Front. Chem. Sci. Eng.

DOI 10.1007/s11705-016-1574-2

COMMUNICATION

Curcumin based combination therapy for anti-breast cancer: from *in vitro* drug screening to *in vivo* efficacy evaluation

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Received April 8, 2016; accepted May 10, 2016

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Supplement Information

Experimental

Cell culture

Human breast cancer MCF-7 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 unit/mL penicillin and 100 μg/mL streptomycinat 37 °C in a humidified atmosphere with 5% CO_2, and were maintained in an exponential growth phase for all subsequent experiments.

The synergistic effect of different chemotherapeutic agents

The synergistic effect between different drug combinations was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT). To assessthe potential synergism of curcumin with other compounds, we firstanalyzed theanti-proliferative effect ofcurcumin(CUR), doxorubicin(DOX), sunitinib(SUN), sorafenib(SOR) and erlotinib(ER) alone. Briefly, approximately 8,000 MCF-7 cells were planted in each well of the 96-well culture platefor 24 h, and were then exposed to the test formulations pre-diluted with phosphate buffer saline (PBS)containing 0.05% DMSO. The molar concentrations of CUR were 0.05, 0.1, 0.5, 1, 2.5, 5, 10, 20, 30 μg/mL; DOX were 0.04, 0.08, 0.2, 0.4, 0.8, 1.6, 2.4, 4, 8 μg/mL; ER were 0.05, 0.25, 0.5, 1.25, 2.5, 5, 10, 20, 40 μg/mL; SUN were 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20 μg/mL; and SOR were 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20 μg/mL. All cells were treated with the test drugs for 24 h and then incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h at 37 °C.The formazan product was quantified by measuring the absorbance at 570 nm using a microplate reader (Microplate spectrophotometer, ThermoFisher).

To evaluate the synergistic potential, we analyzed the anti-proliferative effect of curcumin in combination with other drugs at different combination ratios. Briefly, approximately 8,000 MCF-7 cells were plated in each well of the 96-well culture plate for 24 h, and were then exposed to the drug formulations pre-diluted with phosphate buffer (PBS) containing 0.05% DMSO. The molar ratios of DOX to CUR were 0.04, 0.08, 0.16, 0.24, 0.4, 0.8; of ER to CUR were 0.005, 0.025, 0.05, 0.125, 0.25, 0.5; of SUN to CUR were 0.05, 0.1, 0.25, 0.5, 1, 2; and of SOR to CUR were 0.05, 0.1, 0.25, 0.5, 1, 2. After 24 h, cell viability was measured using the MTT assay. The CI-isobologram developed by Chou and Talalay was applied to analyze the synergistic effect, and the CompuSyn software was used to calculate the combination index (CI). The CI values were accounted for in terms of: $CI > 1$, antagonistic effect; $CI = 1$, additive effect; and $CI < 1$, synergistic effect.

Preparation of SUN and CUR loaded BSA nanoparticles and the *in vitro* therapeutic efficacy

Half milligram of SUN, a milligramof CUR, or half milligram SUN plusa milligram CUR were added to 4 mL of 20 mg/mL BSA solution, respectively, away from light and stirring for 4h at room temperature. The solutions were stirred in dark and at room temperature for 4 h, centrifuged for 30 min twice, and the precipitateswere collected as nanoparticles.

Approximately 8,000 MCF-7 cells were planted in each well of the 96-well culture plate, incubated for 24 h, and then exposed to the free drug or drug-containing BSA nano-particles pre-diluted with phosphate buffer saline (PBS)containing 0.05% DMSO. The concentration of SUN or SUN in BSA nano-particles was 2 μg/mL, whereas the concentration of CUR or CUR in BSA nano-particles was 4 μg/mL. All of the cells were treated with drug formulations for 24 h and then incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)for 4 h at 37 °C. The formazan product was quantified by measuring the absorbance at 570 nm using a microplate reader (Microplate spectrophotometer, ThermoFisher).

Preparation of the tumor xenograft animal model

Female Balb/c nude mice (4 weeks old) were obtained from Chinese Academy of Military Medical Sciences (Beijing, China). All animal experiments were approved by the Animal Management Rules of the Ministry of Health of People's Republic of China.

The nude mice were allowed to accommodate in the environment for 3 days before tumor inoculation. MCF-7 cells (1×10^6 cells in 1640 medium) were injected in situ to a depth of 2.5 mm into the skin of the back near armpit using a hypodermic needle, and tumor volume was monitored for a period of 19 days till the mice were dead or the rumor volume were oversize for experiment.

In vivo therapeutic efficacy of SUN and CUR combination formulations

When the average tumor volume reached 60 mm^3， mice were randomly divided into four groups (n = 8) and received intravenous injection of PBS, SUN, CUR, SUN+CUR (mixture of SUN&CUR) or SUN+CUR-loaded BSA nano-particles, respectively. Treatment with SUN (15 mg/kg) or CUR (30 mg/kg) formulations was initiated at day 11, followed by administration of the test formulation every other day for a period of 19 days. The tumor growth of each mouse was daily monitored, with the volume being calculated as: $V_{tumor}=\frac{1}{2}ab^2$, where a and b represented the length and width of the tumor, respectively. Mice were sacrificed under the criteria when the tumor volume was larger than 2,000 mm^3 or weight loss exceeded 20%, whichever came first. On

day 19 post administration, all mice were sacrificed, and tumors were collected and weighted.

Statistical analysis

All measurements were carried out in triplicate, and the results were expressed as means ± SD. Unpaired Student's t-test was used to determine the difference in the mean values between the two tested groups. Statistically significant differences in multiple comparisons were measured by the two-way analysis of variance (ANOVA). All statistical calculations were performed using the GraphPad Prism 5.0 software.

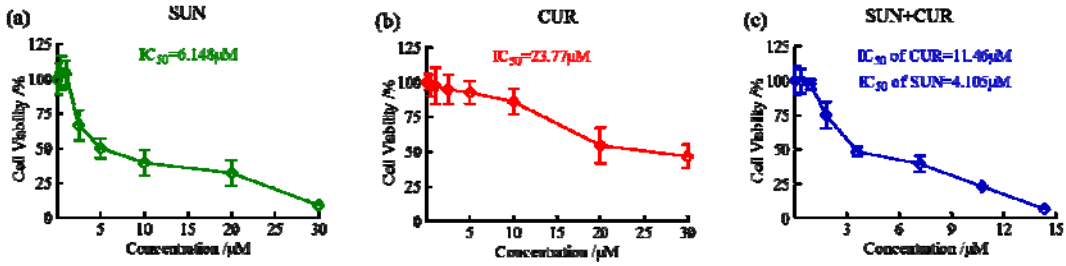

Fig. S1 The IC_{50} values of CUR, SUN and SUN+CUR on MCF-7 cells were used to display the anti-tumor activity *in vitro*

Table S1 The tumor volumes (mm^3) of different treatment groups in MCF-7 tumor xenograft mouse model

Days	Control	SUN	CUR	SUN+CUR	BSA-SC
0	61.34 ± 21.6	61.34 ± 28.3	61.33 ± 39.8	61.33 ± 28.4	61.33 ± 42.9
1	106.0 ± 39.0	86.81 ± 33.6	69.76 ± 36.2	91.25 ± 44.2	49.17 ± 40.0
2	192.3 ± 70.2	95.06 ± 46.8	79.71 ± 48.3	119.3 ± 58.3	71.57 ± 76.8
3	256.1 ± 85.3	135.8 ± 67.5	113.6 ± 65.4	126.7 ± 70.7	94.98 ± 69.2
4	330.5 ± 97.3	157.9 ± 78.9	138.4 ± 74.5	157.6 ± 74.0	138.1 ± 105.8
5	436.3 ± 148.9	206.1 ± 94.8	189.2 ± 108.6	197.7 ± 88.7	161.5 ± 137.9
6	507.9 ± 151.6	238.4 ± 128.3	213.9 ± 102.0	198.7 ± 85.8	174.3 ± 171.5
7	648.7 ± 195.9	325.5 ± 190.7	266.3 ± 98.54	278.7 ± 124.2	250.5 ± 183.5
8	776.3 ± 213.1	361.9 ± 175.6	282.6 ± 118.4	335.4 ± 129.5	280.7 ± 240.5
9	869.3 ± 269.6	428.7 ± 192.6	332.9 ± 133.1	369.4 ± 157.3	300.3 ± 249.4
10	957.7 ± 285.9	525.6 ± 218.6	378.1 ± 139.3	464.7 ± 149.9	354.2 ± 273.1
11	1078 ± 272.0	534.5 ± 258.9	450.8 ± 178.7	469.2 ± 116.2	365.4 ± 325.2
12	1146 ± 327.3	620.1 ± 281.5	579.9 ± 217.7	521.2 ± 129.5	358.7 ± 236.6
13	1299 ± 311.7	731.0 ± 303.3	578.6 ± 222.5	505.0 ± 149.4	475.4 ± 434.5
14	1412 ± 313.1	753.3 ± 305.0	630.2 ± 248.9	584.7 ± 141.3	502.9 ± 438.7
15	1661 ± 417.1	844.0 ± 341.6	754.8 ± 284.9	658.7 ± 198.2	542.1 ± 437.1
16	1673 ± 375.3	1008 ± 371.5	850.7 ± 310.7	720.1 ± 186.9	588.4 ± 511.5
18	1892 ± 311.7	1180 ± 408.1	1030 ± 343.8	784.4 ± 215.1	595.3 ± 253.0
19	2194 ± 279.2	1282 ± 397.6	1029 ± 334.2	871.6 ± 236.9	733.1 ± 350.8