Azaspirene Analogs Inhibit the Growth of Human Uterine Carcinosarcoma *In Vitro* and *In Vivo*

MAKOTO EMOTO[1,6], KYOKO YANO[2], BATSUREN CHOIJAMTS[1], SHINNOSUKE SAKAI[3], SHUN HIRASAWA[3], SHINNOSUKE WAKAMORI[3], MAMORU AIZAWA[4], KAZUKI NABESHIMA[2], KATSURO TACHIBANA[5] and NOBUHIRO KANOMATA[3]

[1]Department of Health and Welfare, International University of Health and Welfare, Fukuoka, Japan; Department of [2]Pathology, and [5]Anatomy, Faculty of Medicine, Fukuoka University, Jonan-ku, Fukuoka, Japan; [3]Department of Chemistry and Biochemistry, School of Advanced Science and Engineering, Waseda University, Shinjuku-ku, Tokyo, Japan; [4]Department of Applied Chemistry, School of Science and Technology, Meiji University, Kawasaki, Japan; [6]Division of Gynecology in Preventive Medicine, Fukuoka Sanno Hospital, Sawara-ku, Fukuoka, Japan

Abstract. *Uterine carcinosarcoma is a highly aggressive gynecological neoplasm that responds poorly to conventional chemotherapy and radiotherapy. Recent studies have shown high angiogenic activities of this tumor, hence anti-angiogenic approaches are expected to provide new treatment strategies for this tumor. In previous work, azaspirene was isolated from Neosartorya sp. fungi, and in vitro anti-angiogenic activities were shown. In the present study, the anti-angiogenic effects of azaspirene analogs, synthetic molecules with a shorter ethyl group replacing a hexadienyl side-chain of the natural compound, were assessed in vitro using human umbilical vein endothelial cells (HUVECs) co-cultured with FU-MMT-3 human uterine carcinosarcoma cells. The anti-tumor and anti-angiogenic effects of these analogs were also evaluated in vivo using FU-MMT-3 xenografted tumors in nude mice. The azaspirene analogs inhibited the tube formation of HUVECs induced by FU-MMT-3 cells in vitro and significantly suppressed tumor growth in vivo compared to the untreated group (control). A significant reduction of the microvessel density in tumors was observed, in comparison to the control. No apparent toxicity, including body loss, was observed in any mice treated in this study. These azaspirene analogs may be effective against uterine carcinosarcoma, possibly acting via potent anti-angiogenic effects.*

Correspondence to: Makoto Emoto, MD, Ph.D., Professor and Director, Division of Gynecology in Preventive Medicine, Fukuoka Sanno Hospital, International University of Health and Welfare, 3-6-45 Momochihama, Sawara-ku, Fukuoka 814-0001, Japan. Tel: +81 928321100, Fax: +81 92 8321102, e-mail: emoto1209@gmail.com

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Uterine carcinosarcoma (UCS) (also called malignant mixed Müllerian tumor) is a highly aggressive and primitive tumor composed of mixed malignant epithelial and mesenchymal components. These tumors are uncommon, accounting for only 2-5% of all uterine cancers, and mostly occur in elderly females. UCS is associated with poor survival rates, even when presenting at an apparently early stage. Most UCSs respond poorly to cytotoxic chemotherapeutic agents and to radiotherapy, thus, the five-year survival estimate for all patients with this tumor is approximately 30%, and for patients with disease confined to the uterus (stage I) is only 50-60% (1-5).

Based on the high frequency of hematogenous and lymphatic spread in patients with UCS, it was hypothesized that most tumors possess extremely high angiogenic activity (4). Extrauterine spread and metastatic disease are common at the time of diagnosis in patients with UCS, and the patterns of failure suggest a pattern of hematogenous spread, supporting the use of an anti-angiogenic approach for treatment. Increased expression of vascular endothelial growth factor (VEGF) and angiopoietin-2 were found to be associated with a poor prognosis in many types of tumors (6). Recent studies have indicated that UCSs highly express VEGF and angiopoietin-2, and have a higher microvessel density (MVD) compared to poorly-differentiated endometrial carcinomas (4, 7, 8). *In vitro* and *in vivo* studies, including our own, showed that anti-angiogenic treatments suppressed the tumor growth and vascularization of UCSs. Thus, anti-angiogenic approaches may represent an important therapeutic strategy for this tumor type (9-16).

Azaspirene, isolated from fungal metabolites by screening with an endothelial cell migration assay, has anti-angiogenic activity *in vitro* and *in vivo*. This agent, which has a characteristic 1-oxa-7-azaspiro[4.4]non-2-ene-4,6-

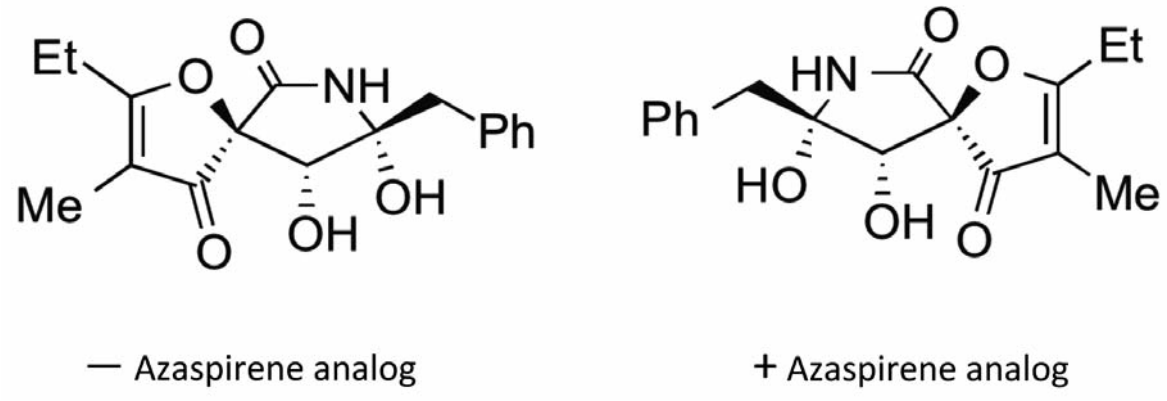

Figure 1. *The structures of the azaspirene analogs used in this study.*

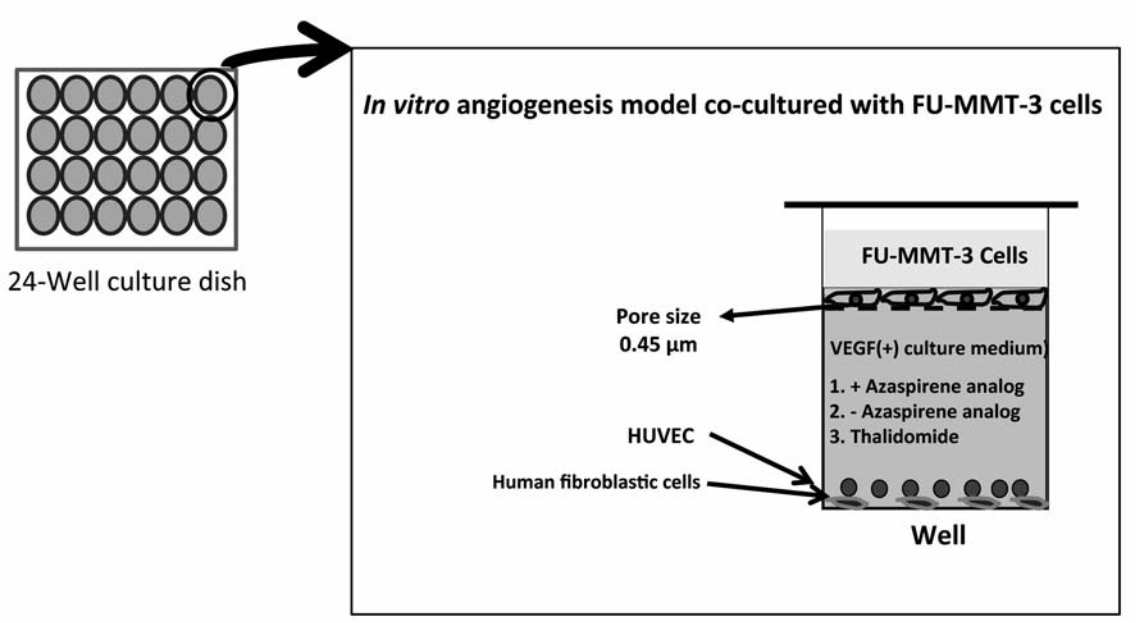

Figure 2. *An illustration of the in vitro angiogenesis model involving the co-culture with FU-MMT-3 cells. FU-MMT-3 cells (1×10^5) were placed in the upper chambers, and human umbilical vein endothelial cells (HUVECs) (2×10^4) with fibroblasts were cultured in the bottom chamber.*

dione skeleton, inhibited the endothelial migration induced by VEGF (effective dosage $(ED)_{100}$=27.1 μM), as did its first analog (17, 18). Subsequent *in vitro* experiments indicated that azaspirene suppressed the Rik1-associated factor (RAF)1 activation induced by VEGF without affecting the activation of kinase insert domain receptor (KDR)/fetal liver kinase (FLK)1 (VEGFR2) (19). We recently designed and synthesized new azaspirene analogs, replacing a hexadienyl side chain of the natural compound with a shorter ethyl group, and reported their *in vitro* anti-angiogenic activity (20).

According to a PubMed analysis, there exist only a few useful cell lines, including FU-MMT-3, derived from human UCS. The FU-MMT-3 cell line has been fully characterized and is commonly used *in vitro* and *in vivo* to study this disease (9-14, 21). As far as we are aware of, no previous study has examined the anti-angiogenic effects of azaspirene analogs against any cells representing tumors of the female genital tract. Therefore, in the present study, the newly-synthesized azaspirene analogs were evaluated *in vitro* and *in vivo* using FU-MMT-3 cells, which provide a highly angiogenic cancer model derived from a primary UCS.

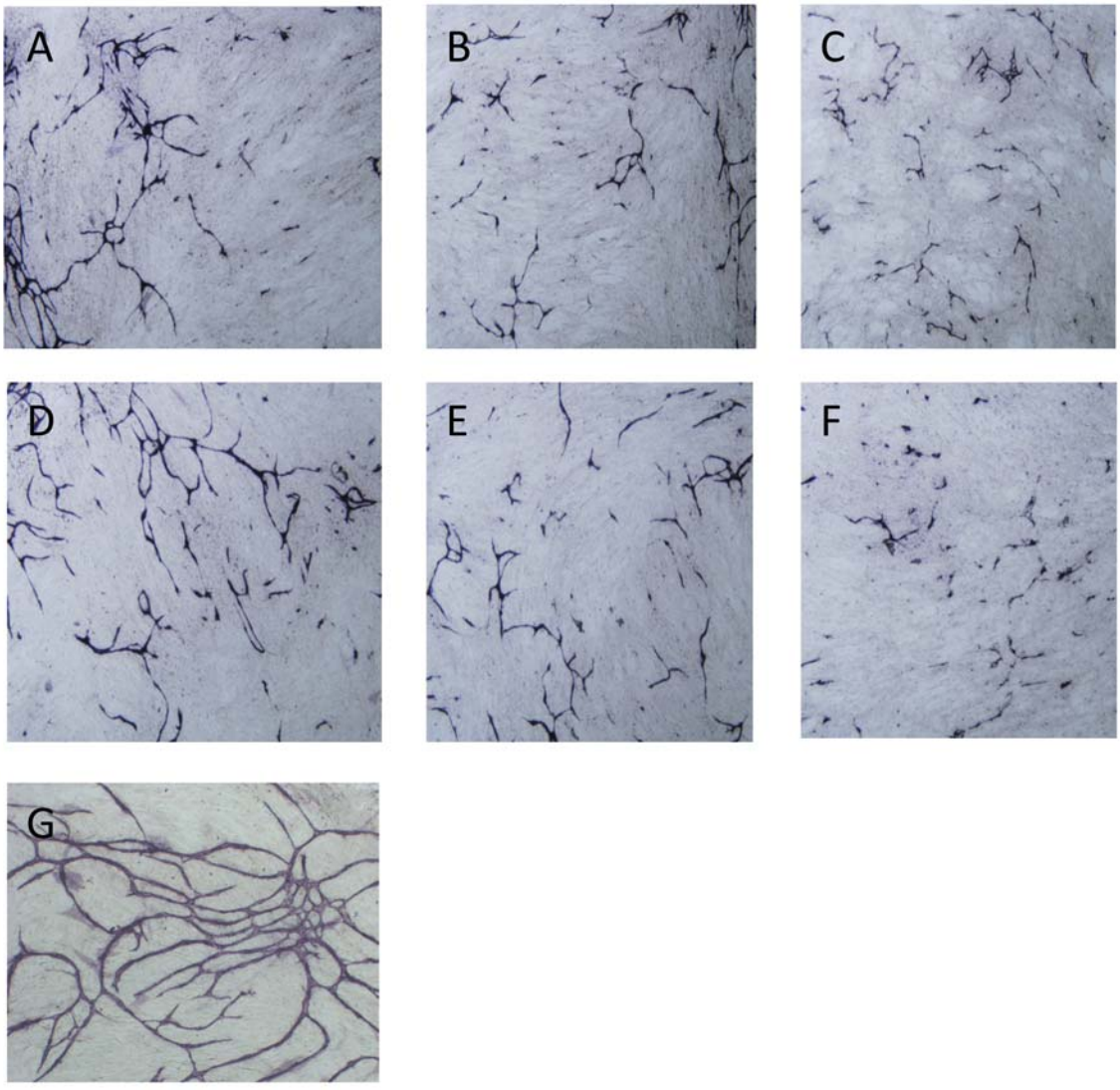

Figure 3. *Continued*

Materials and Methods

Cell lines and nude mice. We previously established a human UCS cell line, FU-MMT-3, from a patient with UCS (21). The FU-MMT-3 cells show highly aggressive characteristics *in vitro* and *in vivo*. The immunophenotype, tumorigenicity, and cytogenetic characteristics of the FU-MMT-3 cells have already been reported (21). Our subsequent studies showed that the FU-MMT-3 cells showed high-angiogenic activity *in vitro* and *in vivo* (13, 14).

Female BALB/cA Jcl-nu athymic nude mice (Clea, Tokyo, Japan) were used in the present study, according to the instructions of the Institute of Experimental Animal Science, Fukuoka University Medical School. The *in vivo* experiments were performed in accordance with the Declaration of Helsinki and the World Medical Association, and were approved by the Institutional Animal Care and Use Committee of Fukuoka University (no. 0803222).

Chemicals. To study structure-activity relationships of the unique spirostructure of azaspirene, we synthesized analogous compounds, including (−)- and (+)-azaspirene, (−)-(5S,8R,9R)- and (+)-(5R,8S,9S)-8-benzyl-2-ethyl-8,9-dihydroxy-3-methyl-1-oxa-7-azaspiro[4.4]non-2-ene-4,6-dione. The (+) and (−) analogs were designed by formally replacing the natural 1,3-hexadienyl side chains with an ethyl group to retain the rest of the structure, including the same and the opposite stereochemistry of the core skeleton of the natural compounds (Figure 1) (20).

In vitro tube-formation assay. Tube formation by the cells was determined in triplicate in 24-well dishes using an angiogenesis kit (Kurabo, Osaka, Japan), according to the manufacturer's instructions, with minor modifications as described previously (14). Briefly, FU-MMT-3 cells (1×10^5 cells/well) were co-cultured with human umbilical vein endothelial cells (HUVECs) and

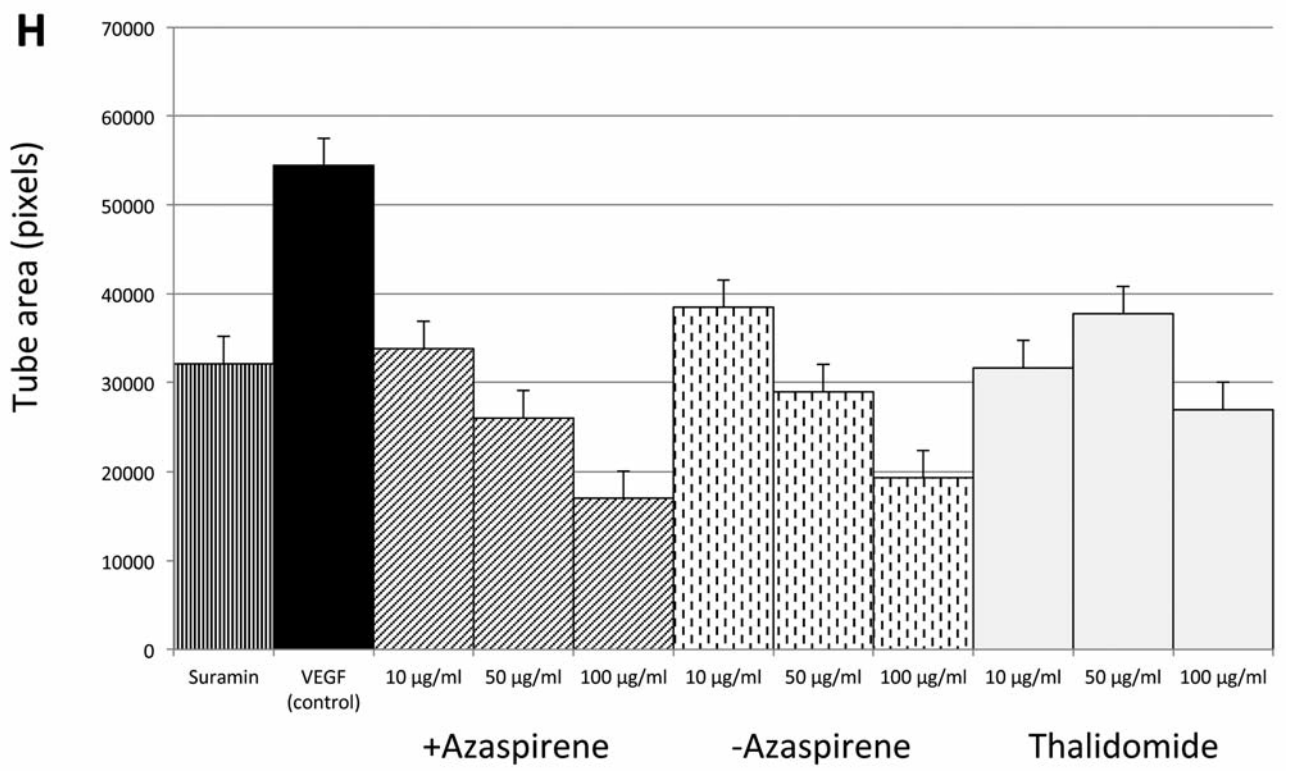

Figure 3. *Continued*

fibroblasts as an *in vitro* model of tumor angiogenesis, in the presence or absence of the azaspirene analogs . FU-MMT-3 cells (1×10^5) were seeded in the upper chambers of 24-well dishes while HUVECs (2×10^4) were seeded on the bottom chambers in the presence of the (+)-azaspirene analog, (−)-azaspirene analog, or thalidomide at 10 μg/ml, 50 μg/ml, or 100 μg/ml, in Ham's Dulbecco's modified Eagle medium (DMEM)-12 with 10% fetal bovine serum (FBS). The azaspirene analogs were dissolved in normal saline containing 0.1% dimethyl sulfoxide (DMSO). The media were changed every three days.

The area and tube length of HUVECs stained by a mouse antibody to human CD31 (Kurabo, Osaka, Japan) were measured using a Kurabo angiogenesis image analyzer (Kurabo) and the results were statistically analyzed essentially as described previously (11-14).

In vivo treatments. The mice (6 weeks after birth, weight; 20g, n=5 in each group) were injected subcutaneously with 2×10^5 FU-MMT-3 cells in 0.2 ml of vehicle. Mice that developed tumors measuring 5-10 mm in diameter by day 7 were randomly separated into three groups and were treated as follows: i) (+)-azaspirene analog; ii) (−)-azaspirene analog; iii) control (untreated). Azaspirene analogs were dissolved in normal saline containing 3.5% DMSO, and these analogs were injected subcutaneously at a dose of 30.0 mg/kg three times per week. Treatments were continued for six weeks. Tumor growth was monitored by measuring the size of the tumor twice per week, and was calculated as $V=\text{length}\times\text{width}^2/2$. The bodyweight of the mice was measured weekly.

Immunohistochemical staining. Cryosections were used to determine the microvessel density (MVD) in each FU-MMT-3 xenograft, which were resected from mice after six-week treatments. The sections were incubated with primary antibody against CD31 (dilution 1:100; PharMingen, San Diego, CA, USA). The MVD was quantified in most highly vascularized area (at ×400) examined in each tumor, as described previously (14).

Statistical analysis. The data are expressed as the means±SD. The Mann–Whitney *U*-test was used to compare tumor growth *in vivo* and bodyweight of mice. Unpaired *t*-tests were used to compare tube formation, and tumor MVD. All statistical analyses were performed using the StatView 5.0 software package (SAS Institute, Inc., Cary, NC, USA) for Macintosh. Values of $p<0.05$ were considered to be statistically significant.

Results

In vitro tube formation. To investigate the anti-angiogenic effects of azaspirene analogs, we performed tube-formation assays using HUVECs co-cultured with FU-MMT-3 cells, as a model of tumor angiogenesis. The (+) and (−) analogs at 10, 50 and μg/ml and 100 μg/ml all significantly inhibited tube formation compared to the untreated control group ($p<0.05$: Figure 3A-G). In a quantitative analysis of the tube area and

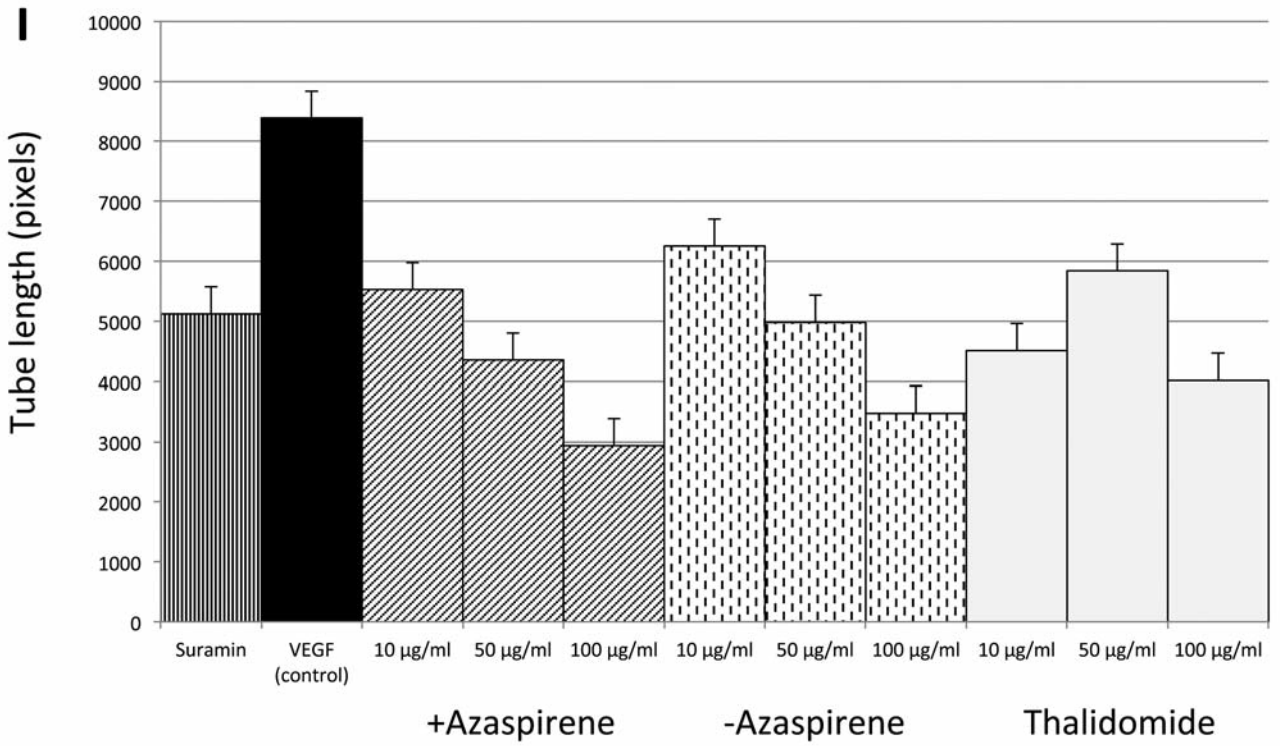

Figure 3. *The effects on tube formation in vitro. When human umbilical vein endothelial cells (HUVECs) were co-cultured with FU-MMT-3 cells, efficient tube formation was observed (G, control). Both the (+)-azaspirene analog (A, 10 μg/ml; B, 50 μg/ml; C, 100 μg/ml) and the (−)-azaspirene analog (D, 10 μg/ml; E, 50 μg/ml; F, 100 μg/ml) significantly inhibited tube formation in vitro compared with the control (A) in a quantitative analysis of both the tube area (H) and length (I) ($p<0.05$).*

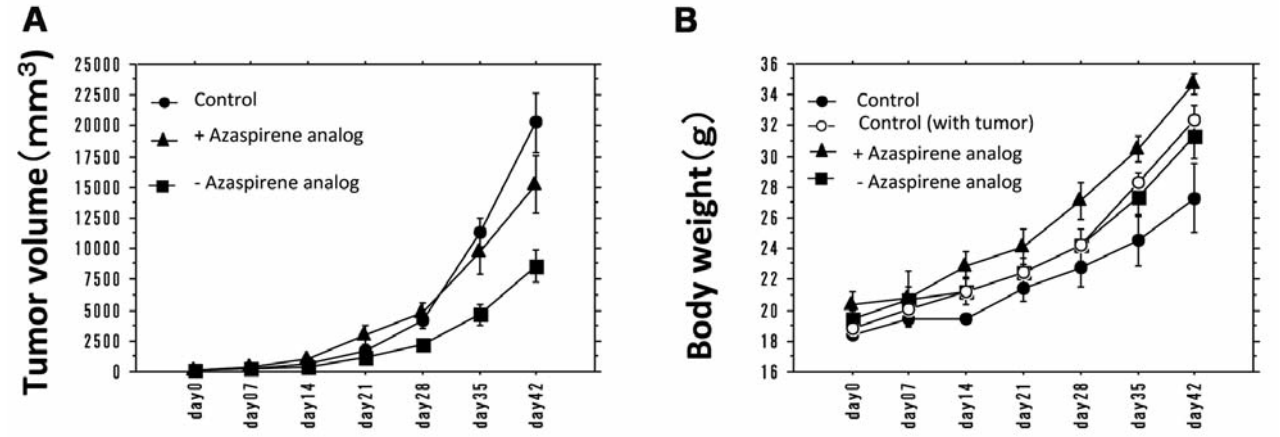

Figure 4. *Both the (+)-azaspirene and (−)-azaspirene analogs significantly suppressed the growth of FU-MMT-3 xenografts compared to the control ($p<0.05$) (A). However, there was no difference in the bodyweight loss following treatment with the (+)-analog, or (−)-analog compared to the control group (B).*

length, the (+) analog at 50 or 100 μg/ml, and the (−) analog at 50 μg/ml, significantly reduced tube formation *in vitro*, compared to thalidomide, an anti-angiogenic agent ($p<0.05$: Figure 3H and I). The inhibitory concentration $(IC)_{50}$ values of both of these analogs was determined to be 10 μg/ml (31.5 μM).

Suppression of tumor growth in vivo. Figures 4A and B show the weekly changes in the mean tumor volume and bodyweight, respectively, in mice bearing FU-MMT-3 xenografts. Both the (+)- and (−)-azaspirene analogs at 30.0 mg/kg significantly inhibited tumor growth compared

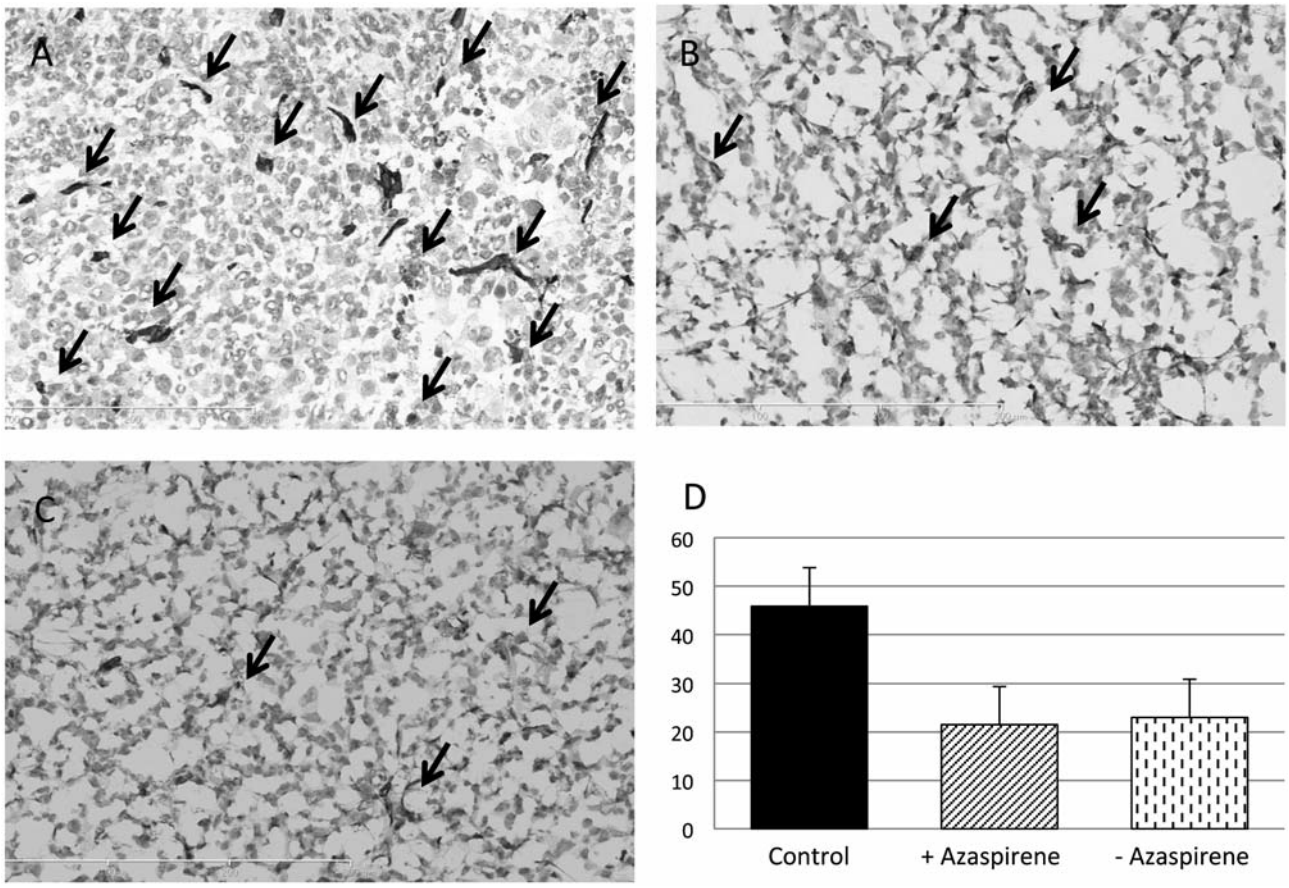

Figure 5. *Representative images of the tumor microvessels [A, control; B, (−)-azaspirene analog; C, (+)-azaspirene analog]. D: The microvessel density (MVD) was significantly lower in both treatment groups compared with the control group ($p<0.0001$).*

with the untreated group (control) ($p<0.05$) (Figure 4A). No differences were observed in the mean tumor volume between mice that received the (+)-analog and (−)-analog treatments. No obvious toxicity, including bodyweight loss, was observed in any mice treated with these analogs (Figure 4B).

MVD. The MVD determined by anti-CD31 immunostaining is shown in Figure 5. The MVD in the tumors was significantly lower in both analog-treatment groups than in the control group ($p<0.001$; Fig were 5). No differences were found in the MVD between mice that received the (+)-analog and (−)-analog treatments.

Discussion

UCS is a highly aggressive neoplasm with a poor prognosis due to unsatisfactory responses to current treatment modalities. No standard treatments exist for these tumors except for surgery during the early stages of disease. The five-year survival rates of patients with this tumor are reported to range between 18% and 39% (1-4). According to a PubMed analysis, there have been only a few clinical trials of treatment for this disease, and investigations into the expression of potential therapeutic targets have been limited (16, 22). Currently, UCSs are considered to be de-differentiated, or metaplastic endometrial adenocarcinomas (4). VEGF-A and angiopoietin-2 were shown to be key factors in the angiogenesis of various tumor types (6, 7). Uterine carcinosarcomas highly express both VEGF-A and angiopoietin-2, in comparison to poorly-differentiated endometrial carcinoma. The high invasiveness and high metastatic potential of this tumor might be associated with its high angiogenic activities (4, 7, 8). Therefore, anti-angiogenic approaches may represent an important therapeutic strategy for this tumor (9-15), and thalidomide was recently evaluated as an anti-angiogenic agent for patients with refractory UCS in a phase II clinical trial by the Gynecologic Oncology Group (16). This clinical trial provided evidence to support further evaluation of more active anti-angiogenic agents for UCS.

There have been only a few research studies, including our own, in which the investigators were able to establish and fully characterize cell strains of this uncommon bi-phasic tumor. Our previous studies showed that the growth and angiogenesis of human UCS cell lines were suppressed by TNP-470 (9-13, 15). TNP-470 is a low-molecular-weight synthetic analog of fumagillin, a natural compound secreted by *Aspergillus fumigatus*, which is known to be a broad-spectrum angiogenesis inhibitor (9). This agent suppressed VEGF production in UCS tumor cells (9, 13). Therefore, an anti-angiogenic strategy may be a useful approach for UCS, which was supported by the recent clinical trial using thalidomide (16).

Azaspirene, isolated from the *Neosartorya sp*. fungi, inhibits the migration of HUVECs induced by VEGF, as did its first reported analog (17, 18). Their subsequent *in vitro* experiments indicated that azaspirene suppressed RAF1 activation induced by VEGF without affecting the activation of VEGFR2. Additionally, azaspirene preferentially inhibited the growth of HUVECs, without affecting that of non-vascular endothelial cells (NIH3T3, HeLa, MSS31 and MCF-7) (19). We designed and synthesized new azaspirene analogs, replacing a hexadienyl side-chain of the natural compound with a shorter ethyl group. The *in vitro* anti-angiogenic activities of these compounds against HUVECs were previously reported, and both (+)- and (−)-azaspirene analogs functioned almost equally to inhibit cell migration (IC_{50}=31.5 μM each) without inducing significant cell damages (20). In the present study, the anti-angiogenic effects of these analogs were evaluated for the first time *in vitro* and *in vivo* against a highly angiogenic cancer model established from the tissue obtained from human UCS (21). Our present findings indicate that the newly synthesized azaspirene analogs inhibited tumor angiogenesis and the growth of FU-MMT-3 cells and tumors without any remarkable toxicity. Thus, these analogs may represent an effective treatment for UCS, possibly acting via their potent anti-angiogenic effects.

Acknowledgements

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Disclosure Statement

The Authors have no conflict of interest in regard to this study.

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