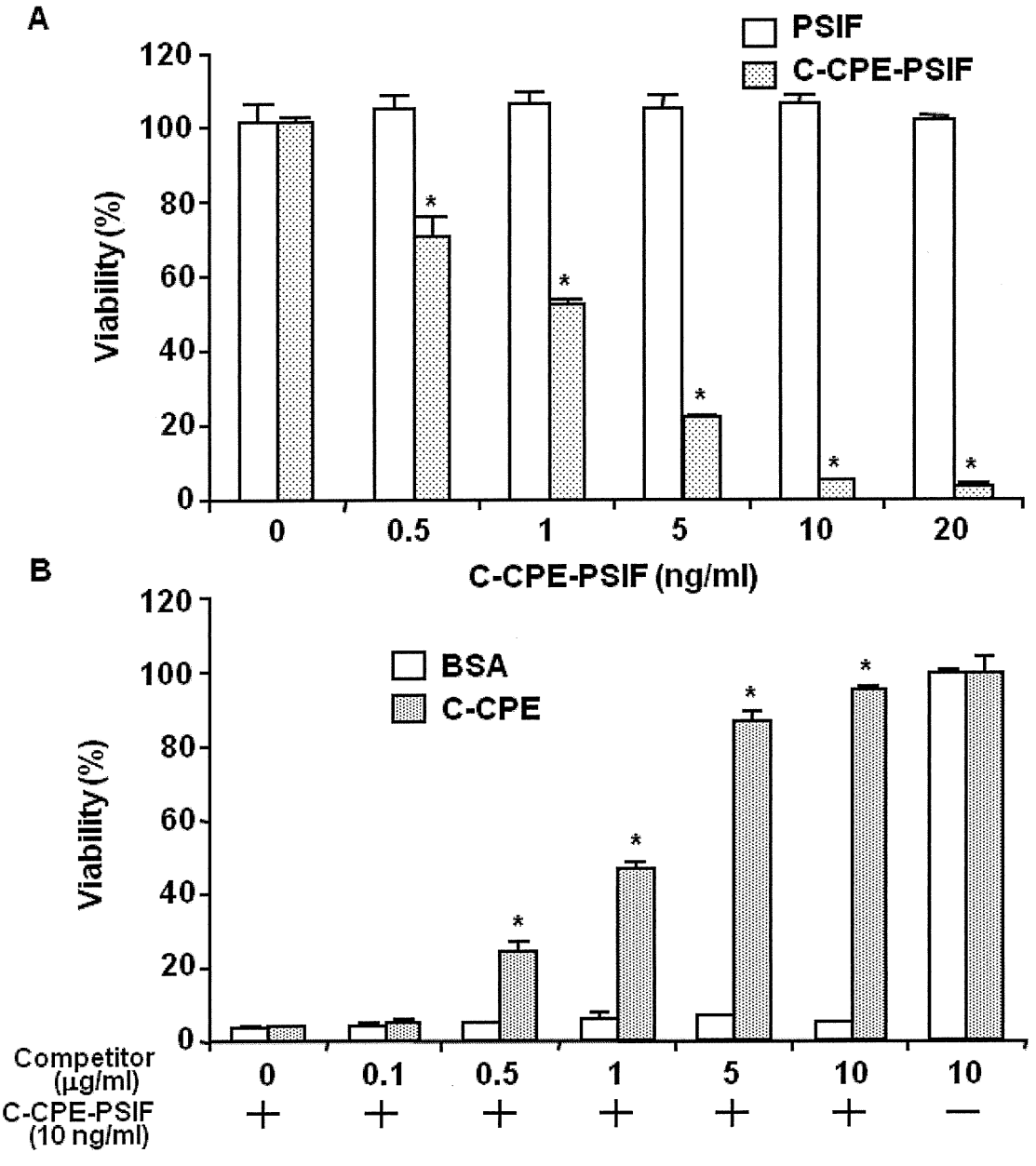


Figure 2. Characterization of C-CPE-PSIF

A) Cytotoxicity of C-CPE-PSIF in claudin-4/L (CL4/L) cells. After a 24 h-treatment of CL4/L cells with PSIF or C-CPE-PSIF at the indicated concentration, the cell viability was measured by WST-8 assay. Viability (%) was calculated as a percentage of the vehicle-treated cells. The data represent the mean ± SD of three independent experiments. *Significantly different from the vehicle-treated group ($p<0.01$). B) Competition assay using C-CPE. CL4/L cells were treated with C-CPE or BSA at the indicated concentration for 2 h, and then the cells were treated with C-CPE-PSIF (10 ng/ml) for 24 h. The cell viability was measured by WST-8 assay, as described above. The data represent the mean ± SD of three independent experiments. *Significantly different between BSA and C-CPE-treated groups ($p<0.01$).

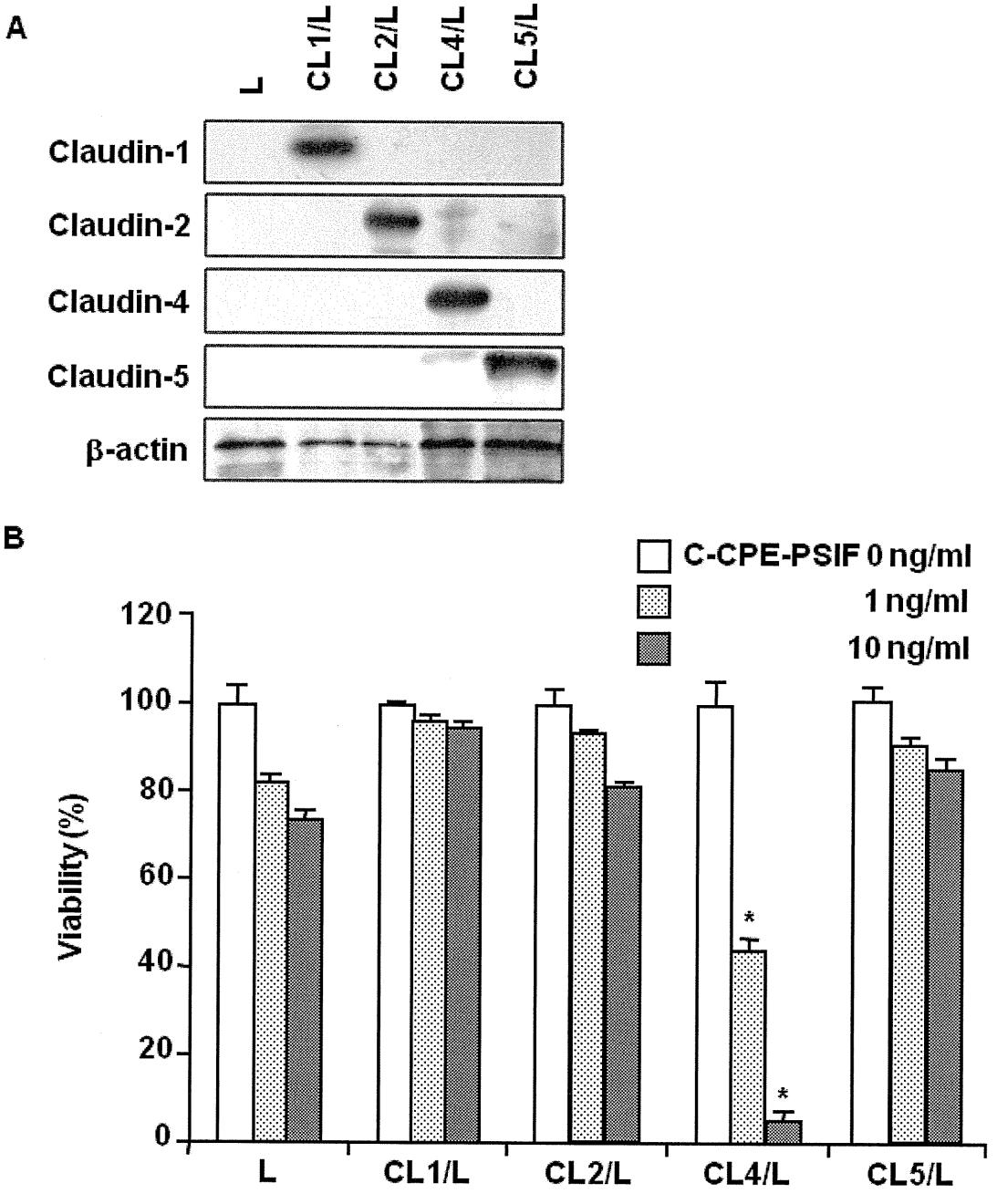


Figure 3. Claudin-specificity of C-CPE-PSIF-induced cytotoxicity
A) Immunoblot analysis. Lysates of L, CL1/L, CL2/L, CL4/L or CL5/L cells were subjected to SDS-PAGE, followed by immunoblotting with antibodies against the indicated CL. B) Specific cytotoxicity of C-CPE-PSIF. L, CL1/L, CL2/L, CL4/L and CL5/L cells were treated with C-CPE-PSIF for 24 h at the indicated concentration. The cell viability was assayed by WST-8 assay. The data represent the mean ± SD of three independent experiments. *Significantly different from the L cells ($p<0.01$).

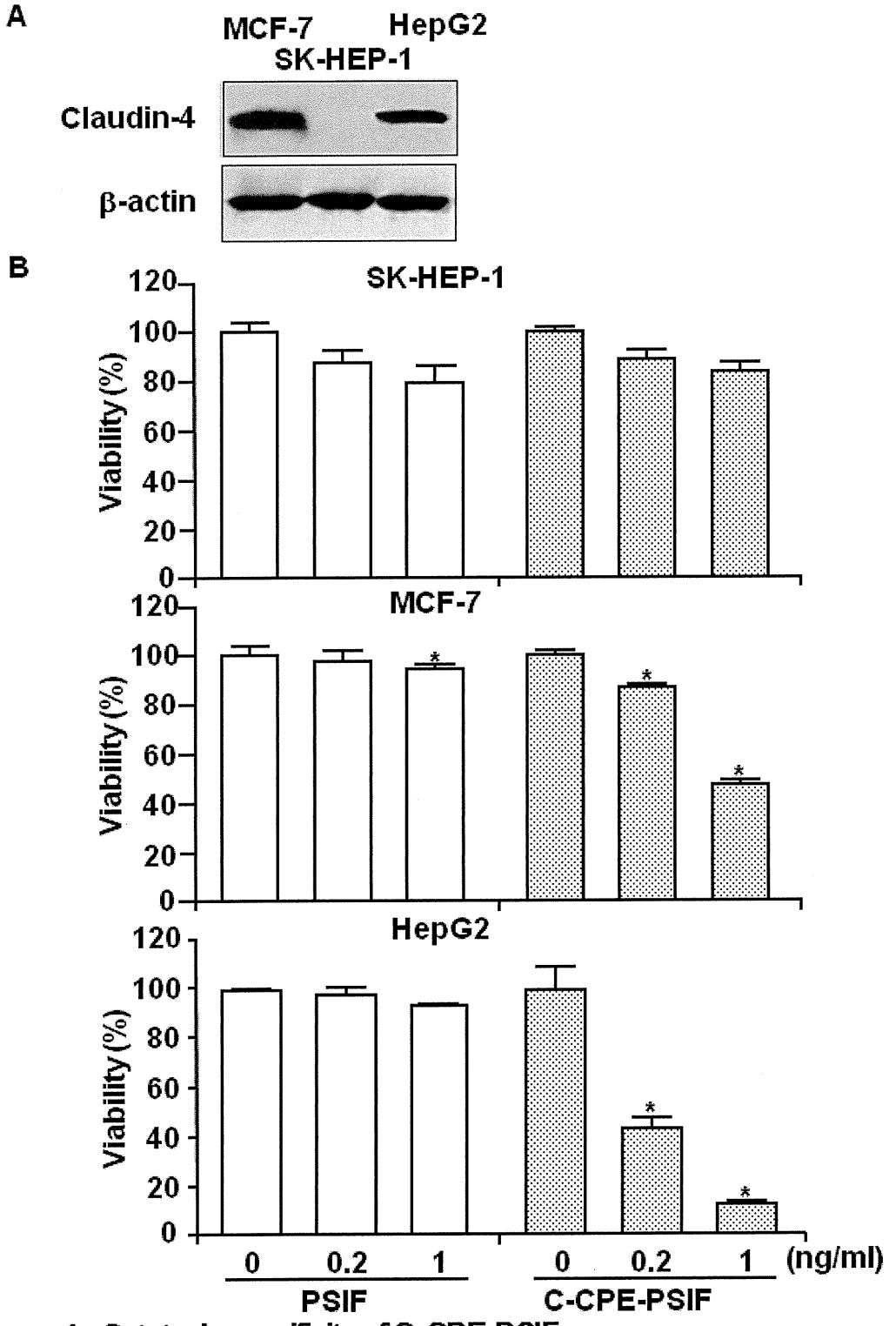


Figure 4. Cytotoxic specificity of C-CPE-PSIF

A) Expression of claudin-4 in SK-HEP-1 and HepG2 cells. The cell lysates were subjected to SDS-PAGE, followed by western blotting with anti-claudin-4 Ab. MCF-7 cells were used as a positive control. B) Cytotoxicity of C-CPE-PSIF in SK-HEP-1 and HepG2 cells. Cells were treated with PSIF or C-CPE-PSIF for 48 h at the indicated concentration. The cell viability was measured by WST-8 assay. The data are representative of at least three independent experiments. Data are the mean ± SD (n=3). *Significantly different from the vehicle-treated cells ($p<0.01$).

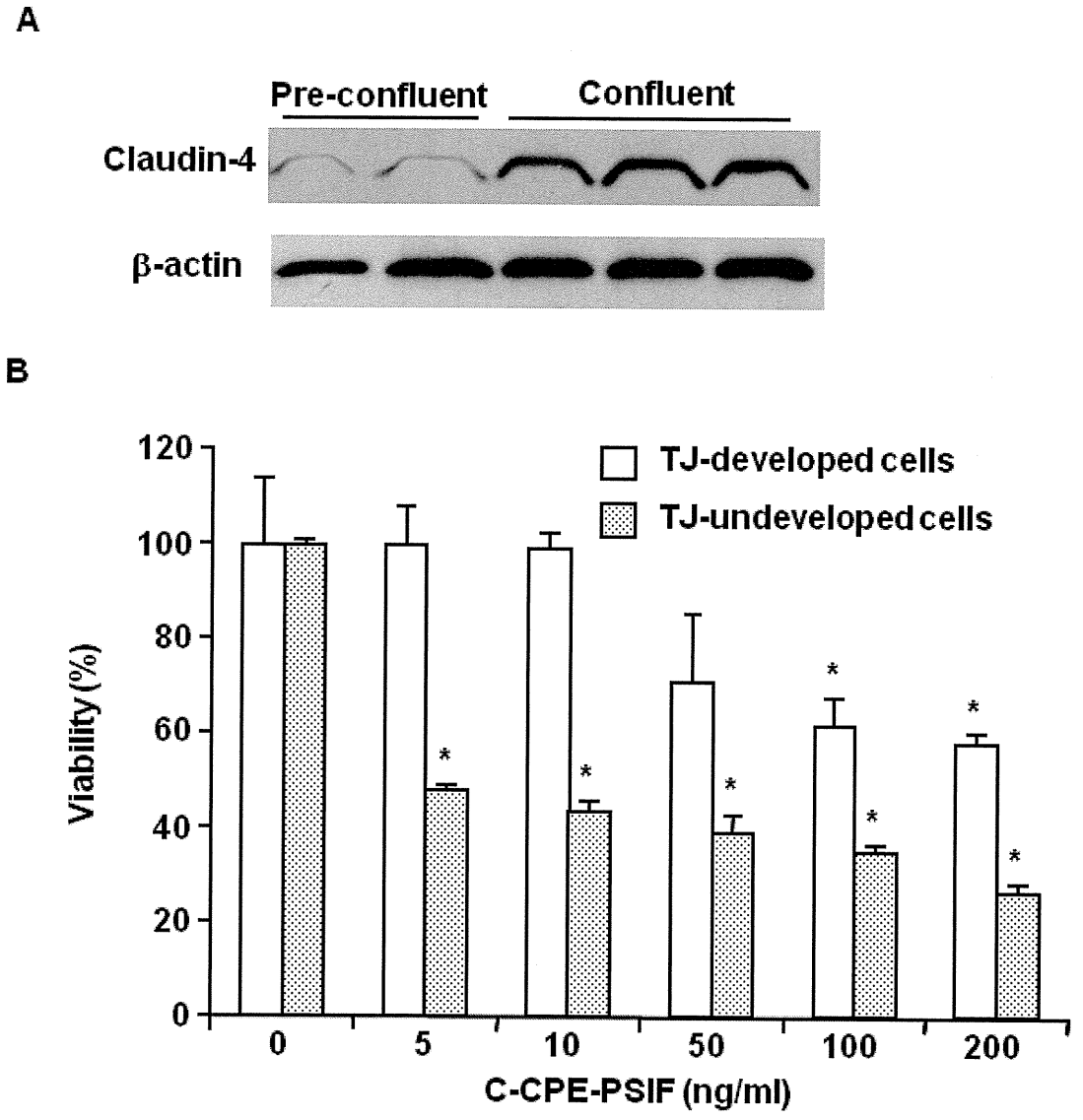


Figure 5. Cytotoxicity of C-CPE-PSIF in TJ-developed or –undeveloped Caco-2 monolayer cells

TJ-developed cells were Caco-2 monolayer cells grown at confluency for 3 days. TJ-undeveloped cells were Caco-2 cells seeded at 1×10^4 cells/well in 96-well plates. The cell lysates were subjected to SDS-PAGE, followed by western blotting with anti-claudin-4 Ab (A). The cells were treated with the indicated concentrations of C-CPE-PSIF for 48 h, and then the cell viability was measured. The data are representative of at least three independent experiments. Data are the mean ± SD (n=3). *Significantly different from the vehicle-treated cells ($p<0.05$).

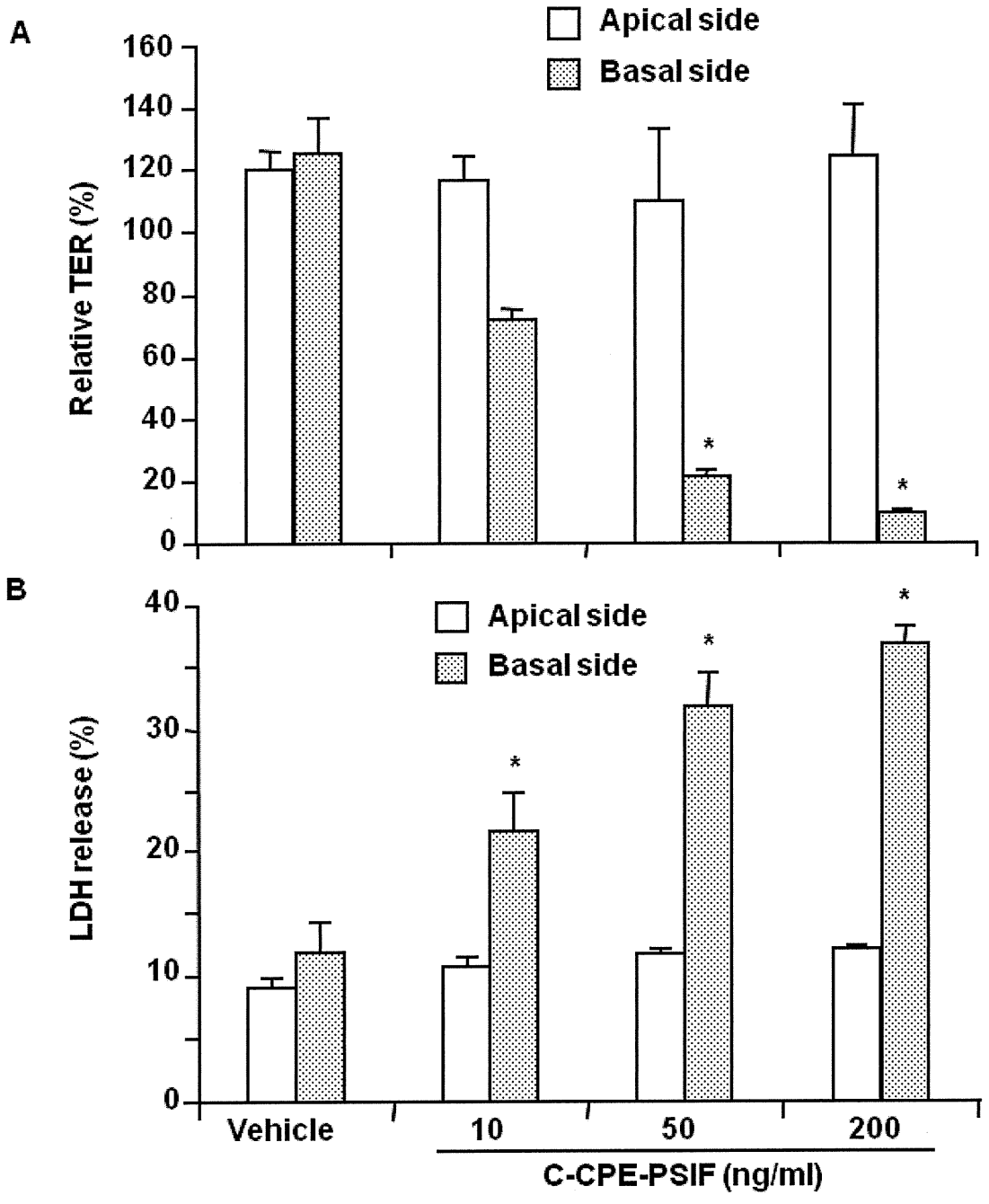
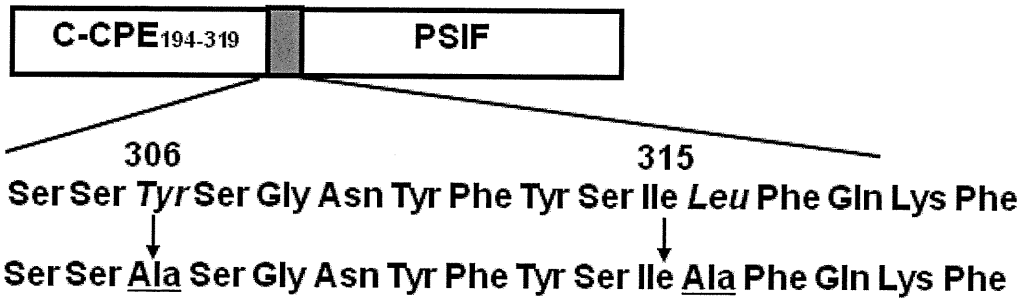


Figure 6. Effect of C-CPE-PSIF on TER and LDH release in Caco-2 monolayer cells
Caco-2 cells were grown on Transwell chambers to form tight junctions. When TER values were constant, C-CPE-PSIF was added to the apical or basolateral side in Transwell chambers at the indicated concentrations. After 0 and 48 h of incubation, TER values were measured (A), and the LDH release from the cell was determined (B). TER values and LDH release were calculated as the ratio of TER values at 0 h and of the total cellular LDH, respectively. Data are the mean ± SD (n=3).
*Significantly different from the vehicle-treated cells ($p<0.05$).

A



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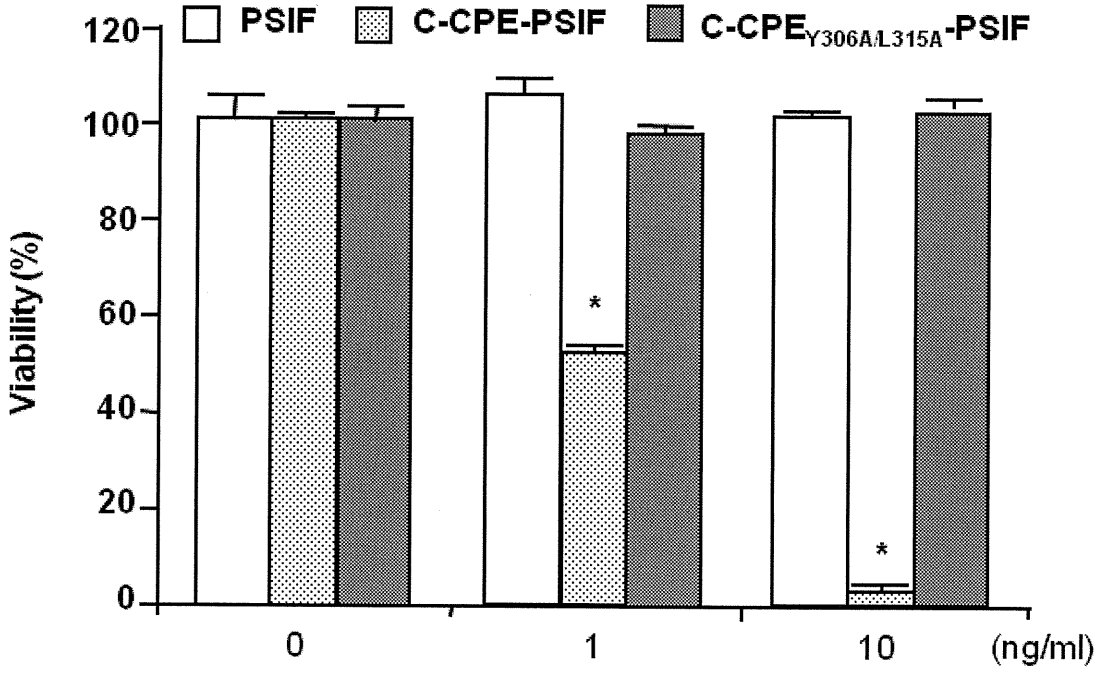

Figure 7. C-CPE$_{Y306A/L315A}$-PSIF

A) Schematic structure of C-CPE$_{Y306A/L315A}$-PSIF. The dark area indicates the putative receptor-binding region of C-CPE in its C-terminus. Among 16 amino acids, Y306 and L315 (indicated by italic letters) play a pivotal role in the binding of C-CPE with claudin-4. B) Cytotoxicity of C-CPE$_{Y306A/L315A}$-PSIF in CL4/L cells. After a 24 h-treatment of CL4/L cells with PSIF, C-CPE-PSIF or C-CPE$_{Y306A/L315A}$-PSIF at the indicated concentration, the cell viability was measured. The data represent the mean ± SD of three independent experiments. *Significantly different from the vehicle-treated group ($p<0.01$).

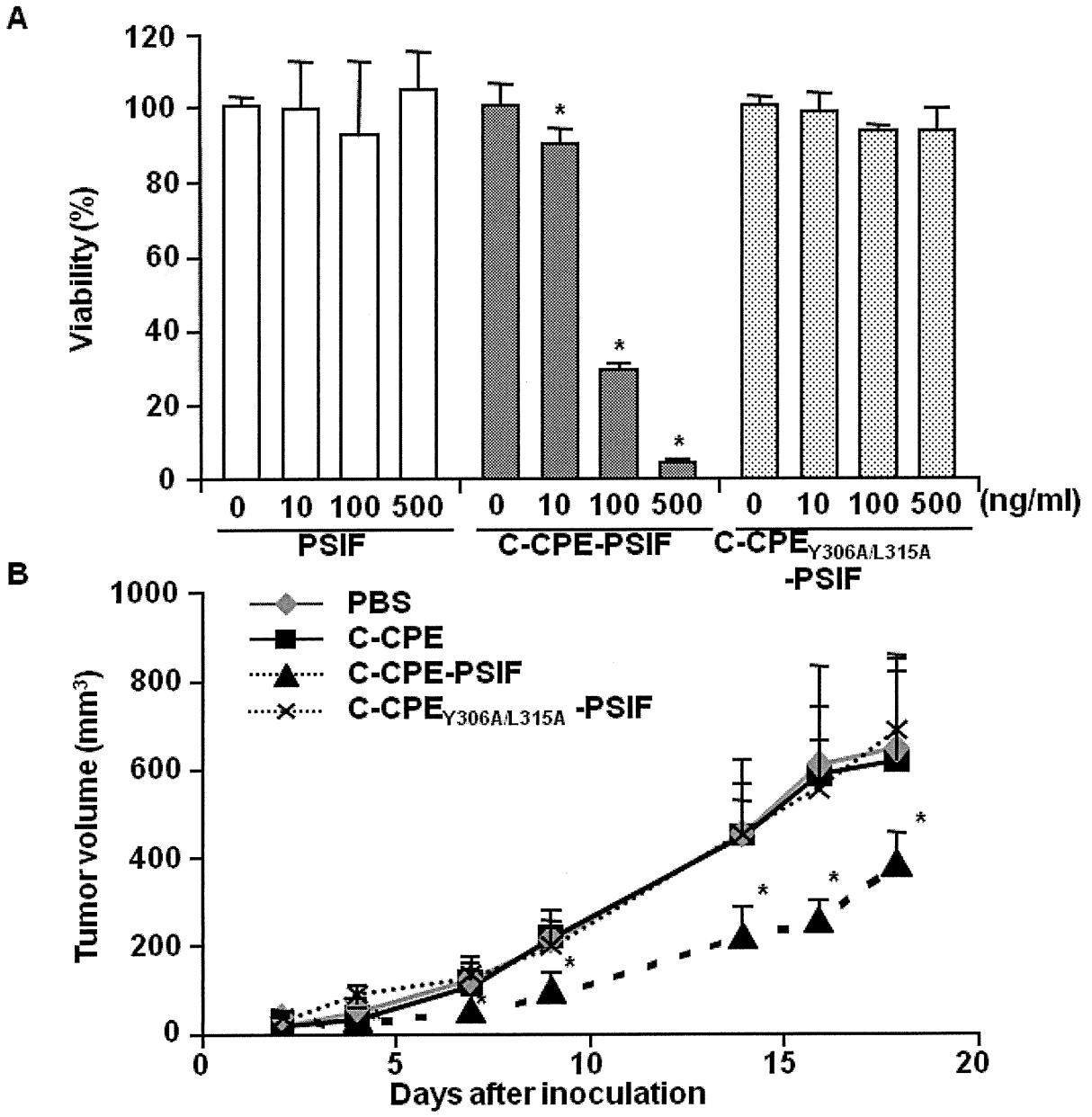

Figure 8. Anti-tumor activity of C-CPE-PSIF

A) Cytotoxicity of C-CPE-PSIFs in 4T1 cells. Cells were treated with PSIF, C-CPE-PSIF or C-CPE$_{Y306A/L315A}$-PSIF for 48 h at the indicated concentration. The cell viability was measured as above. Date are mean ± SD. *Significantly different from the vehicle-treated group ($p<0.05$). B) In vivo anti-tumor activity of C-CPE-PSIF. 4T1 cells (2×10^6 cells) were intradermally inoculated into the right flank of mice on day 0, and each sample (5 mg/kg) was intratumorally injected on days 2, 4, 7, 9, 11 and 14. Tumor growth was monitored by calculating tumor volume. Each point is the mean ± SD from five mice. *Significantly different from the vehicle (PBS)-treated group ($p<0.05$).

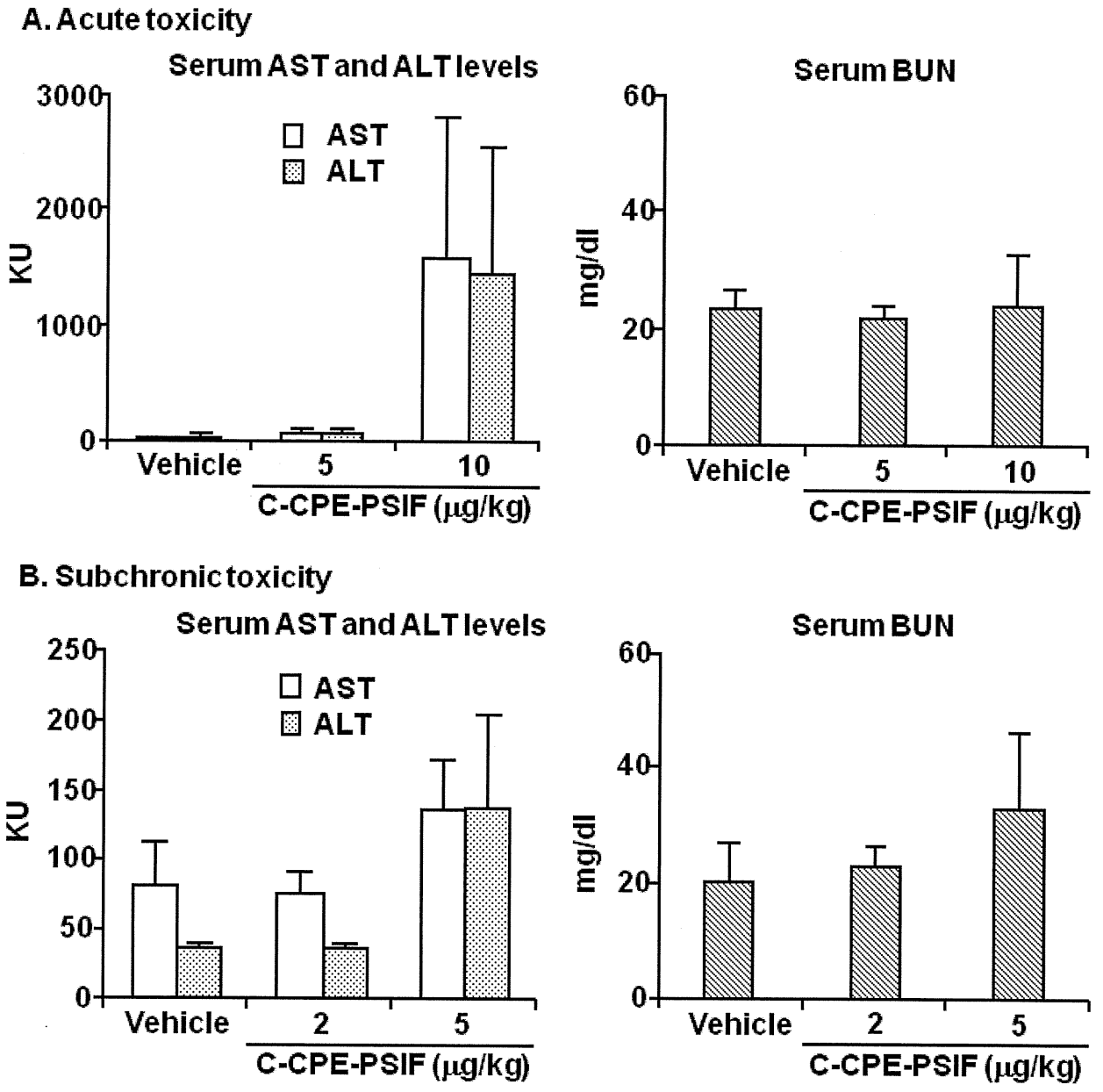

Figure 9. Toxicity of C-CPE-PSIF in liver and kidney
Female BALB/c mice (7 to 8 weeks old) were intravenously injected with C-CPE-PSIF at the indicated dose once (A) or three times per week for 2 weeks (B). Seventy-two hours after the last injection, the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and blood urea nitrogen (BUN) were measured by using a Test-WAKO kit. Data are presented as the mean ± SD (n=5). Data are representative of at least two independent experiments.

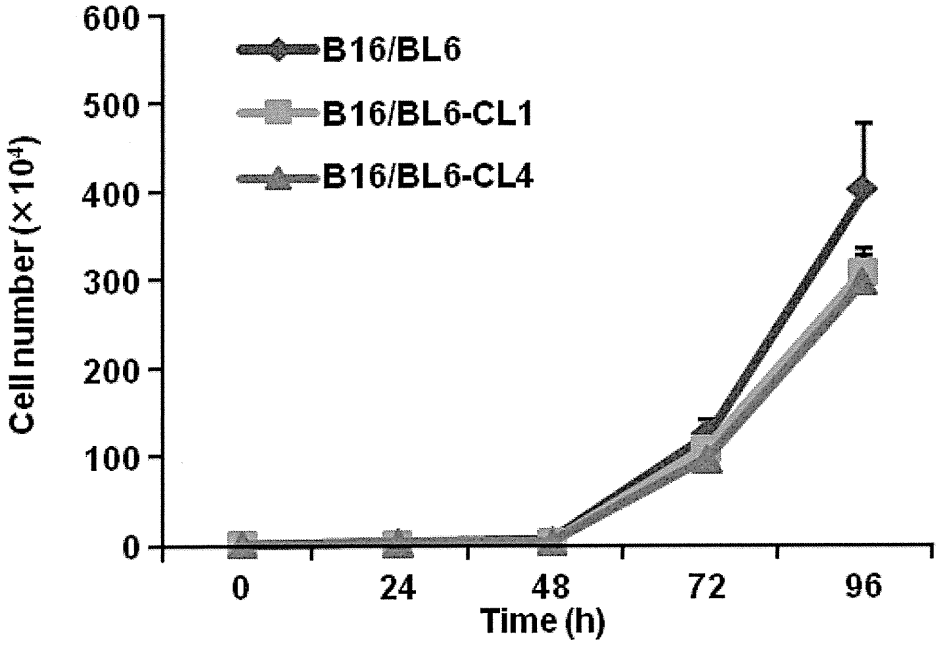

Figure 10. Effect on B16/BL6 cell growth by claudin expression
Claudin-1-expressing B16/BL6(B16/BL6-CL1)cells, claudin-4-expressing B16/BL6(B16/BL6-CL4) cells and B16/BL6 cells were seeded at 1 X 10^4 cells/well in 24-well plate. Cell growth was monitored by counting cells at after 24, 48, 72 and 96 h of incubation. The data represented the means ± SD (n=3).

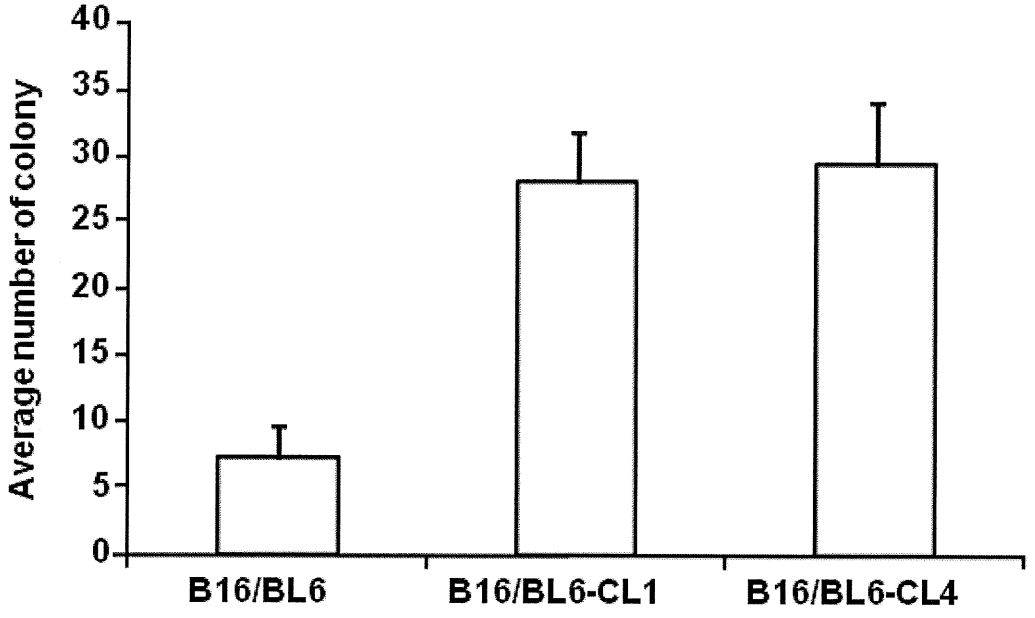

Figure 11. Effect on anchorage-independent growth of B16/BL6 by claudin expression.

Claudin-1-expressing B16/BL6(B16/BL6-CL1)cells, claudin-4-expressing B16/BL6(B16/BL6-CL4)cells and B16/BL6 cells were seeded at 1 X 10^4 cells/well in 0.4 % soft agar. After 9 days of incubation, the number of colonies were counted by MTT assay. The data represented the means ± SD (n=3).

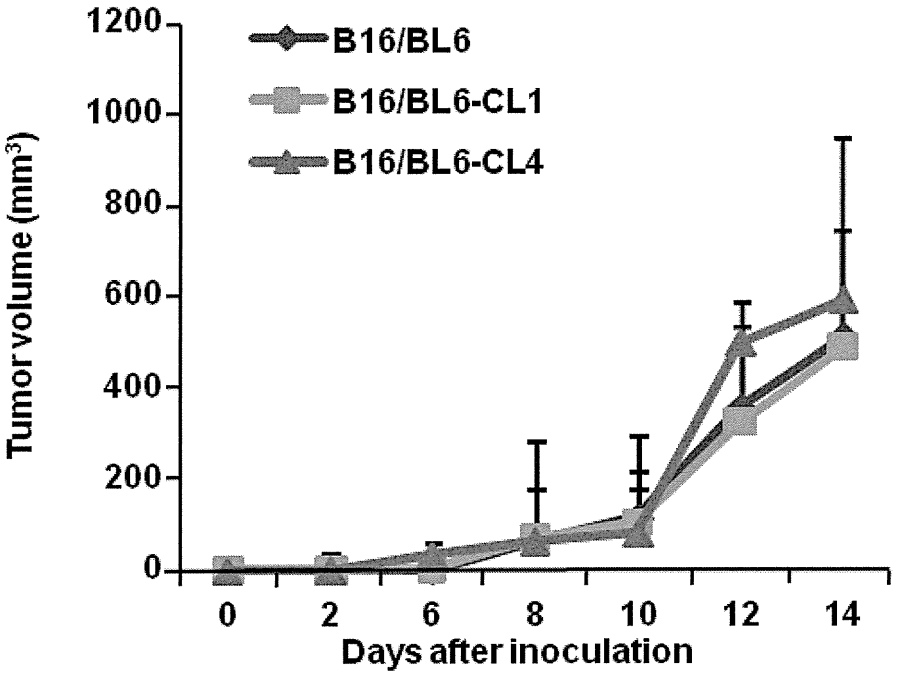

Figure 12. Effect on tumorigenicity of B16/BL6 by claudin expression
Claudin-1-expressing B16/BL6(B16/BL6-CL1)cells, claudin-4-expressing B16/BL6(B16/BL6-CL4)cells and B16/BL6 cells (1×10^5 cells) were subcutaneously inoculated into the left flank of mice. Tumor growth was monitored by calculating tumor volume. The data are representative of two independent experiments. Data are shown as means ± SD (n=5).

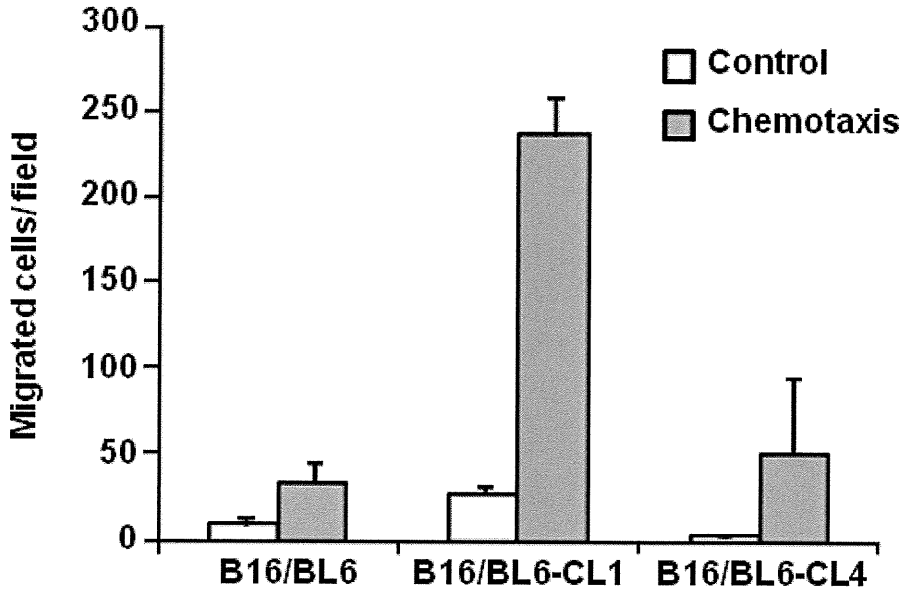

Figure 13. Effect on migration of B16/BL6 by claudin expression.
Claudin-1-expressing B16/BL6(B16/BL6-CL1)cells, claudin-4-expressing B16/BL6(B16/BL6-CL4)cells and B16/BL6 cells were seeded at 1×10^5 cells/well in pore insert. Chemotaxis added 10 mg/ml fibronectin to 0.5 % DMEM in bottom well, and control added only 0.5 % DMEM. After 6 h of incubation, cells were stained by Diff-Quik and were counted the number of migrated cells. The data represented the means ± SD (n=3).

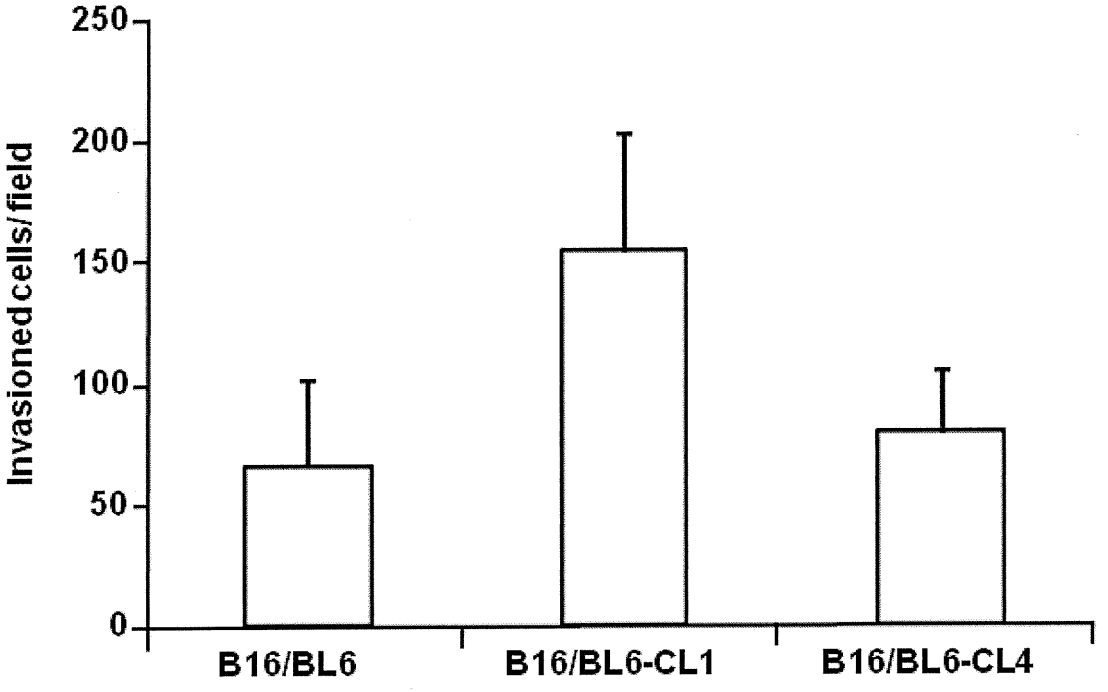

Figure 14. Effect on invasion of B16/BL6 by claudin expression.
Claudin-1-expressing B16/BL6(B16/BL6-CL1)cells, claudin-4-expressing B16/BL6(B16/BL6-CL4)cells and B16/BL6 cells were seeded at 1×10^5 cells/well on matrigel in pore insert. Chemotaxis added 10 mg/ml fibronectin to 0.5 % DMEM in bottom well, and control added only 0.5 % DMEM. After 6 h of incubation, cells were stained by Diff-Quik and were counted the number of migrated cells. The data represented the means ± SD (n=3).

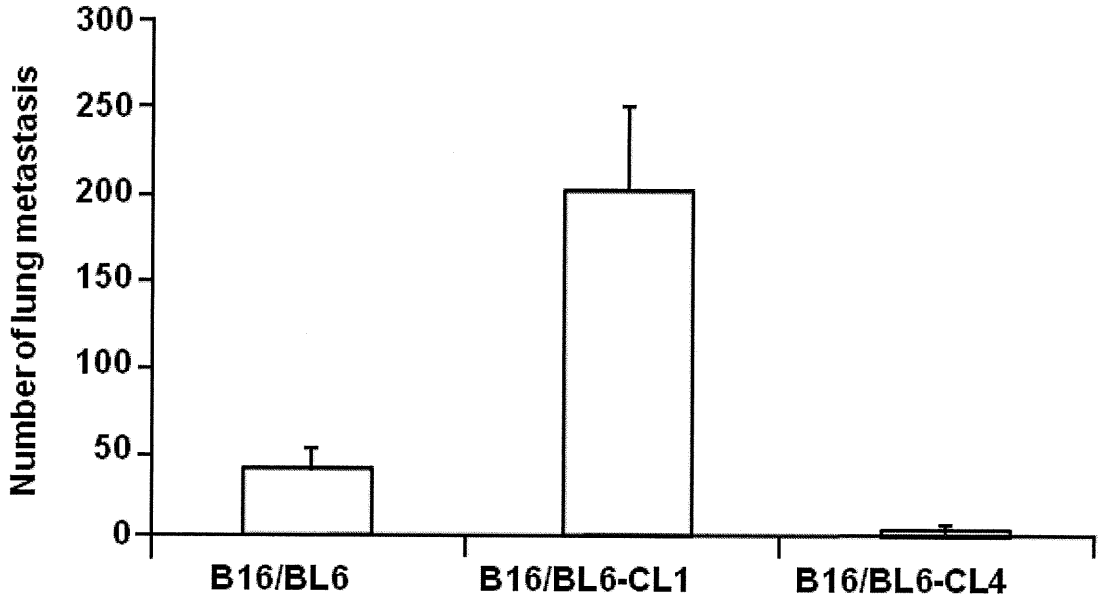

Figure 15. Effect on metastasis of B16/BL6 by claudin expression
Claudin-1-expressing B16/BL6(B16/BL6-CL1)cells, claudin-4-expressing B16/BL6(B16/BL6-CL4)cells and B16/BL6 cells (1×10^5 cells) were intravenously injected. After day 14, the mice were sacrificed, their lungs were counted number of metastatic foci. The data are representative of two independent experiments. Data are shown as means ± SD (n=5).

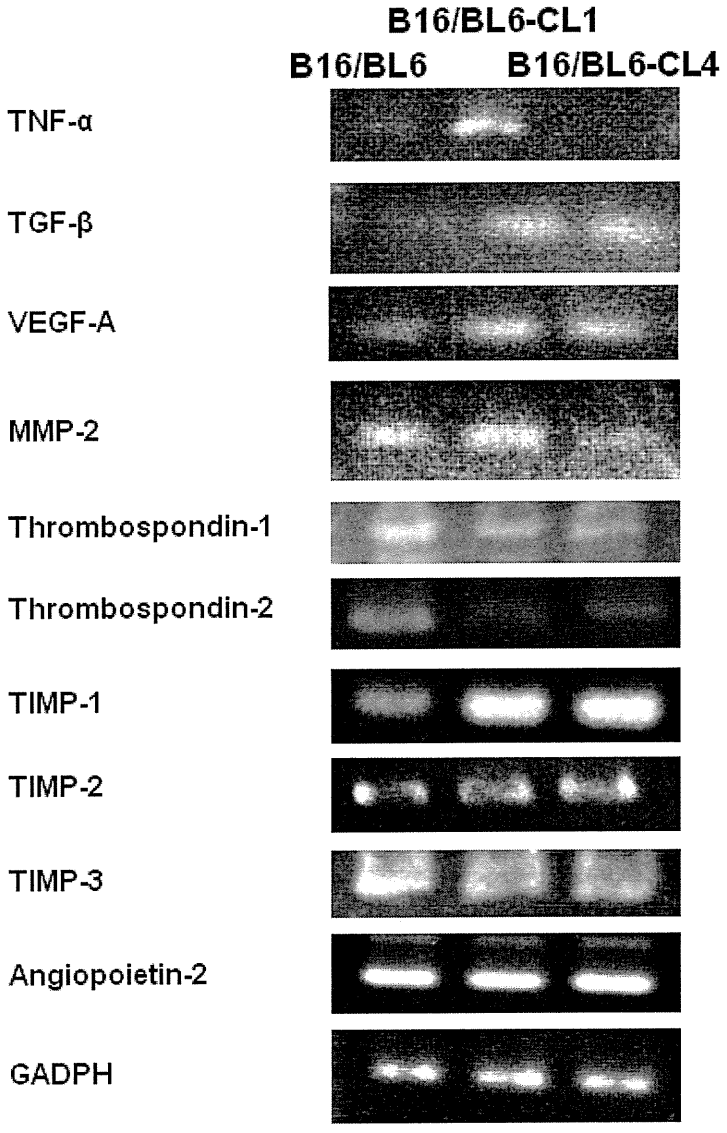

Figure 16. Effect on expression of MMPs and cytokines of B16/BL6 by claudin expression.
Total RNA was extracted from claudin-1-expressing B16/BL6(B16/BL6-CL1)cells, claudin-4-expressing B16/BL6(B16/BL6-CL4)cells and B16/BL6 cells, followed by reverse transcription-polymerase chain reaction analysis.

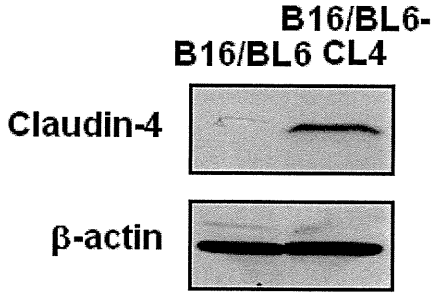

Figure 17. Expression of claudin-4 in B16/BL6-CL4 cell

Cell lysates from B16/BL6 and B16/BL6-CL4 cells were subjected to SDS-PAGE, followed by western blotting with claudin-4 and β-actin.

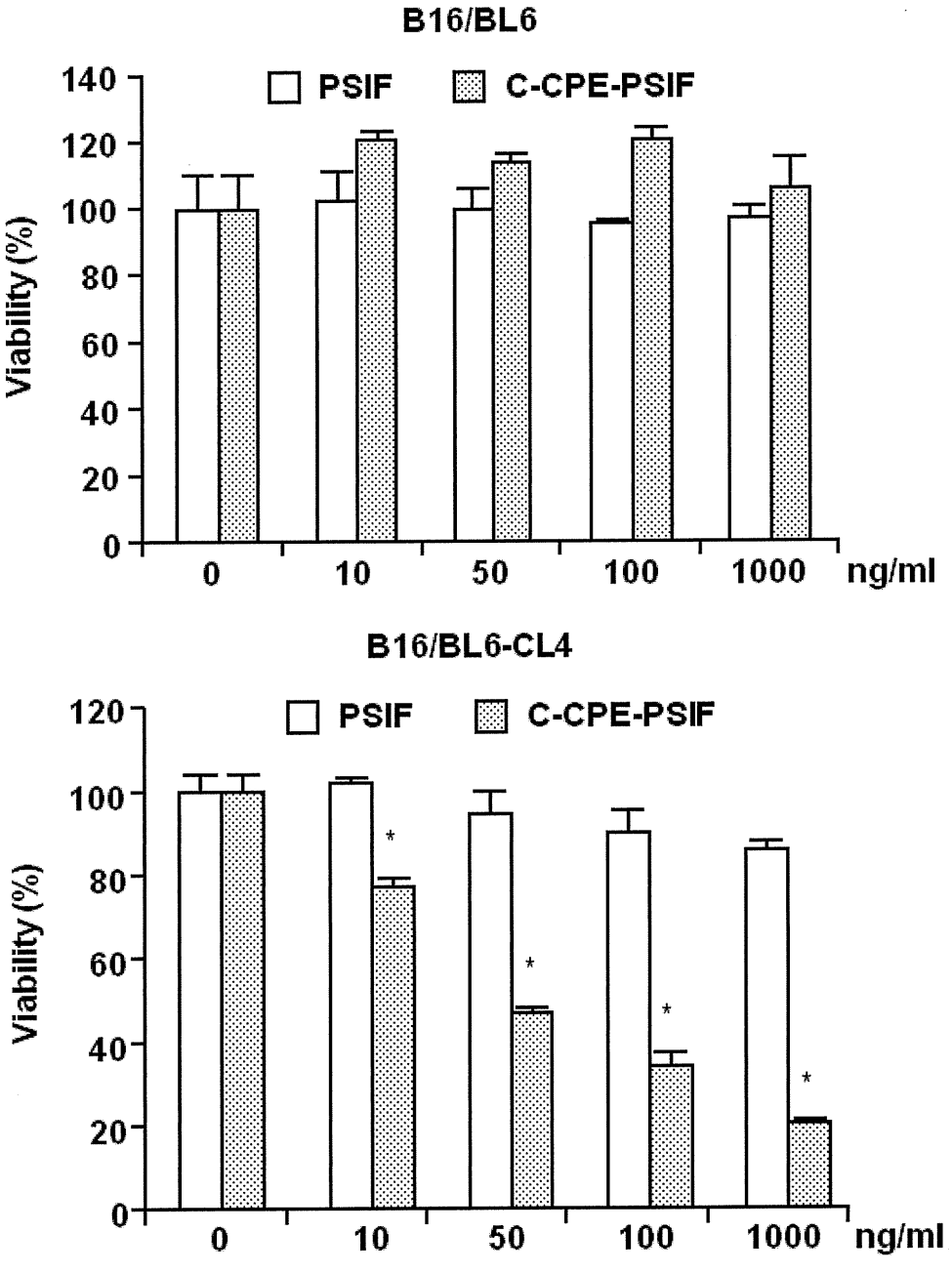

Figure 18. Cytotoxicity of C-CPE-PSIF in B16/BL6-CL4 cells
B16/BL6 cells (upper panel) and B16/BL6-CL4 cells (lower panel) were treated with PSIF or C-CPE-PSIF at the indicated concentration for 48 h. The cell viability (%) was measured by WST-8 assay. Data represent the mean ± SD (n=3). *Significantly different from the vehicle-treated group ($p < 0.05$).

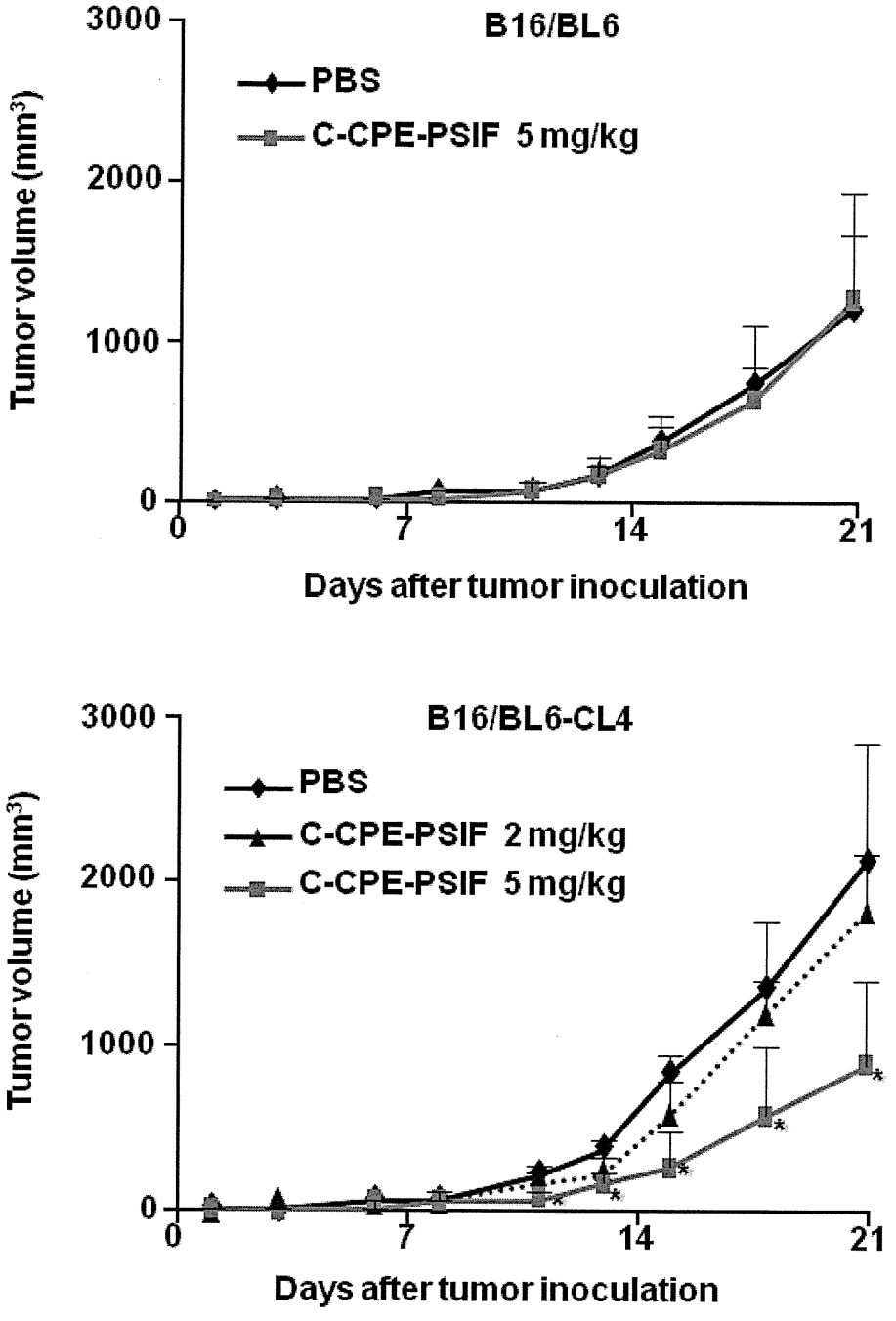

Figure 19. Anti-tumor activity of C-CPE-PSIF on B16/BL6-CL4 subcutaneously inoculated allograft.
B16/BL6 (upper panel) or B16/BL6-CL4 (lower panel) cells (1×10^5 cells) were intradermally inoculated into the right flank of mice, and PBS or C-CPE-PSIF (2 or 5 μg/kg) was intravenously injected three times a week. After inoculation, the tumor volume was monitored. Each point is the mean ± SD (n=5). *Significantly different from the vehicle-treated group ($p < 0.05$).

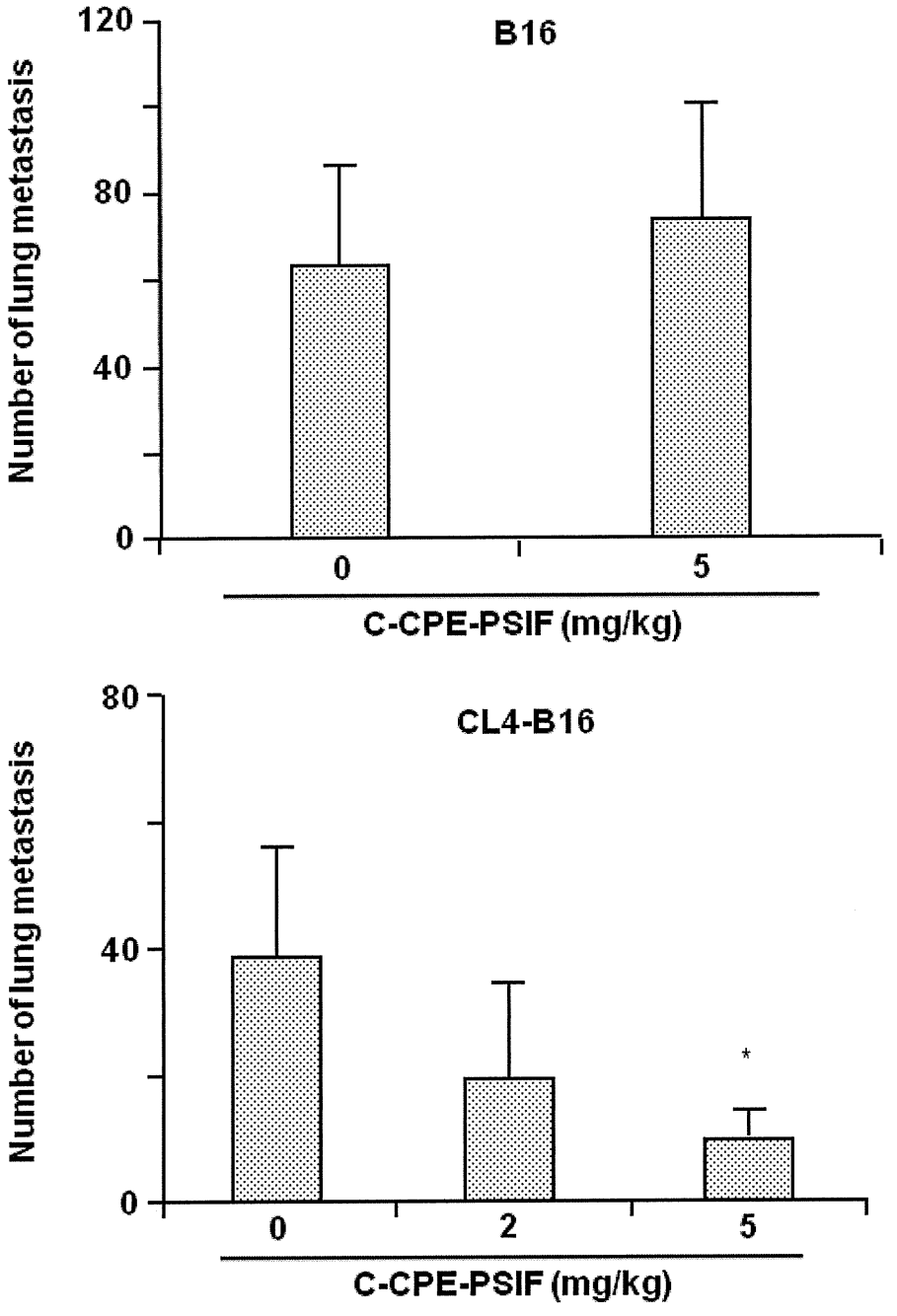

Figure 20. Anti-metastatic activity of C-CPE-PSIF on lung metastasis of B16/BL6 or B16/BL6-CL4 cells

B16/BL6 or B16/BL6-CL4 cells (1×10^6 cells) were injected into the tail veins of mice, and vehicle or C-CPE-PSIF (2 or 5 μg/kg) was intravenously injected on days 0, 2, 4, 7, 9, 11, and 13. On day 14, the mice were sacrificed, their lungs were fixed, and the colonies on the lung surface were counted. Data are shown as means ± SD (n=5).

*Significantly different from the vehicle-treated group ($p < 0.05$).

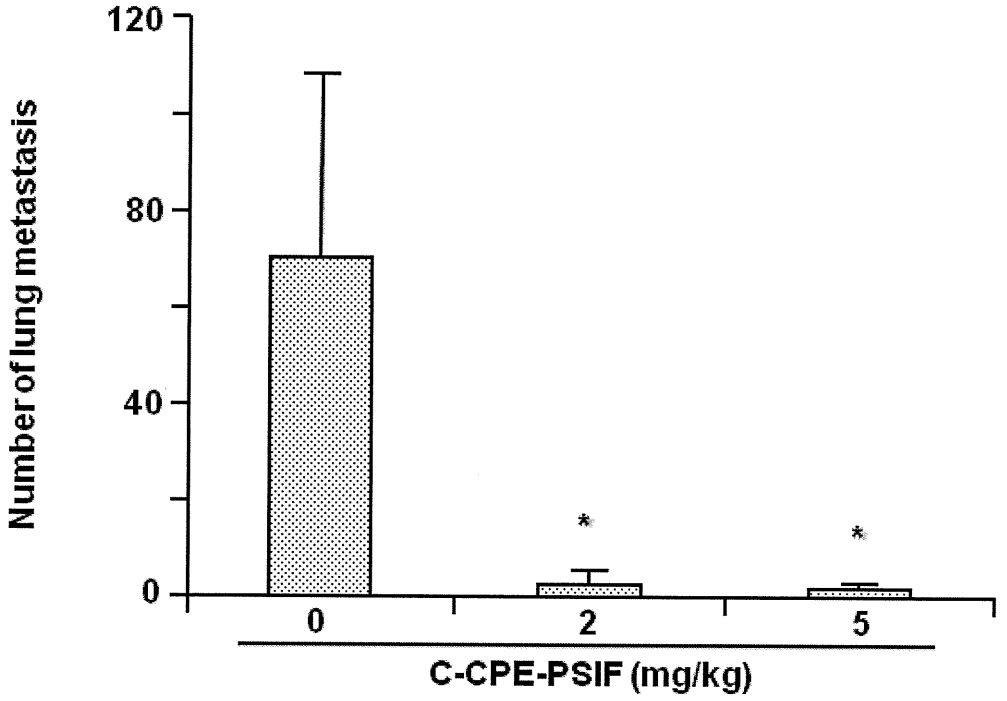

Figure 21. Anti-metastatic activity of C-CPE-PSIF on lung metastasis 4T1 cells
4T1 cells (1×10^5 cells) were intradermally inoculated into the right flanks of mice, and C-CPE-PSIF was intravenously injected three times or two times a week at the indicated dose, respectively. On day 35, the mice were sacrificed, their lungs were stained with india ink, and the number of spontaneous metastases were determined. Data are shown as means ± SD (n=5). *Significantly different from the vehicle-treated group ($p < 0.01$).