Clear cell sarcoma (CCS) is an aggressive soft tissue sarcoma and highly resistant to conventional chemotherapy and radiation therapy. This devastating disease is defined by EWSR1-ATF1 fusion gene resulting from chromosomal translocation t(12;22)(q13;q12) and characterized by melanocytic differentiation. A marine-derived antineoplastic agent, trabectedin, inhibits the growth of myxoid liposarcoma and Ewing sarcoma by causing adipogenic differentiation and neural differentiation, respectively. In this study, we examined the antitumor effects and mechanism of action of trabectedin on human clear cell sarcoma cell lines. We showed that trabectedin decreased the cell proliferation of five clear cell sarcoma cell lines in a dose-dependent manner in vitro and reduced tumor growth of two mouse xenograft models. Flow cytometry and immunoblot analyses in vitro and immunohistochemical analysis in vivo revealed that trabectedin-induced G2/M cell cycle arrest and apoptosis. Furthermore, trabectedin increased the expression of melanocytic differentiation markers along with downregulation of ERK activity in vitro and the rate of melanin-positive cells in vivo. These results suggest that trabectedin has potent antitumor activity against clear cell sarcoma cells by inducing cell cycle arrest, apoptosis, and, in part, by promoting melanocytic differentiation through inactivation of ERK signaling. Our present study indicates that trabectedin is a promising differentiation-inducing agent for clear cell sarcoma.
neural crest cell with the potential for melanocytic differentiation and melanin synthesis [15–17].

Trabectedin (ET-743; Yondelis®) is a marine-derived natural product originally isolated from the Caribbean Sea squirt, Ecteinascidia turbinata and currently prepared synthetically [18]. It has potent antitumor activity in vitro and in vivo against ovarian cancer, breast cancer, non-small-cell lung cancer, melanoma, and soft tissue sarcoma (STS) [19–23]. Clinical trials revealed that trabectedin is particularly active in sarcomas bearing translocations, namely mucoid liposarcoma, Ewing sarcoma, and synovial sarcoma [24, 25]. The mechanisms of action of trabectedin seem to be unique and still poorly understood. It has been reported that trabectedin interferes with several transcription factors, DNA-binding proteins, and DNA repair pathways, likely differing from other DNA-interacting agents [26]. It has also been demonstrated that trabectedin shows antitumor activity by inducing adipogenic differentiation in liposarcoma [27] and neural differentiation in Ewing sarcoma [28]. However, the activity and potential mode of action of trabectedin against CCS is still unknown.

In this study, we examined the antitumor effects of trabectedin on human CCS cell lines in vitro and in vivo. Furthermore, we investigated the mechanisms of action of trabectedin, focusing on the melanocytic differentiation pathway.

Materials and Methods

Cell culture

The human CCS cell lines Hewga-CCS [29] and Senju-CCS were kindly provided by Dr. Moritake (Miyazaki University, Miyazaki, Japan) [30] and Dr. Nakamura (Japanese Foundation for Cancer Research, Tokyo, Japan), respectively [31]. SU-CCS1 was purchased from American Type Culture Collection. We performed reverse transcription–PCR (RT–PCR) and assessed the type of EWSR1-ATF1 chimeric transcripts in all CCS cell lines according to the EWSR1-ATF1 fusion types proposed by Panagopoulos, et al. [11] (Data S1, Fig. S1). All the cell lines were cultured in Dulbecco’s Modified Eagle Medium (Life Technologies, Carlsbad, CA, USA) containing 10% FBS (Sigma-Aldrich, St. Louis, MO, USA) at 37°C with 5% CO2 and 100% humidity.

Quantitative real-time PCR analysis

Real-time PCR was performed, using a Step One Plus Real-Time PCR System (Life Technologies) and Fast SYBR Green Master Mix (Life Technologies), in which each cDNA sample was evaluated and expression values were normalized to GAPDH. PCR primers (forward and reverse, respectively) were as follows: GAPDH (5’-TGCACCACCAACTGCTTAGAC-3’ and 5’-ACTGTGGTCATGAGTCTCTTCCA-3’) and MITF (5’-GAGGCAGTGGTTTGGCCTT-3’ and 5’-AATTTCTGACCACCCGGAATC-3’).

Reagents and antibodies

Trabectedin was gifted by TAIHO Pharmaceutical, Co. LTD (Tsukuba, Japan). Methotrexate (MTX) and doxorubicin (DOX) were purchased by Sigma-Aldrich (St. Louis, MO, USA) and Wako Pure Chemical Industries (Osaka, Japan), respectively. The ERK inhibitor, SCH772984, was purchased from Cayman Chemical (Ann Arbor, MI, USA). The drugs were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. For in vitro examinations, the drugs were diluted in DMEM to the desired concentration. For in vivo experiments, trabectedin was further diluted with phosphate buffer pH 4.0 immediately before administration.

The following primary antibodies were used: anti-cleaved caspase-3 and anti-beta-actin (Cell Signaling Technology, Inc., Danvers, MA, USA); anti-microphthalmia-associated transcription factor (MITF) and anti-activating transcription factor 1 (ATF1) (Abcam, Cambridge, UK); anti-PCNA, anti-tyrosinase (TYR), and anti-tyrosinase-related protein 2 (TRP2) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Horseradish peroxidase (HRP)-conjugated secondary antibody was obtained from GE Healthcare Life Sciences (Pittsburg, PA, USA).

Immunoblot analysis

For the lysate preparation, cells were first washed with phosphate-buffered saline and lysed in radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Waltham, MA, USA). Protein concentrations were measured using the bicinchoninic acid method (Thermo Scientific). The cell lysates were separated on 4–12% Bis-Tris gels (Life Technologies) and transferred to polyvinylidene difluoride membranes (Nippon Genetics, Tokyo, Japan). The membranes were incubated in 5% skim milk in Tris-buffered saline with Tween 20 (TBS-T) at room temperature. Blocked membranes were incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies at room temperature for 1 h. After washing in TBS-T, immunoreactive bands were visualized using enhanced chemiluminescence (GE Healthcare Life Sciences).

Cell proliferation assay

CCS cells were plated into 96-well plates at a density of 2 × 103 and incubated with trabectedin or the vehicle for
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72 h. Cell proliferation rate was measured using the premix WST-1 cell proliferation assay system (Takara Bio, Inc., Otsu, Japan). Absorbance was measured with a microplate reader and relative cell proliferation rate was calculated.

**Flow cytometry**

5 × 10⁵ CCS cells per dish were plated in 10 cm culture dishes and grown overnight, followed by treatment with trabectedin or vehicle solution for 48 h. The cells were harvested and stained with propidium iodide (PI) solution (25 μg/mL PI, 0.03% NP-40, 0.02 mg/mL RNase A, 0.1% sodium citrate) for 30 min at room temperature. For cell cycle analysis, we used a BD FACSCount II flow cytometer (Becton Dickinson (BD) Biosciences, San Jose, CA, USA).

**In vivo mouse xenograft model**

Five-week-old male BALB/c nu/nu mice (SLC, Shizuoka, Japan) were housed at the Institute of Experimental Animal Sciences, Osaka University Medical School, in accordance with a guideline approved by the Institutional Animal Care and Use Committee of the Osaka University Graduate School of Medicine. For the xenograft tumor growth assay, 1 × 10⁷ CCS cells were injected subcutaneously into the left side of the back. Tumor volumes were measured twice per week with calipers and calculated by the formula (A × B²)/2, where A was the longest diameter and B was the shortest diameter of the tumor. When the average diameter of tumors reached 5 mm, therapy was initiated. The mice were randomized and divided into trabectedin-treated or vehicle-treated groups. Trabectedin was intravenously injected through the tail vein at the dose of 0.15 mg/kg, every 7 days for three cycles. After 18 days of treatment, all mice were euthanized and the tumor weight was measured. The tumors were resected for histological analysis. Body weight of each mouse was measured once a week for the evaluation of side effects.

**Statistical analysis**

All data are expressed as means ± SDs. We used Student’s t-test for biological assays and Mann–Whitney’s U test for animal experiments to determine significant differences. Values of \( P < 0.05 \) were considered significant.

**Results**

**Trabectedin suppressed the growth of CCS cell lines in vitro**

Cell proliferation assays were performed to examine the antitumor activity of trabectedin against 5 CCS cell lines in vitro. Trabectedin suppressed proliferation of all CCS cell lines in a dose-dependent manner (Fig. 1). The 50% inhibitory concentration (IC₅₀) values of trabectedin were as follows: Hewga-CCS: 0.48 nmol/L, Senju-CCS: 0.87 nmol/L, SU-CCS1: 0.30 nmol/L, MP-CCS-SY: 0.47 nmol/L, KAS: 0.42 nmol/L (Fig. 1).

**Trabectedin induced G2/M cell-cycle arrest and apoptosis in CCS cell lines**

Flow cytometry analyses showed that 1 nmol/L trabectedin induced G2/M cell-cycle arrest, and 10 nmol/L trabectedin increased the number of cells in sub-G1 phase in Hewga-CCS and KAS cells (Fig. 2A). Furthermore, cleavage of caspase-3 was enhanced dose-dependently after trabectedin exposure by immunoblot (Fig. 2B). These findings indicated that trabectedin inhibited the cell proliferation in

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**Histological analysis**

Specimens of xenograft tumors were fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned in 4 μm thicknesses. Paraffin-embedded sections were deparaffinized and dehydrated. After antigen retrieval (boiled at 95°C for 20 min in a 10 mmol/L citrate buffer), endogenous peroxidase activity was blocked for 10 min with methanol containing 3% H₂O₂. The sections were reacted for 1 h with TBS containing 2% bovine serum albumin at room temperature. The sections were incubated with designated antibodies at 4°C overnight, followed by 1 h incubation with secondary antibodies and stained with 3,3′-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark). Finally, the sections were counterstained using hematoxylin. To detect melanin, paraffin sections were stained using Fontana–Masson stain as described previously [32].

Figure 1. Trabectedin inhibits CCS cell growth in vitro. The CCS cells were incubated with various concentrations of trabectedin for 72 h. Cell proliferation was determined by WST-1 assay. The IC₅₀ values were calculated and shown in the table. Bars: SD.
all CCS cell lines by inducing G2/M cell cycle arrest and apoptosis in vitro.

The expression of melanocytic differentiation markers was upregulated by treatment with trabectedin in vitro

We examined the expression of melanocytic differentiation markers such as MITF, TYR, and TRP2 in Hewga-CCS and KAS cells treated with trabectedin, MTX, and DOX by immunoblot. It was reported that MTX increased MITF expression and induced melanocytic differentiation in melanoma cells [33]. DOX is a conventional chemotherapeutic drug and was used as a control. All three drugs induced cleavage of caspase-3 in both cell lines (Fig. 3). Interestingly, prior to cleavage of caspase-3, expression of melanocytic differentiation markers was upregulated by treatment with trabectedin and MTX (Fig. 3B).
Treatment with DOX did not upregulate these markers (Fig. 3C). These results suggested that trabectedin might inhibit the growth of CCS cells by inducing melanocytic differentiation and apoptosis.

**Trabectedin caused inactivation of ERK and increased MITF protein level**

To elucidate the reason why trabectedin increased MITF protein level, we examined whether the drug induced MITF gene transcription. Unexpectedly, trabectedin did not upregulate the mRNA level of MITF in Hewga-CCS and KAS cells (Fig. 4A). Further, the expression of the EWSR1-ATF1 fusion protein was not affected by the treatment of trabectedin in both cells (Fig 4B). These results suggested that the upregulation of MITF protein levels by trabectedin treatment was neither attributable to the transcriptional activation of MITF nor mediated by the interaction between trabectedin and EWSR1-ATF1.

Previous studies showed that the activation of ERK was followed by MITF ubiquitination and degradation [34, 35], thus we evaluated the phosphorylation status of ERK in response to trabectedin treatment. Consistent with those studies, the reduction in ERK phosphorylation via
trabectedin exposure was accompanied by the upregulation of MITF (Fig. 4B). Since trabectedin appeared to induce melanocytic differentiation by deregulating the activation of ERK, we investigated whether its similar effect on CCS was promoted by the treatment of SCH772984, a specific inhibitor of ERK signaling. Similar to trabectedin, SCH772984 suppressed ERK activation and upregulated MITF protein level in Hewga-CCS and KAS cells (Fig. 4C). These findings suggested that the inhibition of the ERK signaling might play a pivotal role in trabectedin-induced melanocytic differentiation.

### Trabectedin suppressed the growth of CCS xenograft tumors

We evaluated the antitumor effects of trabectedin against Hewga-CCS and KAS xenograft tumors. Trabectedin at a dose of 0.15 mg/kg was intravenously injected once every 7 days for three cycles. Administration of trabectedin notably suppressed the growth of CCS xenograft tumors compared to the vehicle control (Fig. 5A). Body weight loss of the mice was not observed and drug treatment was well-tolerated with no toxicity in this study. Consistent with the in vitro findings, trabectedin suppressed the growth of CCS xenograft tumors.

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**Figure 4.** Trabectedin did not enhance the mRNA expression of MITF. Moreover, the drug did not affect the expression of EWSR1-ATF1 fusion protein. Both trabectedin and a selective ERK inhibitor, SCH772984, decreased the ERK signaling and increased the protein level of MITF. Hewga-CCS and KAS were treated with 5 nmol/L trabectedin or 100 nmol/L SCH772984 for 0–24 h. (A) Total RNA was extracted, and MITF transcription was quantified by qRT-PCR. Values mean ± SD. (B, C) The protein expressions were assessed by Immunoblot analyses.

**Figure 5.** Trabectedin abrogated the growth of Hewga-CCS and KAS xenograft tumors. (A) Hewga-CCS (n = 8/group each) and KAS (n = 7/group each) xenograft tumors were treated with 0.15 mg/kg trabectedin intravenously injected. (B) The rate of PCNA-positive tumor cells, cleaved caspase-3-positive cells, and melanin-positive cells were counted in Hewga-CCS xenograft tumors. *P < 0.01.
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A) Hewga-CCS

- Tumor Volume (mm³)

Day 0  Day 4  Day 7  Day 11  Day 14  Day 18

B) KAS

- Tumor Volume (mm³)

Day 0  Day 4  Day 7  Day 11  Day 14  Day 18

B) Immunohistochemical analysis

- PCNA
- Cleaved caspase3
- Melanin

Vehicle

Trabectedin

Scale Bars :100 μm

- PCNA positive rate (%)

Vehicle  Trabectedin

- Cleaved caspase3 positive rate (%)

Vehicle  Trabectedin

- Melanin positive rate (%)

Vehicle  Trabectedin
data, a decrease in the rate of PCNA-positive tumor cells and an increase in that of cleaved caspase-3-positive cells and melanin-positive cells were observed in trabectedin-treated CCS xenograft tumors (Fig. 5B). These data suggested that trabectedin showed antineoplastic activity against CCS cells, and supported the hypothesis that one of the mechanisms of action of trabectedin might be associated with promotion of melanocytic differentiation in addition to the induction of cell cycle arrest and apoptosis.

Discussion

Prior publications suggest that maintaining an undifferentiated state is a crucial aspect of cancer tumorigenesis since cancer cells display suspended differentiation properties compared to normal cells [36, 37]. Extended from those studies, several investigators presented an idea that the lineage differentiation block associated with both fusion protein expression and sarcoma-associated genetic abnormalities played a critical role in translocation-based sarcomagenesis [38–40]. For instance, Tirode, et al. demonstrated that EWSR1-FLI1 fusion protein blocked terminal differentiation of mesenchymal stem cells, leading to Ewing sarcoma tumorigenesis [38]. Similarly, Charytonowicz, et al. reported that PAX3/PAX7-FOXO1 translocation promoted the differentiation arrest in the myogenic lineage, resulting in alveolar rhabdomyosarcoma sarcomagenesis [39]. These results suggest that suspension of undifferentiated state is required for tumorigenic process and targeting impaired terminal differentiation could be a novel therapeutic strategy for translocation-based sarcomas.

Differentiation therapies aim to force the cancer cell to resume the process of maturation and tend to have less toxicity than conventional cancer treatments [41]. Many approaches for differentiation therapy in cancer using histone deacetylase inhibitors [42], retinoids [43], or peroxisome proliferator-activated receptors γ agonists [44] exhibited encouraging results in both in vitro and in vivo experiments. Among them, the highly successful clinical application of differentiation therapy was all-trans-retinoic acid-based therapy against acute promyelocytic leukemia [45].

CCS is characterized by a chromosomal translocation, t(12;22)(q13;q12), that leads to the fusion of EWSR1 gene with a CREB-family transcription factor gene (ATF1, or more rarely CREB1) [11–14]. These translocations provided a means of defining CCS and distinguishing it from malignant melanoma. Recent studies showed that CCS was a neural crest-derived malignancy as malignant melanoma [15–17]. Several lines of evidence indicated that trabectedin was more effective against translocation-related sarcoma, such as myxoid liposarcoma, Ewing sarcoma and synovial sarcoma [24, 25]. It was hypothesized that the greater sensitivity of these sarcomas was related to the ability of trabectedin to interact with the fusion gene product. Beyond expectations, we found that trabectedin did not influence the expression of EWSR1-ATF1 protein.

This study demonstrated that treatment of CCS with trabectedin suppressed cell proliferation, increased the number of cells in G2/M and sub-G1 phase in vitro, and induced cleavage of caspase-3 both in vitro and in vivo. Previous studies revealed that trabectedin exerted differentiation inducing and antitumor effects for a subset of translocation-related sarcomas, including myxoid liposarcoma [27] and Ewing sarcoma [28]. In this study, we also noticed that trabectedin treatment upregulated the expression of melanocytic differentiation markers including MITF in vitro and melanin-positive cells in vivo, even though it did not enhance the transcriptional activity for MITF. Intriguingly, recent works suggested that MITF protein levels were regulated by ERK-induced ubiquitination and degradation in melanoma cells [34, 35]. In agreement with this, we noted that the reduction in ERK signaling was associated with the increase in MITF protein levels by the treatment with trabectedin or an ERK inhibitor. These findings suggested that trabectedin might induce melanocytic differentiation on CCS as a result of the reduction in ERK activity, aside from the interaction with the EWSR1-ATF1 fusion gene product.

Our findings strongly indicate that trabectedin exerts antitumor effects via induction of G2/M cell cycle arrest, apoptosis, and, in part, the acceleration of melanocytic differentiation against CCS cell lines. Taken together, we conclude that trabectedin should be a promising therapeutic option and might be a novel differentiation therapy agent for CCS.

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Conflict of Interest

We declare no conflicts of interest.
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Supporting Information

Additional supporting information may be found in the online version of this article:

**Data S1.** EWSR1-ATF1 cDNA was identified by PCR. PCR primers were as follows: EWSR1 forward primer 5'-TCCTACAGCCAAGCTCAAAGTC-3' and ATF1 reverse primer 5'-ACTCGGTTTTCCAGGCATTTCAC-3'.

**Figure S1.** RT–PCR with EWSR1 forward primer and ATF1 reverse primer that amplifies a 779-base pair (bp) PCR product in the type 1 EWSR1-ATF1 transcript, a 464-bp PCR product in type 2, and a 713-bp PCR product in type 3. The type 1 transcript of EWSR1-ATF1 was detected in Senju-CCS, SU-CCS1, and MP-CCS-SY cells. The type 2 and the type 3 transcripts of EWSR1-ATF1 were detected in Hewga-CCS and KAS cells, respectively.