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

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ABSTRACT

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

Introduction

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

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

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

Materials and Methods

Cerulenin

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

Cell culture

Two murine CRC cell lines, Colon 26 and CMT 93, were used and tested for mycoplasm-free cell lines. All cancer cell lines were subdivided in multiple tubes for stock in liquid nitrogen immediately after possession. All cell lines were subjected to present experiment within 6 months of resuscitation. Stock cultures were grown in high glucose DMEM containing 10% FBS and 1% antibiotics. The cells were grown in growth medium at 37°C in a 95% air, 5% CO_2-humidified incubator.

Cell viability assay

To measure the cytotoxicity of cerulenin against these cancer cells, 3×10^3 cells were plated per well onto 96-well plates. Following overnight culture, cerulenin were added at specified concentrations. After 24 hours of incubation, cell viability was measured by the mitochondrial activity in reducing 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) to formazan using Cell Counting kit-8 (Dojindo Laboratories, Kumamoto, Japan). Cells were incubated with a reagent as per manufacturer's instructions. Plates were read at A_{450} on a spectrometer.

Cell Proliferation Assay

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

Apoptosis assay

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

Western blot analysis

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

Animals

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

Liver metastasis

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

Histochemical examination

To assess the liver metastatic area, left and middle lobes of liver were divided in 5mm slice, fixed with 10% formaldehyde, and embedded in paraffin. Thin sections (4μm) were stained with hematoxylin-eosin, and the percentage of liver metastatic area of Colon 26 cells was calculated using image-processing software WinROOF (Mitani

Co., Fukui, Japan).

Serum parameter

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

Statistical analysis

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

Results

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

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

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

In subsequent experiments, we determined the mechanism of the observed

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

Inhibition of general lipogenesis by cerulenin

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

Inhibition of tumor growth of liver metastasis of CRC by cerulenin

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

Discussion

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

The effect of an intermediate metabolite of fatty acid synthesis on cancer cells is likely mediated through cell signaling pathways.[12] For example, inhibiting FAS decreases phosphorylation of Akt in ovarian cancer cells and suppresses HER2 over expression in breast cancer cells. [12, 13] The mechanism by which FAS inhibitor decreases Akt activation is still unclear, but one mechanism that is advocated is that fatty acids synthesized by FAS are incorporated into membrane phospholipids, which are known modulators of Akt activation. [14, 15] FAS has a major role in the synthesis of phospholipids. [16] When FAS expression is decreased by the treatment of cerulenin, less fatty acid will be synthesized and less phospholipid will be available. One of the important phospholipid is PIP_3. PIP_3 binds to Akt and its activating kinase PDK-1 with high affinity, and the phosphorylation of Akt is dependent on PIP_3. [17] In this study, a decrease of phospholipids may result in inhibition of Akt activity in Colon 26 and CMT 93 cell lines after cerulenin administration. Recently, Liu X et al. reported that inhibition

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

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

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

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

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

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

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Figure Legends

Figure 1

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

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

Figure 2

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

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

Figure 3

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

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

Figure 4

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

Figure 5

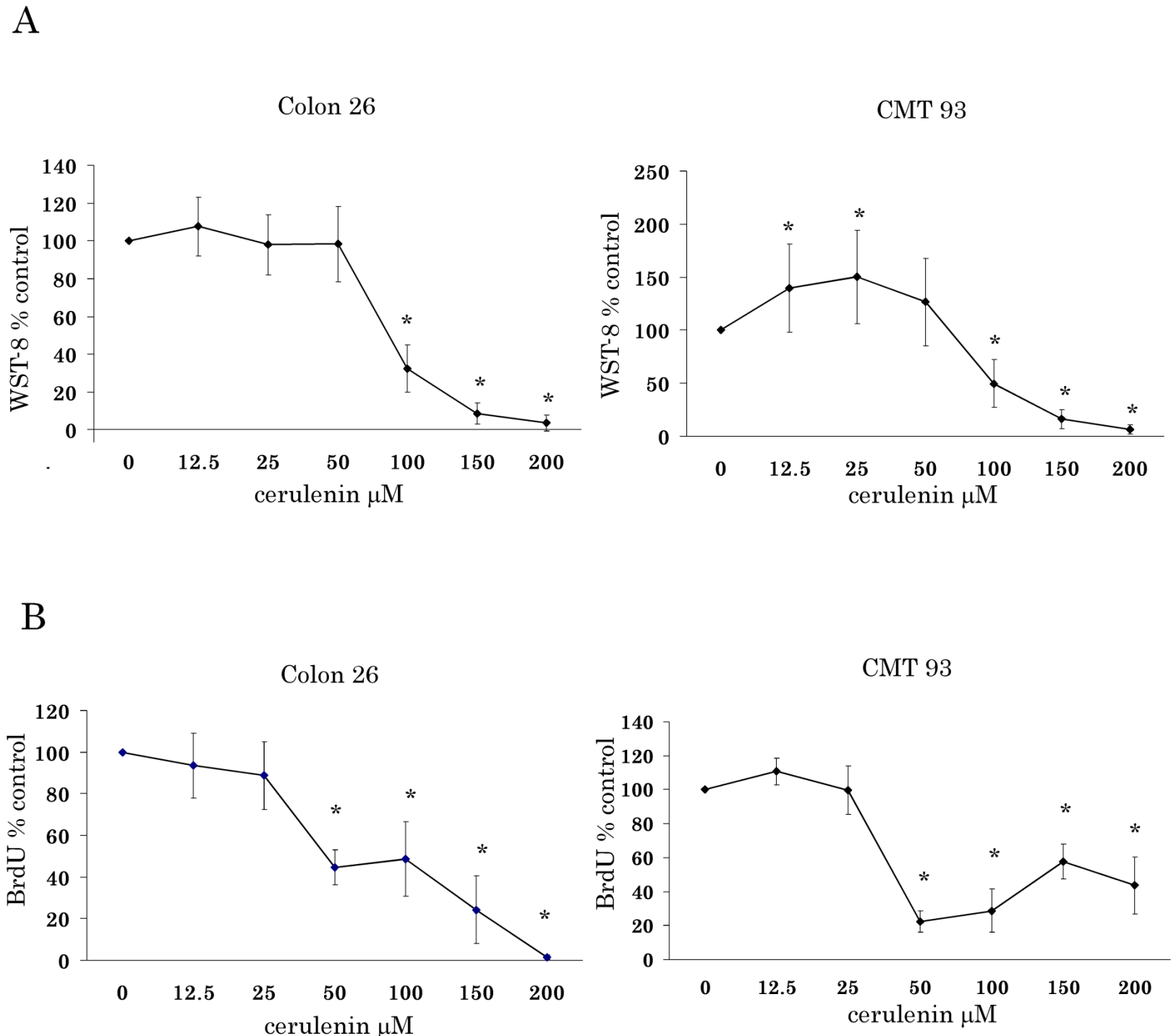
Cerulenin inhibits tumor growth of liver metastasis of CRC cell line in mice.

A. Histologic cross sections of livers of control group (left) and cerulenin group (right). Arrows, metastatic tumors. Original magnification ×100.

B. Tumor area in the liver of all animals in the experiment. Columns, mean; bars, SD. White bar, control group; black bar, cerulenin group. *p<0.05 vs control group, *t*-test.

C. TUNEL staining of tumor tissues. Arrows indicated apoptotic tumor cells in cerulenin group. Original magnification ×200.

Figure 1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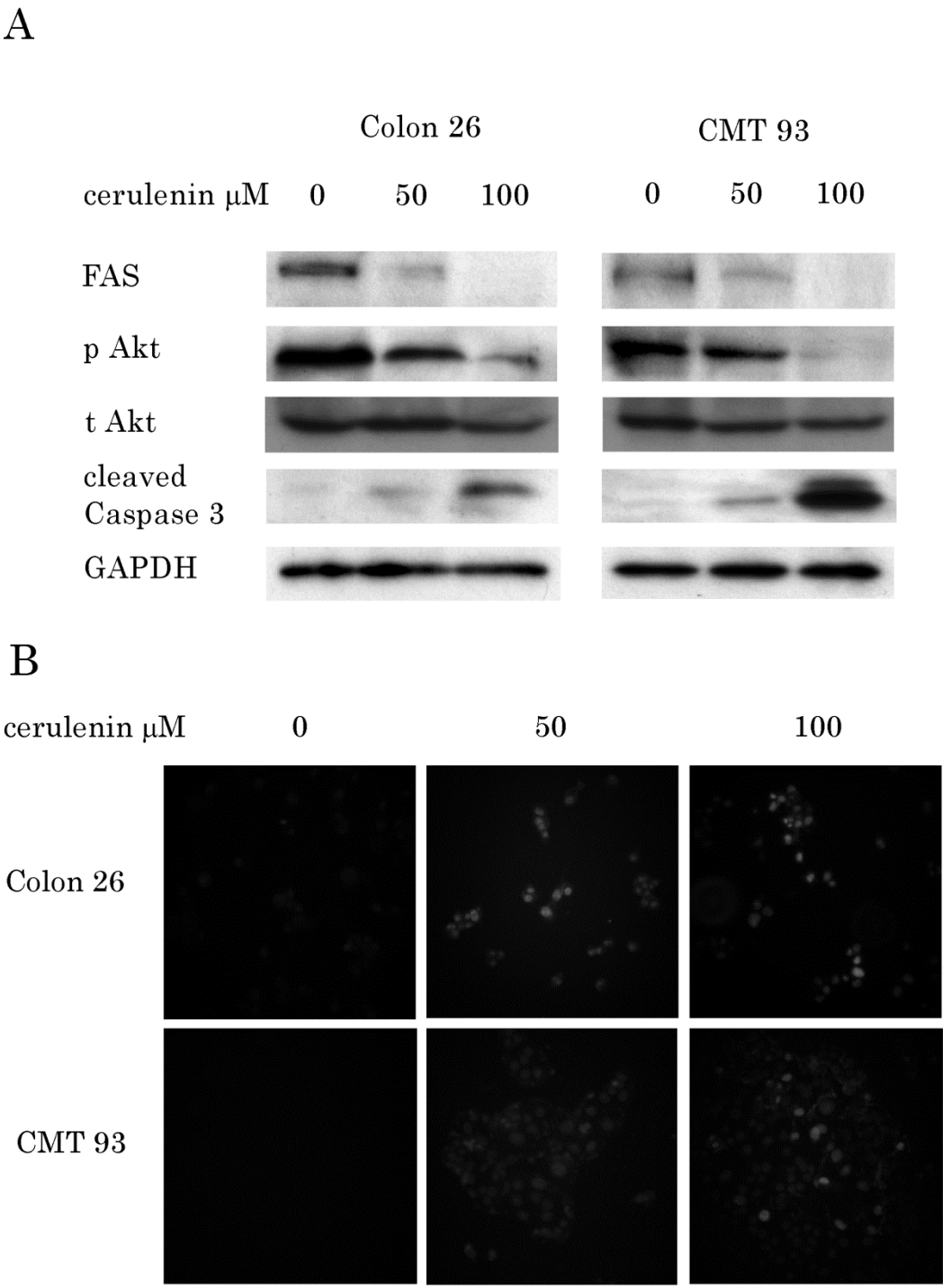
Colon 26
CMT 93
WST-8 % control
cerulenin μM
0
12.5
25
50
100
150
200
B
Colon 26
CMT 93
BrdU % control
cerulenin μM

Figure 2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Colon 26
CMT 93
cerulenin μM
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50
100
0
50
100
FAS
p Akt
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cleaved
Caspase 3
GAPDH
B
cerulenin μM
0
50
100
Colon 26
CMT 93

Figure 3

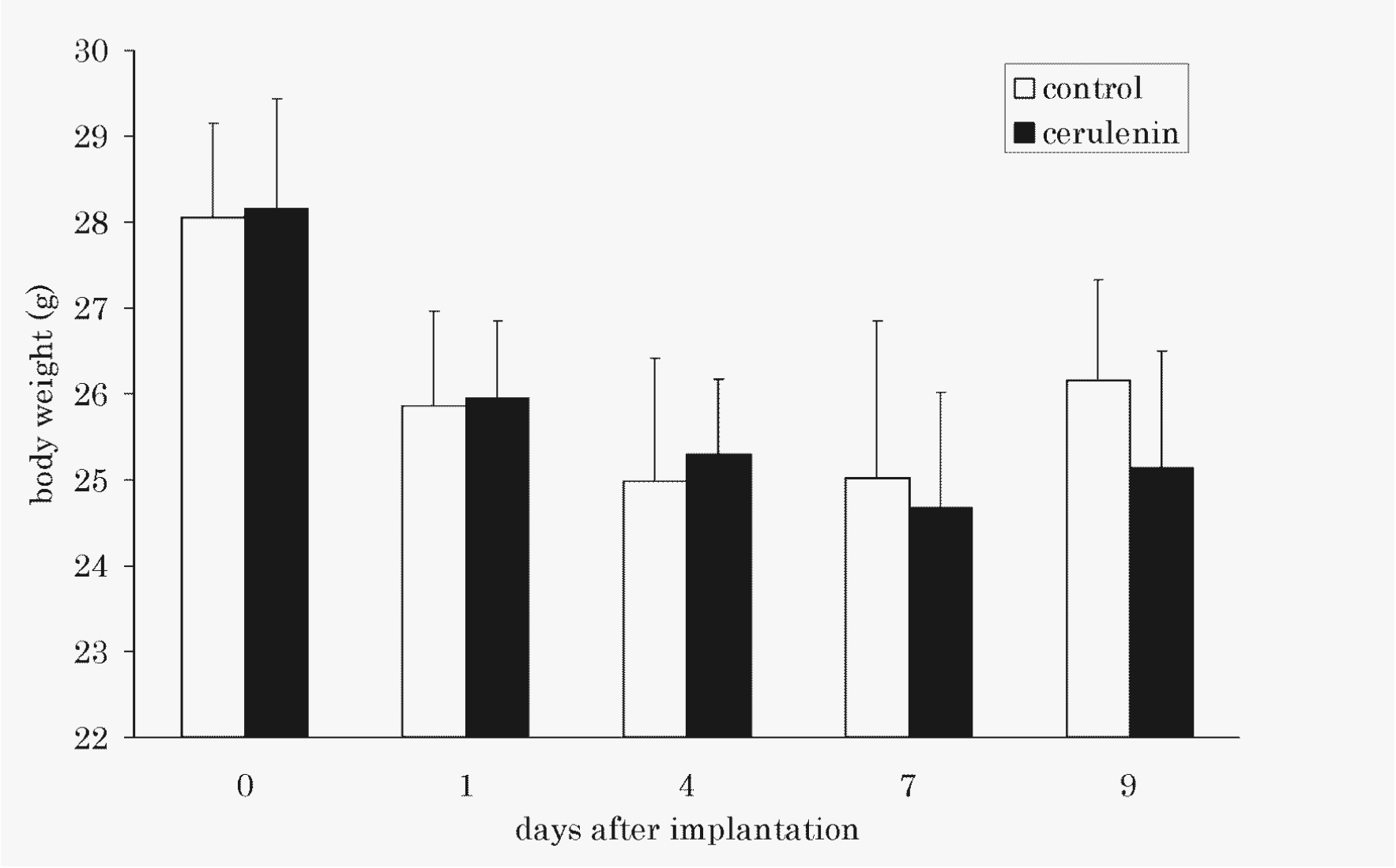

30
29
28
27
26
25
24
23
22
body weight (g)
control
cerulenin
0
1
4
7
9
days after implantation

Figure 4

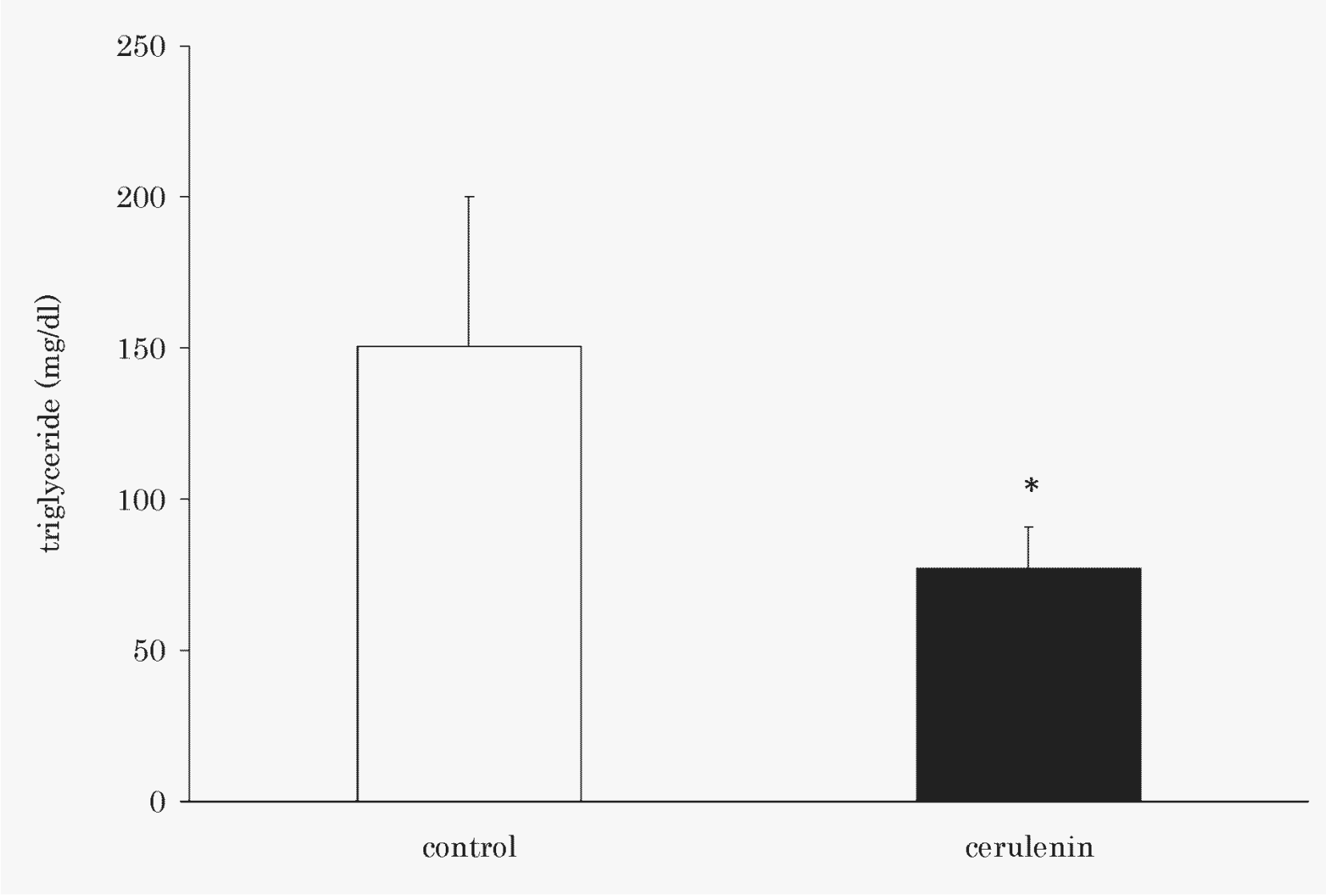
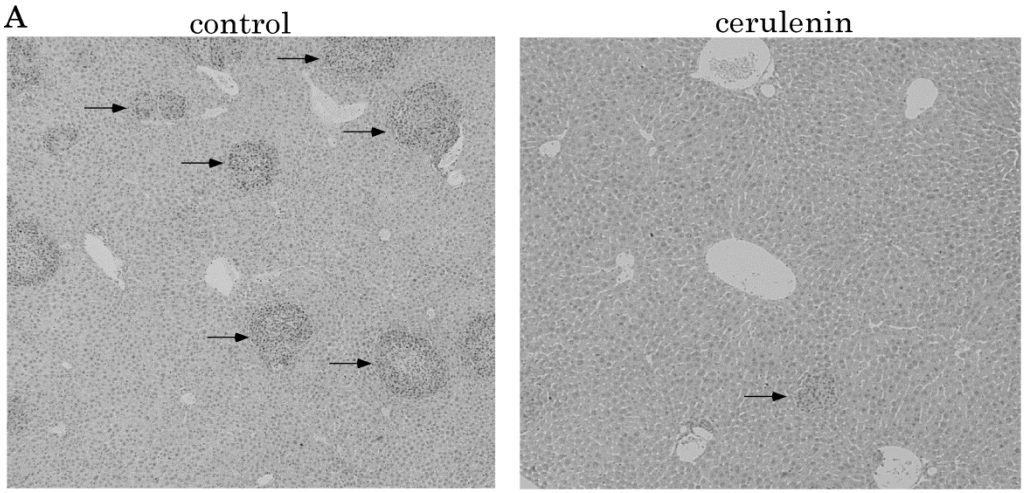
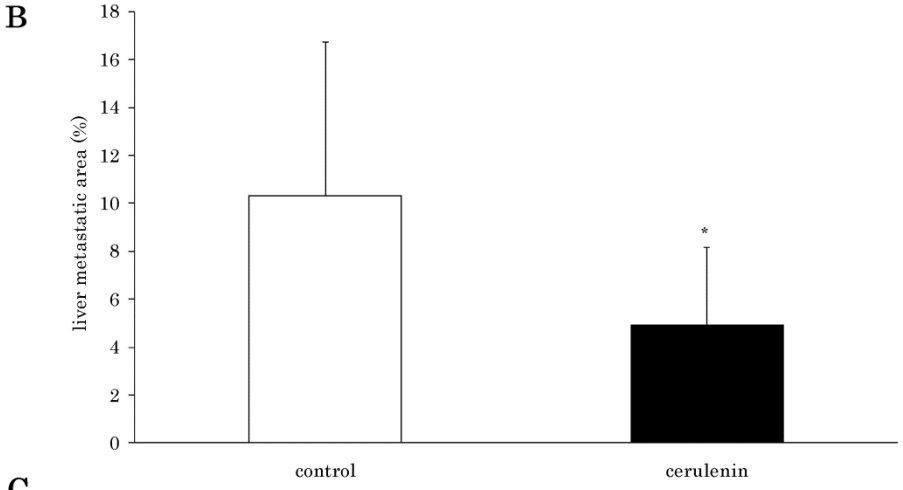


Figure 5


A
control
cerulenin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liver metastatic area (%)
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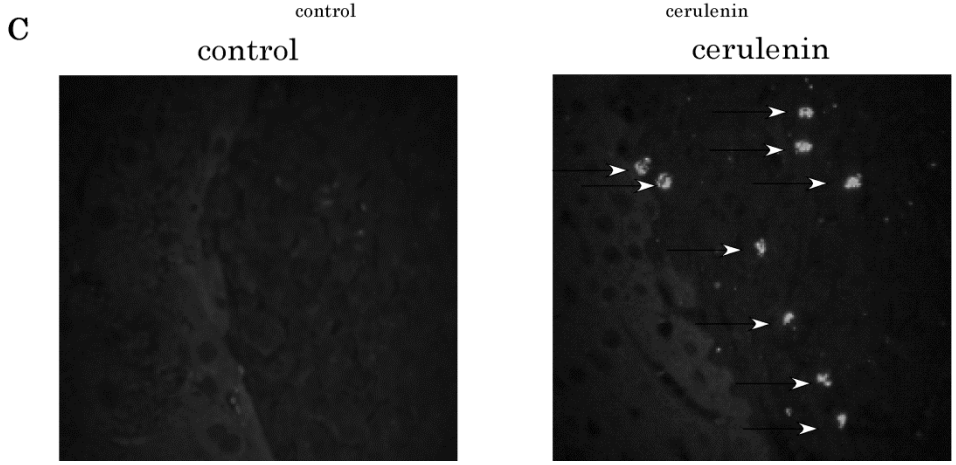
16
14
12
10
8
6
4
2
0
*
control
cerulenin


C
control
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