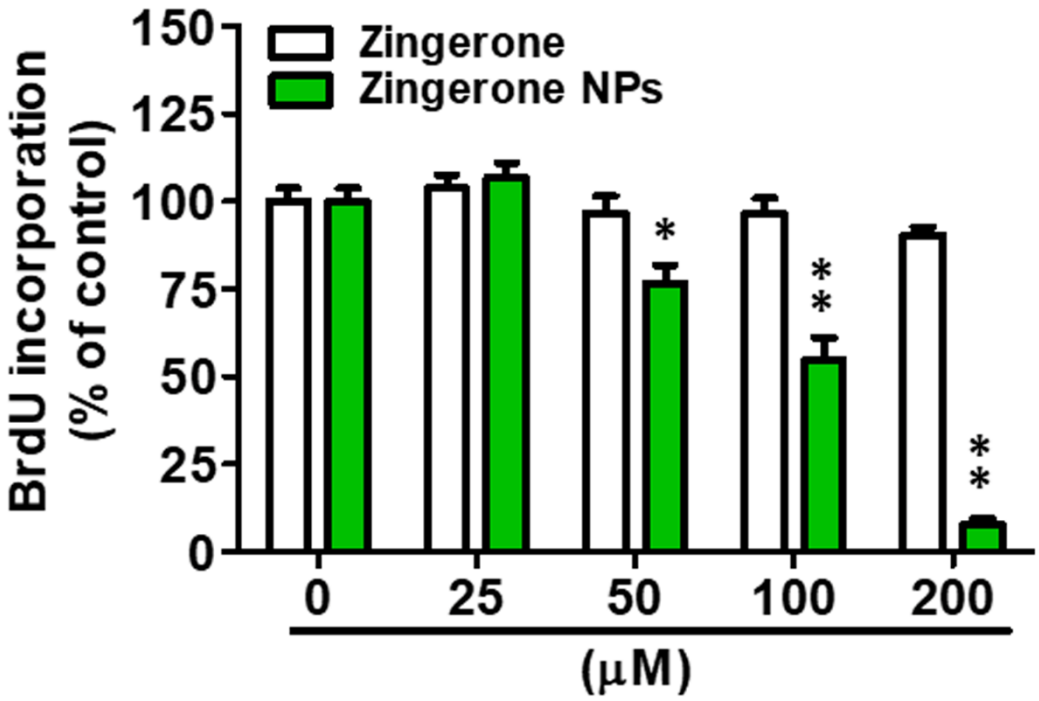

Figure S2. Zingerone NPs inhibit cell proliferation using BrdU incorporation assay. SK-Hep-1 cells were incubated with zingerone and/or zingerone NPs. BrdU incorporation was determined after 24 hours. The values of the control were set to 100%. Each bar represents the mean of five independent experiments (*P < 0.005 and *P < 0.001 vs zingerone control).

Table S1. The effects of zingerone and zingerone NPs on the food uptake, liver weight and liver function index *in vivo*.

	Food uptake (g/day)			Body weight gain (g)	Liver weight of body (%)	Liver function index	
	1st administration	The last administration	FCR			GOT (U/L)	GPT (U/L)
Control	5.33	7.14	1.10±0.51	5.87±2.69	5.50±0.25	54.50±1.50	22.33±3.79
Zingerone	5.37	7.86	0.28±0.09*	28.31±7.70**	5.19±0.25	86.50±8.50[a]	23.00±2.65
Zingerone NPs	5.91	6.20	0.04±0.01**	22.76±3.70**	4.69±0.35	57.67±7.23	19.25±2.06

Values are means ± SD for five mice in each group. **FCR**, Feed conversion ratio, indicates "the amount of feed needed for each additional gram of body weight"; **GOT**, Glutamate oxaloacetate transaminase; **GPT**, Glutamate pyruvate transaminase. *P < 0.05 and **P < 0.01. a: No significance.

Table S2. Flow cytometric analysis of various doses treatment of zingerone and zingerone NPs in human hepatoma SK-Hep-1 cell lines.

	SK-Hep-1	Zingerone (μM)				Zingerone NPs (μM)			
Cell cycle (%)	0	25	50	100	200	25	50	100	200
SubG0	0.54±0.07	0.60±0.03	0.60±0.06	0.83±0.04	1.05±0.08*	1.02±0.11*	1.16±0.05*	1.66±0.06*	8.29±0.14**
G0/G1 phase	56.31±0.67	57.17±1.02	54.76±0.80	52.92±0.64	45.73±2.05*	48.79±1.07*	37.83±1.0**	61.11±1.96*	32.87±0.88**
S phase	18.81±0.91	18.56±0.84	19.30±0.43	19.30±1.40	27.28±1.87*	22.94±0.66*	34.60±1.51**	17.59±1.48	13.96±1.20**
G2/M phase	24.23±0.58	23.46±0.74	25.13±0.47	26.87±0.83	25.43±1.02	26.67±1.60	26.10±1.52	19.36±1.29*	44.53±0.81**

Data are expressed as mean ± SEM of three experiments. *P < 0.05; **P < 0.001

Table S3. Flow cytometric analysis of various doses treatment of zingerone and zingerone NPs in human hepatoma Huh7 cell lines.

	Huh7	Zingerone (μM)				Zingerone NPs (μM)			
Cell cycle (%)	0	25	50	100	200	25	50	100	200
SubG0	0.82±0.11	1.01±0.06	1.03±0.10	1.07±0.12	1.52±0.10*	1.37±0.10*	1.54±0.05*	2.29±0.13**	9.29±0.40**
G0/G1 phase	56.53±0.27	57.23±0.90	57.60±0.11	57.82±0.44	54.72±0.28	52.65±0.80*	51.87±1.37*	68.30±0.63**	56.26±0.51
S phase	19.99±0.29	19.84±0.52	19.99±0.17	19.92±0.19	20.76±0.22	23.38±0.46**	27.15±0.61**	16.55±0.52*	13.12±0.57*
G2/M phase	22.60±0.57	21.84±0.56	21.28±0.15	21.07±0.40	22.91±0.40	22.48±0.40	19.35±0.70*	12.64±0.39**	20.37±0.40*

Data are expressed as mean ± SEM of three experiments. *P < 0.05; **P < 0.001

Materials and methods

Animal model

Male ICR mice (4 weeks old) were purchased from BioLASCO Experimental Animal Center, Taiwan. Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of National Kaohsiung university of science and technology. We ensured that all animals received humane care and that study protocols complied with the institution's guidelines. The mice were maintained under a 12:12 h light/dark cycle in a temperature controlled (25 ± 2℃) room. The mice were provided with a normal commercial laboratory rodent diet 5001 and water ad libitum throughout the experiment. To validate the effects of both zingerone and zingerone NPs on liver function, the mice were divided into three groups (n=5 in each group) and received sterile water, zingerone or zingerone NPs (16 mg/kg) via oral gavage in route of 2 times /week and lasting for eight weeks. The animal food intake and growth character were recorded during experiment execution. Further, the liver weight was obtained when mice were sacrificed at the eighth week. To verify the effects of both zingerone and zingerone NPs on hepatic toxicity, Sera were collected for determination of biochemical parameters, including aspartate aminotransferase (AST or GOT) and alanine aminotransferase (ALT or GPT) using commercially available test kits (Randox, UK). The data are presented as the mean ± standard deviation (SD). Statistical analysis was performed with Tukey's test statistical analysis. $P < 0.05$ was considered statistically significant.

Human hepatocellular carcinoma cell -Huh7 xenograft in NOD/SCID mice.

NOD/SCID mice (5-6 weeks old) were purchased from The National Laboratory Animal Center, Taiwan. Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Academia Sinica and ensured that all animals received humane care and that study protocols complied with the institution's guidelines. Huh7 cells (3×10^6) were suspended in PBS and subcutaneously inoculated into the two flanks of the dorsal of NOD/SCID mice and allowed to grow for 7-10 days to reach a tumor volume of approximately 50-100 $mm^{3.}$ The mice were randomly divided into three groups that included PBS, zingerone, and zingerone NPs, which were administrated with an equal volume of compounds (50 µl of 100 µM) or vehicle (PBS) by intratumor injection. Tumor growth and body weight were monitored every 3.5 days throughout the treatment period. The tumor volume was calculated using the formula of $0.5 \times a \times b^2$, where a

and b are the long diameter and short diameter of the tumor, respectively. Tumor weight of xenografts was obtained after the mice were euthanized at the end of the experiment. All data are presented as the mean ± standard error of the mean (SEM). GraphPad Prism 5.0 (Graph Pad Software, La Jolla, CA, USA) was used for statistical analysis. Statistical analysis was performed with t test statistical analysis. *P*-values of < 0.05 were considered as statistically significant.

BrdU incorporation assay

SK-Hep-1 cells (4×10^4 cells) were seeded on a coverslip/well in 12 well culture plate for overnight. After cells were treated with zingerone and/or zingerone NPs for 24 h and removed the cultured medium, cells were incubated with BrdU (a final concentration of 10 μM, Sigma) for 3 h at 37℃ with 5% CO_2. Cells were then fixed with 4% paraformaldehyde and permeability with 0.2% Triton X-100. Next, Cells were incubated with 2 M HCl for 20 min, washed, and switched to $Na_2B_4O_7$ (100 mM) for 2 minutes. After blocked in 10% calf serum for 1 hour, the cells were then incubated with anti-BrdU antibody (1:500, Santa Cruz) at 4 ℃ for overnight. Followed washing with PBS-T, the secondary fluorescent antibody (1:1000, Alexa Fluor 488) was used for the detection of BrdU positive cells and nuclear DNA was counterstained with DAPI (Invitrogen). Counting was based on randomly chosen five fields for each coverslip.