

Fatty acid synthase inhibitor cerulenin suppresses liver metastasis of colon cancer in mice

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1 **Fatty acid synthase inhibitor cerulenin suppresses liver**
2 **metastasis of colon cancer in mice**

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1 **ABSTRACT**

2 Fatty acid synthase (FAS) is highly expressed in many kinds of human cancers,
3 including colorectal cancer (CRC), and we have investigated the potential use of FAS
4 inhibitors for chemoprevention of liver metastasis of CRC in mice. Expression of FAS
5 was evaluated in murine CRC cell line Colon 26 and CMT 93. Cerulenin, a natural
6 inhibitor of FAS induced apoptosis in these cell lines. The ability of cerulenin to prevent
7 development of liver metastatic lesion of Colon 26 was evaluated. The numbers and
8 sizes of liver metastatic CRC tumors are significantly reduced by treating mice with
9 cerulenin. Cerulenin treatment is associated with reduced levels of phosphorylated Akt
10 in Colon 26 cells, suggesting that inhibition of this signal transduction pathway might
11 be involved in the chemo preventive activity of this compound. Based on studies in
12 mouse models, inhibiting FAS would be an effective strategy in preventing and
13 retarding growth of liver metastatic tumors of CRC that have high expression of this
14 enzyme.

15

1 **Introduction**

2 Colorectal cancer (CRC) is one of the most common cancers in the world with
3 more than million new cases⁽¹⁾. The liver is the commonest site of distant metastasis in
4 CRC, and approximately 50% of patients ultimately develop liver metastasis in the
5 course of CRC.^(1,2) Despite recent advances, systemic chemotherapy for metastatic
6 disease is considered palliative, and we rarely see long-term survivors treated only by
7 chemotherapy.⁽¹⁾ Hepatic resection, the only curative treatment for liver metastasis of
8 CRC, has become the standard treatment, but most cases of liver metastases are
9 inoperable and approximately 50% of the patients treated with hepatectomy have a
10 tumor recurrence in the liver.⁽³⁾

11 Fatty acid synthase (FAS) is highly expressed in many human cancers including
12 CRC,⁽⁴⁻⁷⁾ and previous studies have shown that cancer cell growth can be suppressed by
13 inhibiting the activity of this enzyme with a natural antibiotic cerulenin,⁽⁸⁾ orlistat which
14 is a pancreatic lipase inhibitor developed for obesity treatment,⁽⁹⁾ and C75, which is a
15 stable synthetic small-molecule developed specifically for inhibiting FAS.⁽¹⁰⁾ But there
16 are no previous studies in which FAS inhibitors suppress liver metastasis of CRC.

17 The aim of this study extends the investigation of the potential use of a FAS
18 inhibitor for chemoprevention of liver metastasis of CRC in mice. In this study, we
19 examined the effect of cerulenin on the murine CRC cell line, Colon 26 and CMT 93
20 cell proliferation and apoptosis. Then, the effect of cerulenin on the prevention of
21 growth of liver metastasis lesion of Colon 26 was investigated.

22

1 **Materials and Methods**

2 **Ceruleinin**

3 Ceruleinin was obtained from Sigma(St. Louis, MO). For cell culture and i.p.
4 injections, ceruleinin was dissolved in acetone at a concentration of 20 mg/ml and stored
5 at -20°C. In vitro experiment, 50 to 200 µM of ceruleinin was added to the medium and
6 cell viability assay and Western blot experiments were performed 24 hours later. In vivo
7 experiments, treatment with ceruleinin at 30 mg/kg was given i.p. at day 1, 4, and 7 after
8 tumor inoculation in ceruleinin group.

9 **Cell culture**

10 Two murine CRC cell lines, Colon 26 and CMT 93, were used and tested for
11 mycoplasm-free cell lines. All cancer cell lines were subdivided in multiple tubes for
12 stock in liquid nitrogen immediately after possession. All cell lines were subjected to
13 present experiment within 6 months of resuscitation. Stock cultures were grown in high
14 glucose DMEM containing 10% FBS and 1% antibiotics. The cells were grown in
15 growth medium at 37°C in a 95% air, 5% CO₂-humidified incubator.

16 **Cell viability assay**

17 To measure the cytotoxicity of ceruleinin against these cancer cells, 3×10³ cells
18 were plated per well onto 96-well plates. Following overnight culture, ceruleinin were
19 added at specified concentrations. After 24 hours of incubation, cell viability was
20 measured by the mitochondrial activity in reducing
21 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium
22 monosodium salt (WST-8) to formazan using Cell Counting kit-8 (Dojindo Laboratories,
23 Kumamoto, Japan). Cells were incubated with a reagent as per manufacturer's
24 instructions. Plates were read at A₄₅₀ on a spectrometer.

1 **Cell Proliferation Assay**

2 To measure the cell proliferation activity of cerulenin against these cancer cells,
3 3×10^3 cells were plated per well onto 96-well plates. Following overnight culture,
4 cerulenin were added at specified concentrations. After 24 hours of incubation, cell
5 proliferation was measured by BrdU assay kit (Roche Diagnostics GmbH, Penzberg,
6 Germany). Cells were incubated with a reagent as per manufacturer's instructions.
7 Plates were read at A_{450} on a spectrometer.

8 **Apoptosis assay**

9 The In situ Cell Death Detection kit (Roche diagnostics Corp., Basel,
10 Switzerland) was used for the demonstration of apoptotic cell death of cell culture and
11 liver tissue. 3×10^4 /ml cells were plated per well onto Lab-Tek II Chamber Slide (Nalge
12 Nunc International K. K., Tokyo, Japan) and paraffin-embedded liver samples were
13 incubated with the terminal deoxynucleotidyl transferase-mediated dUTP nick end
14 labeling (TUNEL) reaction mixture according to manufacture's recommendations.

15 **Western blot analysis**

16 For Western blot analysis, total protein extracts of Colon 26 and CMT 93 were
17 obtained 24 hours after cerulenin treatment, and separated by 10% SDS-PAGE and
18 transferred to nitrocellulose membrane (Millipore, Bedford, MA). The following
19 antibodies were used as primary antibodies; total Akt (9272), phosphoserine 473 Akt
20 (9271), p mTOR, cleaved caspase 3, and GAPDH (2118) (Cell Signaling, Beverly, MA).
21 Purified mouse anti fatty acid synthase antibody (610962) was purchased from BD
22 Biosciences (San Jose, CA). Secondary goat anti rabbit antibody conjugated with
23 horseradish peroxidase was purchased from Zymed Laboratories Inc. (San Francisco,
24 CA). Immunoblots were analyzed by enhanced chemiluminescence.

1 **Animals**

2 Eight-week-old male BALB/c mice (Clea, Japan), weighing 24–28g were utilized.
3 The mice were kept in a temperature-controlled room on a 12-hour light-dark cycle.
4 They had free access to water and standard chow throughout the experiment. After an
5 acclimation period of at least 7 days, the mice were separated into two groups as
6 follows: control group, mice without any treatment (n=8); and cerulenin group, mice
7 with cerulenin treatment (n=6). All animal experiments were carried out in a humane
8 manner after receiving approval from the Institutional Animal Experiment Committee
9 of the University of Tsukuba and in accordance with the Regulation for Animal
10 Experiments in our university and Fundamental Guideline for Proper Conduct of
11 Animal Experiment and Related Activities in Academic Research Institutions under the
12 jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology.

13 **Liver metastasis**

14 Under ether anesthesia, laparotomy was performed with mid line incision, and
15 hepatic ischemia was induced by clamping the portal triad (the hepatic artery, portal
16 vein, and bile duct) with a microclip (Aesculap, Tuttlingen, Germany) for 1 min. After
17 1min reperfusion of the liver, 1×10^5 cells of colon 26 cells were injected into the lower
18 splenic pole with a 27-gauge needle. Nine days after inoculation, mice were sacrificed
19 and livers were removed for examination.

20 **Histochemical examination**

21 To assess the liver metastatic area, left and middle lobes of liver were divided in
22 5mm slice, fixed with 10% formaldehyde, and embedded in paraffin. Thin sections
23 ($4\mu\text{m}$) were stained with hematoxylin-eosin, and the percentage of liver metastatic area
24 of Colon 26 cells was calculated using image-processing software WinROOF (Mitani

1 Co., Fukui, Japan).

2 **Serum parameter**

3 Blood was collected from the plexus of the retro-orbital vein of mice. Blood was
4 centrifuged for 10 min at 4°C at 3500 rpm. Supernatants were collected and stored at
5 -80°C until tested by a serum multiple biochemical analyzer (Fuji Drichem; Fuji Film
6 Inc., Tokyo, Japan) for measuring triglyceride, cholesterol, and ALT levels.

7 **Statistical analysis**

8 All data are expressed as the mean \pm standard deviation of samples. Unpaired
9 *t*-test was used for the comparison between the two groups. Comparisons between
10 various points were using one-way ANOVA. Significant data were examined by
11 Bonferroni-Dunn multiple comparisons post hoc test. In all cases, a P value <0.05 was
12 considered significant.

13

14 **Results**

15 **Dose-dependent inhibition of proliferation of CRC cell lines by cerulenin**

16 We initially determined whether cerulenin treatment led to inhibit CRC cell
17 proliferation. CRC cells were treated with various doses of cerulenin for 24 h, and cell
18 viability was assayed using WST-8 assay (Figure 1A) and BrdU assay (Figure 1B).
19 Figure 1 A and B showed that as the dose of cerulenin increased from 100-200 μ M, cell
20 growth inhibition increased in a dose-dependent fashion in CRC cell lines.
21 Cerulenin-induced growth inhibition was found to be statistically significant ($p < 0.05$)
22 (one way ANOVA) in 100, 150, and 200 μ M of cerulenin compared to 0 μ M.

23 **Induction of apoptosis via activation of caspase-dependent pathway by cerulenin**

24 In subsequent experiments, we determined the mechanism of the observed

1 suppressive effect of cerulenin by WST-8 assays. The overexpression of FAS has been
2 observed to cooperate with survival pathways, including the PI3K/Akt pathway. These
3 two cell lines expressed FAS and p-AKT constitutively, and treatment of CRC cells with
4 cerulenin suppressed FAS expression, dephosphorylated constitutive activated Akt, and
5 increased cleaved caspase-3 in both Colon 26 and CMT 93 cells (Fig. 2A). TUNEL
6 staining of CRC cells revealed that cerulenin induced apoptosis both in Colon 26 and
7 CMT 93 cells in 50 to 100 μ M (Fig. 2B).

8 **Inhibition of general lipogenesis by cerulenin**

9 To investigate the physiological consequences of in vivo inhibition of FAS, we
10 administered cerulenin (30mg/kg body weight every 3 days) to mice by i.p. injection.
11 We observed slight weight loss following treatment with no significant difference in
12 comparison to the control group (Fig. 3). Serum triglyceride was significantly decreased
13 in the cerulenin group compared to the control group. This suggests general lipogenesis
14 in the liver was reduced by cerulenin (Fig. 4).

15 **Inhibition of tumor growth of liver metastasis of CRC by cerulenin**

16 We evaluated the potential effectiveness of cerulenin for metastatic liver tumors
17 of the CRC cell line. Figure 5A showed histological cross sections of livers removed
18 from representative control group and cerulenin groups. Growth reduction of metastatic
19 liver tumors was recognized in the cerulenin group. Figure 5B indicates tumor areas of
20 the liver in both groups. Tumor growth was significantly reduced by cerulenin
21 administration. Figure 5C showed TUNEL staining of liver sections of control group
22 and cerulenin group. In cerulenin group, apoptotic tumor cells were observed in the
23 metastatic liver tumor.

24

1 **Discussion**

2 The present study extends the investigation of the potential use of a FAS inhibitor
3 for chemoprevention of liver metastasis of CRC in mice. In this study, we revealed the
4 effect of cerulenin on the murine CRC cell line, Colon 26 and CMT 93, on growth
5 inhibition by inducing apoptosis. Also, the effect of cerulenin on the prevention of
6 growth of liver metastasis lesions of Colon 26 was revealed. This is the first study on
7 inhibiting liver metastasis of CRC by administrating FAS inhibitor, cerulenin. Many
8 kinds of human cancer cells have high activities of FAS and numerous studies have
9 described the cytotoxic effects of FAS both in vitro and in vivo.⁽⁴⁾ The role of increased
10 FAS in cancer cells and the mechanisms of cell killing by inhibitors of FAS are still not
11 fully understood.⁽¹¹⁾

12 The effect of an intermediate metabolite of fatty acid synthesis on cancer cells is
13 likely mediated through cell signaling pathways.⁽¹²⁾ For example, inhibiting FAS
14 decreases phosphorylation of Akt in ovarian cancer cells and suppresses HER2 over
15 expression in breast cancer cells.^(12, 13) The mechanism by which FAS inhibitor
16 decreases Akt activation is still unclear, but one mechanism that is advocated is that
17 fatty acids synthesized by FAS are incorporated into membrane phospholipids, which
18 are known modulators of Akt activation.^(14, 15) FAS has a major role in the synthesis of
19 phospholipids.⁽¹⁶⁾ When FAS expression is decreased by the treatment of cerulenin, less
20 fatty acid will be synthesized and less phospholipid will be available. One of the
21 important phospholipid is PIP₃. PIP₃ binds to Akt and its activating kinase PDK-1 with
22 high affinity, and the phosphorylation of Akt is dependent on PIP₃.⁽¹⁷⁾ In this study, a
23 decrease of phospholipids may result in inhibition of Akt activity in Colon 26 and CMT
24 93 cell lines after cerulenin administration. Recently, Liu X et al. reported that inhibition

1 of PI3K/Akt by cerulenin induces apoptosis in breast cancer cell lines via release of
2 cytochrome and activation of caspase. ⁽¹⁸⁾ In our study it was clarified that inhibition of
3 PI3K/Akt by cerulenin induces apoptosis in CRC cell lines via activation of caspase.

4 Colon 26 is chemically derived colon cancer from BALB/c mice. ⁽¹⁹⁾ It is highly
5 metastatic and many researchers use this cell line for study of liver metastasis of CRC in
6 mice. ⁽²⁰⁾ From these reports we used colon 26 for this study. P53 expression of colon 26
7 is not fully understood, but reacts to IFN alpha to increase p53, and induce apoptosis.
8 ⁽²¹⁾ Functional p53 reacts to interferon alpha or beta. ⁽²²⁾ That means p53 in colon 26
9 would be functional. Further investigation of p53 in colon 26 should be required.

10 Many efforts to treat xenograft cancers with cerulenin or its derivative of C75 were
11 reported in ovary, ⁽²³⁾ prostate, ⁽²⁴⁾ mesothelioma, ⁽²⁵⁾ breast cancer, ⁽²⁶⁾ and CRC. ^(27, 28)
12 But there is no study of treatment of liver metastasis of CRC using cerulenin. In this
13 study we report that cerulenin inhibits liver metastasis of CRC in a mouse model. In the
14 prior studies, the use of cerulenin or C75 has been hampered by transient but severe
15 anorexia and weight loss. Therefore, this compound could also limit the use in the
16 clinical setting. ^(29, 30) Loftus et al ⁽²⁹⁾ reported that cerulenin treatment 60mg/kg daily for
17 7days caused severe weight loss. Pizer ES et al ⁽²³⁾ reported that cerulenin treatment
18 80mg/kg/daily for 7 days caused severe weight loss. Uddin S et al ⁽¹⁵⁾ reported that C-75
19 treatment 10mg/kg or 20mg/kg twice weekly did not cause severe weight loss. In our
20 experiments we obtained adequate liver metastasis 9 days after tumor injection. We
21 treated cerulenin 10mg/kg or 30mg/kg twice weekly (every 3 days). In our preliminary
22 study cerulenin treatment of 10mg/kg had no effect (data not shown). By treating
23 30mg/kg of cerulenin every 3 days, inhibition of liver metastasis was observed. At this
24 amount, serum triglyceride was significantly decreased in cerulenin group. That means

1 FAS was really inhibited in our animal model. But we could not observe significant
2 weight loss in this group compared to control group. This result indicated the amount of
3 cerulenin in our experiment was appropriate for prevention of side effects of FAS
4 inhibitor. We could not promote complete remission of metastatic tumors by cerulenin
5 at this dose and the appropriate dose of cerulenin or other FAS inhibitors needs to be
6 studied. Also, the effect of FAS inhibitors to the chemically induced colon cancer
7 should be evaluated.

8 One of the reasons for anorexia brought on by cerulenin or C75 is fatty acid
9 oxidation by activating carnitine O palmitoyltransferase 1.⁽³⁰⁾ In recent years, it was
10 reported that C93, which inhibits FAS but has no significant effect on fatty acid
11 oxidation, is effective for treatment of human lung cancer xenografts. As a result, C93
12 does not cause anorexia or weight loss.⁽¹¹⁾ In near future analogues of FAS inhibitors
13 such as C93 would represent promising new treatments for cancer, including liver
14 metastasis of CRC.

15 In conclusion, an FAS inhibitor of cerulenin inhibits CRC cell line survival in vitro
16 and in vivo models with liver metastasis. FAS would be a new drug target for liver
17 metastasis of CRC.

18

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21

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1 **Figure Legends**

2

3 **Figure 1**

4 A. Effect of cerulenin on the WST-8 assay of murine CRC cell lines. CRC cell lines
5 were treated with 0 to 200 μ M cerulenin for 24h. Left, Colon 26; right, CMT93. *
6 $p < 0.01$ compared to 0 μ M cerulenin. The values indicate ratio compared to 0 μ M
7 cerulenin as 100%.

8 B. Effect of cerulenin on the BrdU assay of murine CRC cell lines. CRC cell lines were
9 treated with 0 to 200 μ M cerulenin for 24h. Left, Colon 26; right, CMT93. * $p < 0.01$
10 compared to 0 μ M cerulenin. The values indicate ratio compared to 0 μ M cerulenin as
11 100%.

12 **Figure 2**

13 A. Cerulenin treatment causes down regulation of FAS and dephosphorylation of
14 constitutive phosphorylation of Akt in CRC cell lines. Colon 26 and CMT 93 cell lines
15 were treated with 0, 50, and 100 μ M cerulenin for 24h. After cell lysis, equal amounts
16 of proteins were separated by SDS-PAGE, transferred to Immobilon membrane, and
17 immunoblotted with antibodies against FAS, p-Akt, cleaved caspase-3, and GAPDH as
18 indicated.

19 B. Cerulenin treatment causes apoptosis in CRC cell lines. TUNEL staining of Colon 26
20 and CMT 93 cell lines which were treated with 0, 50, and 100 μ M cerulenin for 24h.

21

22 **Figure 3**

23 Weight of animals for cerulenin and control groups in the experiment. Columns, mean;

1 bars, SD. White bar, control group; black bar, cerulenin group.

2

3 **Figure 4**

4 Serum triglyceride 9 days after tumor inoculation. Columns, mean; bars, SD. White bar,
5 control group; black bar, cerulenin group. * $p < 0.05$ vs control group, *t*-test.

6

7 **Figure 5**

8 Cerulenin inhibits tumor growth of liver metastasis of CRC cell line in mice.

9 A. Histologic cross sections of livers of control group (left) and cerulenin group (right).

10 Arrows, metastatic tumors. Original magnification $\times 100$.

11 B. Tumor area in the liver of all animals in the experiment. Columns, mean; bars, SD.

12 White bar, control group; black bar, cerulenin group. * $p < 0.05$ vs control group, *t*-test.

13 C. TUNEL staining of tumor tissues. Arrows indicated apoptotic tumor cells in

14 cerulenin group. Original magnification $\times 200$.

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A

Figure 1

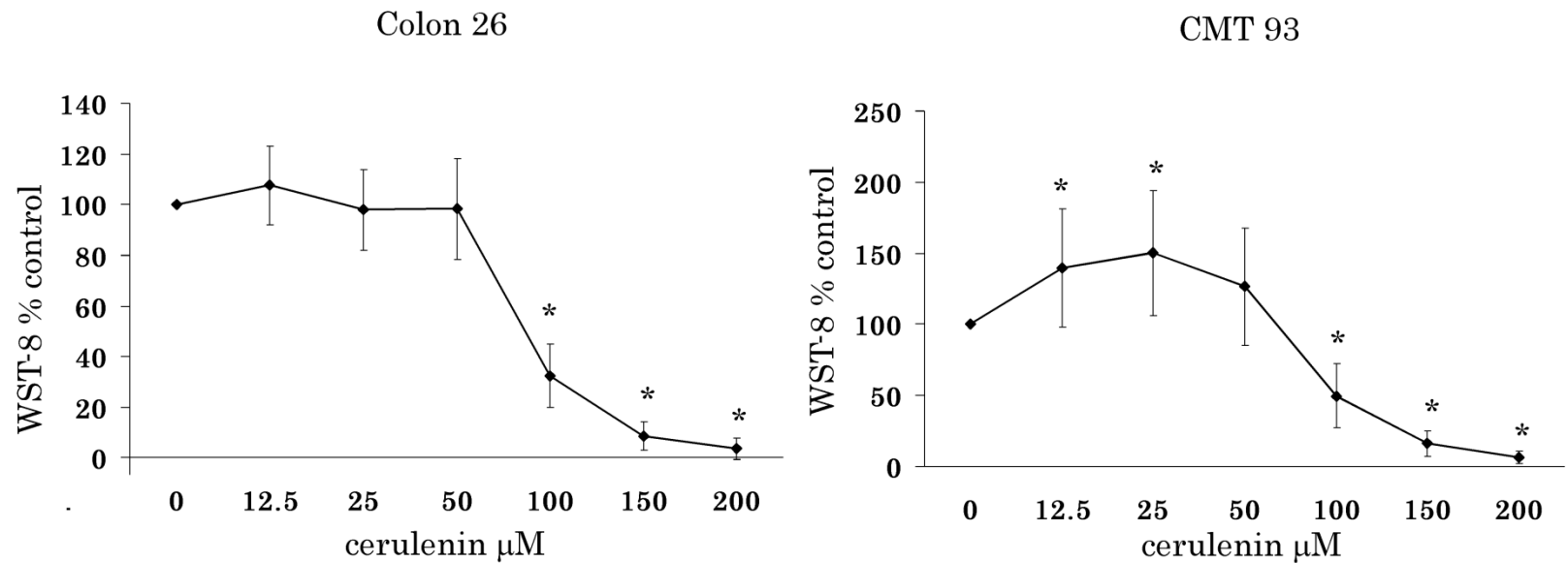
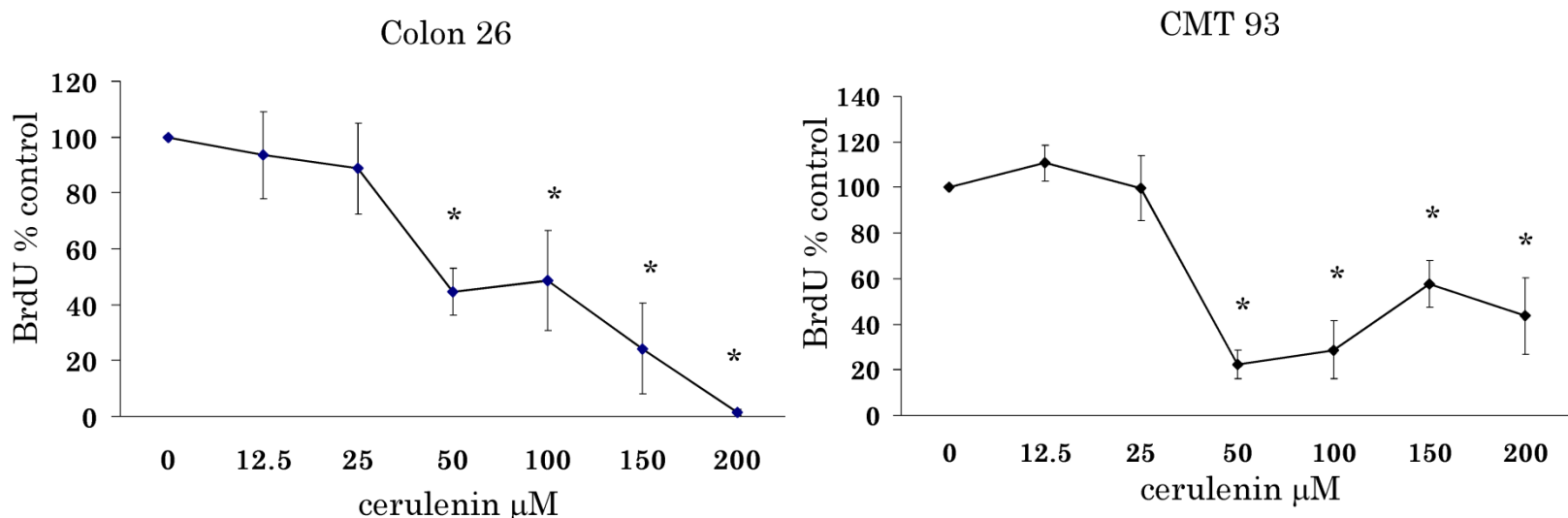
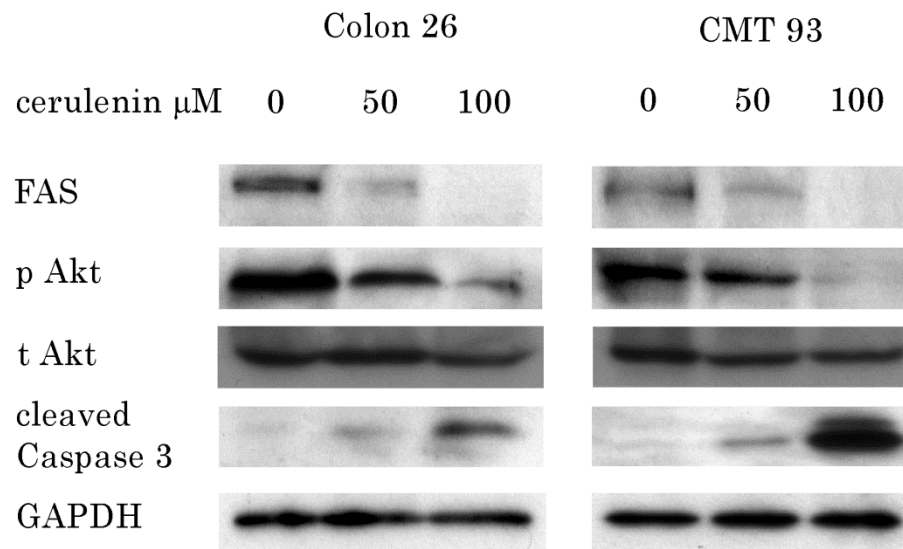
**B**

Figure 2

A



B

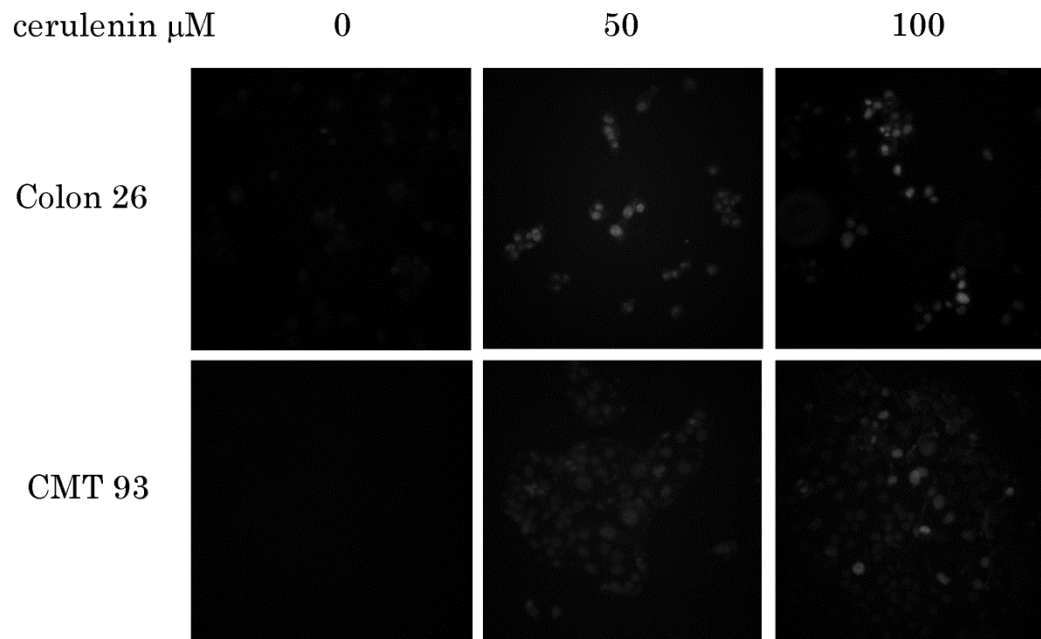


Figure 3

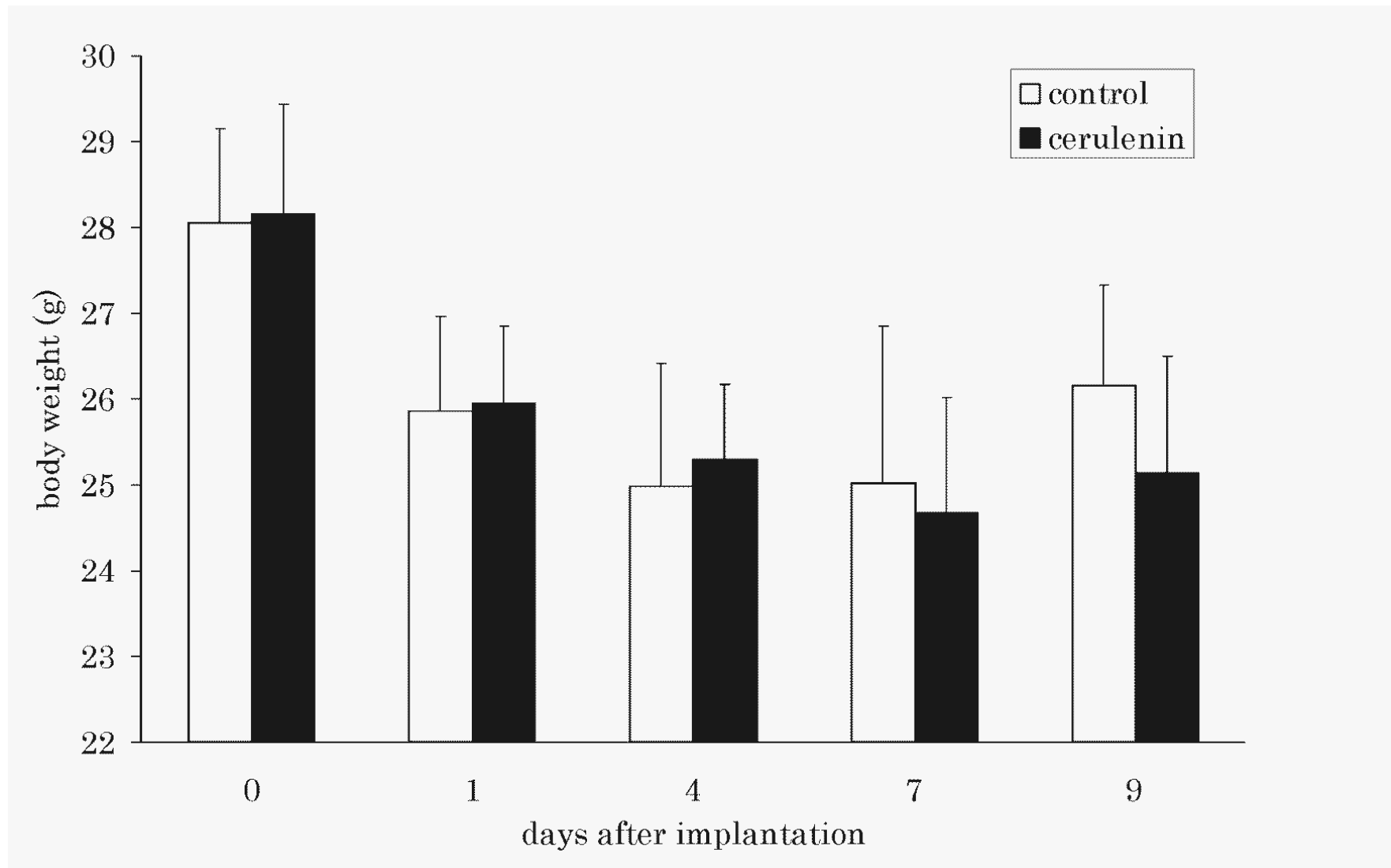


Figure 4

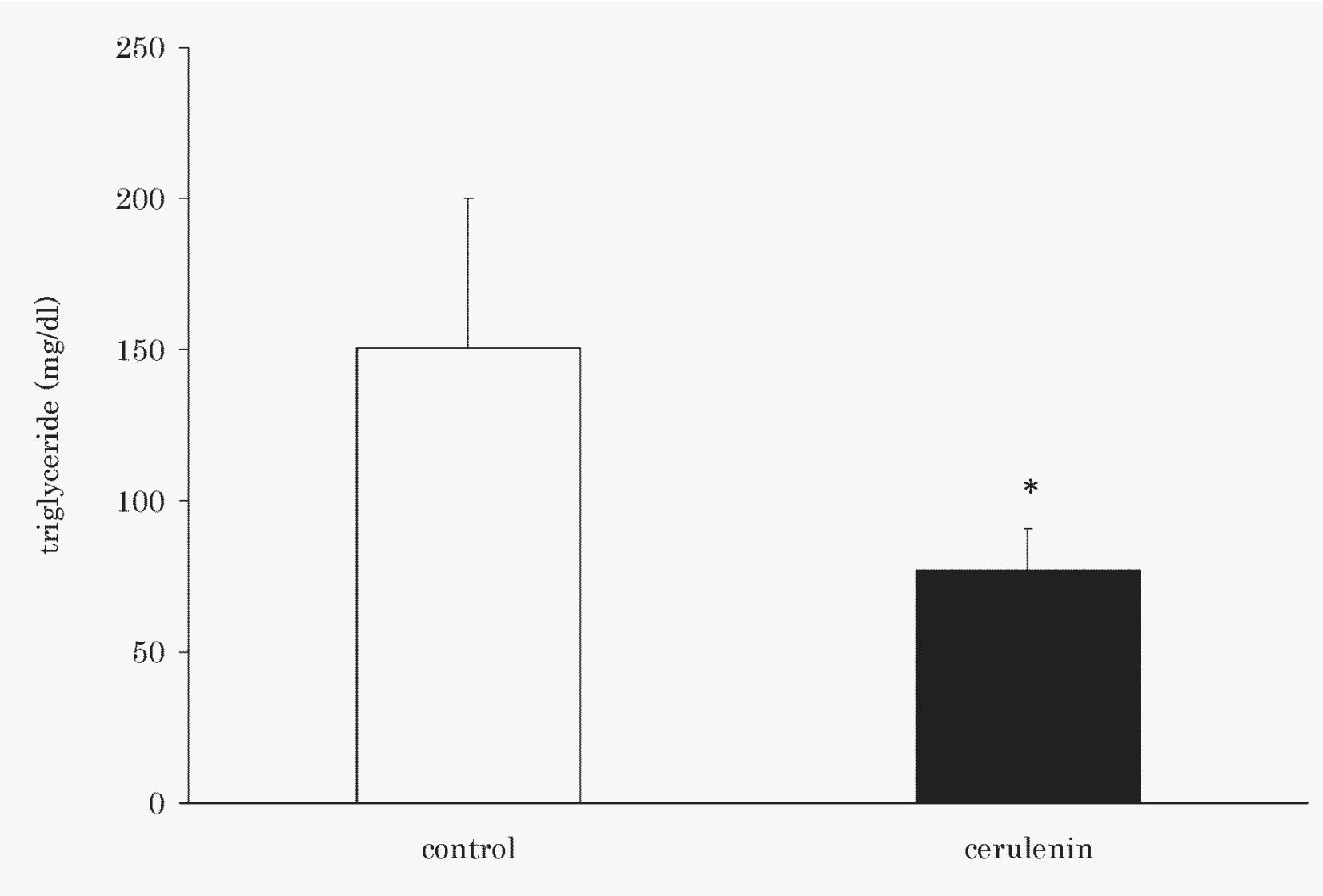


Figure 5

