Investigational New Drugs (2019) 37:818–827
https://doi.org/10.1007/s10637-018-0686-6

PRECLINICAL STUDIES

Lurbinectedin (PM01183), a selective inhibitor of active transcription, effectively eliminates both cancer cells and cancer stem cells in preclinical models of uterine cervical cancer

Eriko Yokoi[1] · Seiji Mabuchi[1] · Kotaro Shimura[1] · Naoko Komura[1] · Katsumi Kozasa[1] · Hiromasa Kuroda[1] · Ryoko Takahashi[1] · Tomoyuki Sasano[1] · Mahiru Kawano[1] · Yuri Matsumoto[1] · Michiko Kodama[1] · Kae Hashimoto[1] · Kenjiro Sawada[1] · Tadashi Kimura[1]

Received: 20 September 2018 / Accepted: 16 October 2018 / Published online: 30 October 2018

Summary

Objective The objective of this study was to evaluate the antitumor effects of lurbinectedin on cervical cancer with a special focus on its effects on cancer stem cells (CSCs). *Methods* Using two cervical cell lines (ME180 and CaSki cells), the antitumor effects of lurbinectedin were assessed in vitro using the MTS assay and colony formation assay. The growth inhibitory effects of paclitaxel and cisplatin were also evaluated as controls. By employing ALDH1 activity as a marker of CSCs, the antitumor effects of lurbinectedin on cervical CSCs and non-CSCs were individually evaluated. Finally, we investigated the mechanisms by which lurbinectedin eliminated cervical CSCs. *Results* Lurbinectedin had significant antitumor activity toward cervical cancer cells at low nanomolar concentrations in vitro. Mouse xenografts of cervical cancer revealed that lurbinectedin significantly inhibits tumor growth. The growth-inhibitory effect of lurbinectedin was greater than that of cisplatin and paclitaxel. ALDH-high CSCs were observed in both cervical cancer cell lines (4.4% and 2.4% in ME180 and CaSki cells, respectively). Lurbinectedin downregulated stem cell-related gene expression (Oct4, Nanog, and SOX2), inhibited HDAC1 activity, and effectively eliminated ALDH-high CSCs. *Conclusions* Lurbinectedin is highly effective on uterine cervical cancer because it eliminates CSCs, and lurbinectedin is a promising agent to overcome platinum resistance in cervical cancer.

Keywords Chemotherapy · Cervical cancer · Cancer stem cells · Lurbinectedin

Introduction

Cervical cancer is the second most common female-specific cancer after breast cancer; an estimated 527,600 new cervical cancer cases and 265,700 deaths were reported worldwide in 2012 [1]. Cervical cancer is the second leading cause of cancer death in women aged 20 to 39 years [2]. Although most cervical cancer can be cured with treatments based on surgery and concurrent chemoradiotherapy, it is estimated that approximately 30–35% of patients with invasive cervical cancer will develop recurrent disease after primary treatment [1].

Chemotherapy has been the main treatment for patients with recurrent or advanced cervical cancer, except for those who are amendable to curative treatments with surgery or radiotherapy. On the basis of phase III trials, platinum-based combination regimens such as cisplatin plus paclitaxel and carboplatin plus paclitaxel with or without bevacizumab have become standard regimens for patients with recurrent or advanced cervical cancer [3, 4]. However, with rare exceptions, recurrent or advanced cervical cancer patients have a painful prognosis, with a reported 1-year survival rate of roughly 15–20% [3, 4]. Platinum resistance is the main reason for the poor prognosis of recurrent or advanced cervical cancer, especially

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10637-018-0686-6) contains supplementary material, which is available to authorized users.

✉ Seiji Mabuchi
smabuchi@gyne.med.osaka-u.ac.jp

[1] Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

those previously treated with platinum-based chemotherapy or radiosensitizing cisplatin [4, 5].

A possible strategy to overcome platinum-resistance is targeting cancer stem-like cells. Cancer stem-like cells (CSCs) are a small rare fraction (in most solid tumors <1%) of tumor cells [6]. Because of their unlimited capacity for self-renewal, differentiation, and tumorigenesis, CSCs contribute to tumor initiation, progression, metastasis, and therapeutic resistance. Experimental evidence supporting the existence of CSCs was first reported in 1997 [7]. Since then, an increasing number of investigators have identified CSCs in various human malignancies including uterine cervical cancer [8–10]. Because of the nature of CSCs, treatment failures after platinum-based chemotherapy may be explained, at least in part, by the CSCs hypothesis. Thus, the development of novel agents that can effectively eliminate CSCs is needed.

Another strategy is to develop a non-platinum regimen for recurrent, persistent, or advanced cervical cancer. Lurbinectedin (PM01183) is a novel synthetic alkaloid derived from trabectedin. Lurbinectedin binds covalently to the minor groove of DNA, which leads to G_2-M cell-cycle arrest and ultimately apoptosis. Replacing the tetrahydroisoquinoline moiety in trabectedin with a tetrahydro β-carboline in lurbinectedin results in reduced toxicity because of structural differences, which allows the development of treatment regimens with higher doses. Consequently, lurbinectedin has increased antitumor activity compared with trabectedin [11]. Because of these encouraging results from preclinical studies [12, 13] and phase I-II clinical trials [14], the activity of lurbinectedin is currently being evaluated in a phase III study involving patients with platinum-resistant ovarian cancer [15] or platinum-resistant small-cell lung cancer [15]. However, the anti-tumor effects of lurbinectedin on uterine cervical cancer have never been investigated.

In this preclinical study, we investigated the therapeutic efficacy of lurbinectedin on uterine cervical cancer with a special focus on its effects on both CSCs and non-CSCs.

Materials and methods

Reagents, antibodies, and drug preparation

PM01183 (lurbinectedin) was obtained from PharmaMar (Madrid, Spain). Cisplatin and paclitaxel were purchased from Sigma (St Louis, MO, USA). Lurbinectedin was prepared as a 1–10 μmol/L stock solution in dimethyl sulfoxide (DMSO). Cisplatin and paclitaxel were dissolved in DMSO to final concentrations of 100 and 1 mmol/L, respectively. Enhanced chemiluminescence western blotting detection reagents were purchased from Perkin Elmer (Boston, MA, USA). Antibodies recognizing β-actin and anti-rabbit and anti-mouse secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies recognizing Histone deacetylase 1 (HDAC1), p53, and anti-goat secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell lines and cell culture

The human cervical cancer cell lines ME180 and CaSki were purchased from the American Type Culture Collection. The cell lines were passaged soon after they were received from the cell bank and stored in liquid nitrogen. Thawed cells were used for experiments without further authentication. The human ovarian CCC cell line RMG1 was kindly provided by Dr. H. Itamochi (Tottori University, Tottori, Japan). RMG1 was extensively characterized in previous studies [16]. We authenticated these cell lines in our laboratory based on morphological observations. No further cell line authentication was conducted by the authors. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All cell lines were maintained in a humidified incubator at 37 °C in 5% CO2.

Cell proliferation assay

The MTS assay was used to analyze the effects of each drug. Cells were plated in 96-well plates and exposed to the drugs at different concentrations. After a 72-h incubation, the number of surviving cells was assessed by determining the A_{490nm} of the dissolved formazan product after the addition of MTS for 2 h according to the manufacturer's instructions (Promega). Cell viability was calculated as follows: $A_{exp\ group}/A_{control} \times 100$. The experiments were repeated at least three times, and representative results are shown.

Aldefluor assay

The Aldefluor Assay Kit (Stem Cell Technologies, Vancouver, Canada) was used to determine the percentage of tumor cells expressing high levels of ALDH (ALDH-high cells) according to the manufacturer's instructions. Briefly, 1×10^6 cells were incubated with the Aldefluor substrate for 45 min at 37 °C with or without the ALDH inhibitor diethylaminobenzaldehyde (DEAB). After incubation, ALDH-high cells were detected in the FITC channel by flow cytometry. Flow cytometric data were acquired on FACS Canto II flow cytometer and analyzed using FACSDiva software (BD Biosciences, San Jose, CA).

Colony formation assay

ALDH-high or ALDH low cells were sorted by FACS Aria II. Five-hundred cells were seeded per well in 6-well-plates 24 h before the addition of drugs. After 1–2 weeks, colonies were

fixed with methanol and stained with Giemsa stain solution as reported previously [17]. The colony was defined to consist of at least 50 cells. The experiments were repeated at least three times, and representative results are shown.

Quantitative real-time RT-PCR (qRT-PCR)

RNA was extracted from cells using Trizol (Life Technologies, Grand Island, NY). The resultant total RNA (1 μ g) was used to synthesize cDNA with ReverTraAce qPCR RT Master Mix (Toyobo, Osaka, Japan). The cDNA was amplified by qRT-PCR using the included TaqMan Gene Expression Master Mix and the specific TaqMan primer/probe assay designed for the investigated genes: SOX2 (Hs01053049_s1), NANOG (Hs02387400_g1), OCT4 (Hs03005111_g1), and GAPDH (Hs99999905_m1) (Applied Biosystems, Tokyo, Japan). The gene expression levels were normalized to the expression of the housekeeping gene GAPDH and were expressed as a fold change relative to the expression of untreated cells. Quantification was performed by the delta/delta Ct calculation method.

Western blot analysis

Cell lines were treated with the indicated concentrations of lurbinectedin or other anti-cancer agents, washed twice with ice cold phosphate-buffered saline (PBS), and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer. The protein concentrations of the cell lysates were determined using the Bio-Rad protein assay reagent. Equal amounts of protein were applied to 5–20% polyacrylamide gels, and then the electrophoresed proteins were transblotted onto nitrocellulose membranes. After the membranes were blocked, they were incubated with various primary antibodies. The immunoblots were visualized with horseradish peroxidase (HRP)-coupled immunoglobulins using the enhanced chemiluminescence western blotting system (PerkinElmer, CA, USA).

Subcutaneous xenograft model

All procedures involving animals and their care were approved by the animal care and usage committee of Osaka University (Osaka, Japan), in accordance with the relevant institutional and National Institutes of Health guidelines. Experiments were conducted to examine the effects of lurbinectedin on cervical cancer. Five- to 7-week-old nude mice ($n = 10$) had 1×10^7 ME180 cells in 150 μL of PBS s.c. injected into their right flanks. After one week of the inoculation procedure, the mice were randomly assigned into two treatment groups. The first group ($n = 5$ was i.v. administered PBS, and the second group (n = 5) was i.v. administered lurbinectedin (0.180 mg/kg) each week for 3 weeks. The dose of lurbinectedin (0.180 mg/kg) used was based on that employed in a previous preclinical study of ovarian cancer, in which it showed significant in vivo antitumor activity [18]. Caliper measurements of the longest perpendicular diameter of each tumor were obtained twice a week and used to estimate tumor volume according to the following formula: $V = L \times W \times D \times \pi / 6$, where V is the volume, L is the length, W is the width, and D is the depth.

Statistical analysis

Continuous data were compared between groups using a Student's t test or Tukey's honestly significant difference test. *P*-values <0.05 were considered significant. All analyses were performed with JMP software version 13.0 (SAS Institute Inc.).

Data availability All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Results

The in vitro growth inhibitory effects of lurbinectedin on cervical cancer cell lines

To examine the effects of lurbinectedin on the proliferation of cervical cancer cells, we conducted the MTS assay using two human cervical cancer cell lines (ME180 and CaSki). Lurbinectedin is highly effective on ovarian clear cell carcinoma cells [12], and an ovarian clear cell carcinoma cell line (RMG1) was used as a reference. As shown in Fig. 1a, treatment for 72 h with lurbinectedin inhibited the proliferation of ME180 and CaSki cells in a dose-dependent manner. The antiproliferative effects of lurbinectedin on cervical cancer cells were similar to its effects on RMG1 cells (Fig. 1a). Using these cell lines, we next compared the antitumor effects of lurbinectedin with those of paclitaxel and cisplatin (Fig. 1b), which are the key agents in the clinical treatment of recurrent, persistent, and advanced cervical cancer. The IC50 values obtained in each experiment are summarized in Table 1. Lurbinectedin had a significantly greater antitumor activity in these cell lines than other anticancer agents.

The in vitro growth-inhibitory effects of lurbinectedin on CSCs and non-CSCs

Because high ALDH activity was reported to be a marker of CSCs in various cancers [19, 20], we defined ALDH-high cells as CSCs. We first investigated the ALDH activity of ME180 and CaSki cells. Both cervical cancer cell lines contained ALDH-high cells: the frequency of ALDH-high

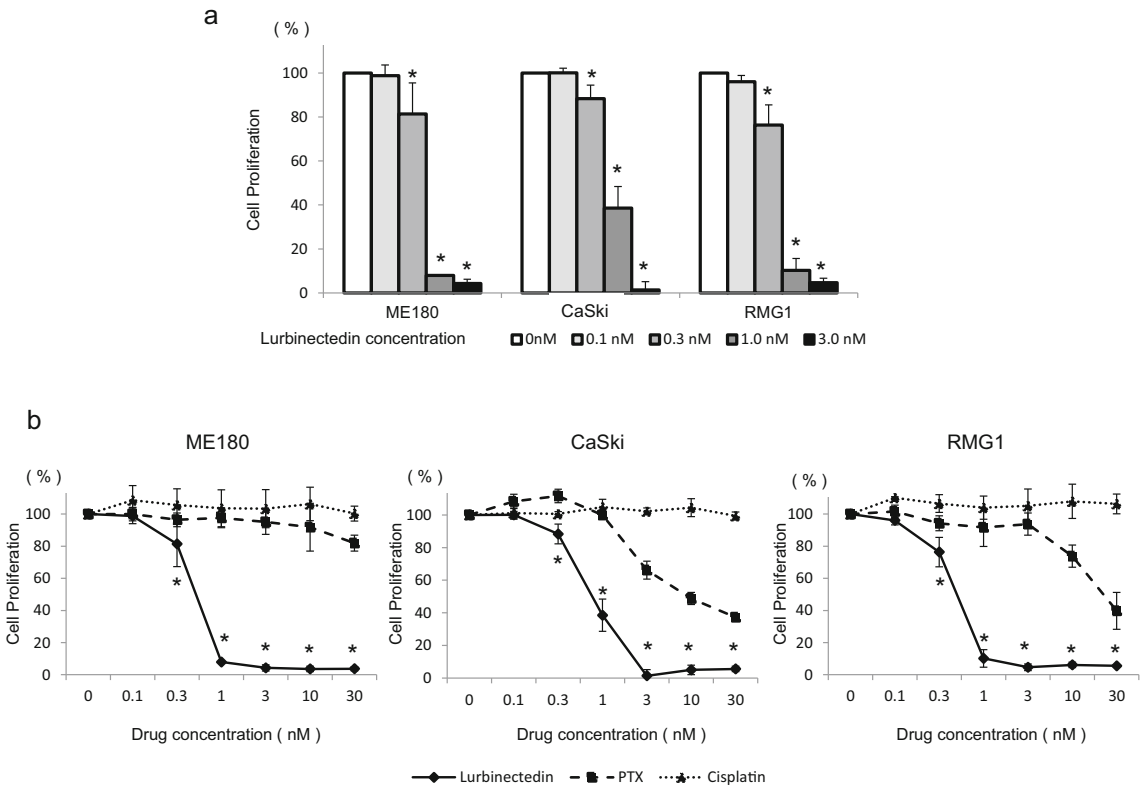

Fig. 1 In vitro growth-inhibitory effects of lurbinectedin. a Sensitivity of cervical cancer cells and ovarian cancer cells to lurbinectedin. Cervical cancer cells (ME180 and CaSki) and ovarian clear cell carcinoma cells (RMG1) were treated with the indicated concentrations of lurbinectedin in the presence of 10% FBS for 72 h. Cell proliferation was assessed using the MTS assay. Data points, mean values; bars, SD (*, significantly different from the control; $P < 0.05$). b. Comparison of the growth-inhibitory effects of three anti-cancer agents. Cells were treated with the indicated concentrations of paclitaxel, cisplatin, or lurbinectedin in the presence of 10% FBS for 72 h. Cell proliferation was assessed using the MTS assay. Data points, mean values; bars, SD (*, significantly different from paclitaxel; $P < 0.05$)

cells was 4.4% and 2.4% in ME180 and CaSki cells, respectively (Fig. S1).

To investigate the impact of ALDH activity on the therapeutic efficacy of lurbinectedin using ALDH-high (CSCs) and ALDH-low cells (non-CSCs), we performed the colony formation assay. Lurbinectedin treatment decreased the colony forming activity of both ALDH-high and ALDH-low cells in a dose-dependent manner (Fig. 2b). However, the colony forming activity of ME180 and CaSki cells after the treatment with lurbinectedin was significantly greater in ALDH-high cells than in ALDH-low cells. Similar results were obtained with RMG1 cells (Fig. S2). Collectively, these results indicate the involvement of ALDH activity in the sensitivity of lurbinectedin. To further demonstrate the chemoresistant nature of ALDH-high CSC cells, ALDH-high and ALDH-low cells were mixed together at various ratios in subsequent MTS assays and clear differential sensitivity to lurbinectedin was observed (Fig. 2c). The inhibitory effect of lurbinectedin was inversely associated with the proliferation of ALDH-high cells, indicating the relatively chemoresistant nature of ALDH-high cells when compared to ALDH-low cells. However, 1–3 nM lurbinectedin completely abolished the ability of cervical cancer cells to form colonies.

Table 1 IC50 values of anticancer drugs in human cervical cancer cell and ovarian cancer cell lines

Drugs		IC 50		
		ME180	CaSki	RMG1
Cisplatin	μM	3.44 (1.05)	48.17 (6.84)	40.96 (7.66)
Paclitaxel	nM	55.36 (3.51)	10.05 (2.06)	23.88 (4.21)
Lurbinectedin	nM	0.49 (0.07)	0.80 (0.12)	0.48 (0.07)

The IC50 values represent the mean of at least 3 independent experiments (SD)

We next compared the inhibitory effect of lurbinectedin on ALDH-high cells with those of paclitaxel and cisplatin using the colony formation assay. In this experiment, we employed three different concentrations: 1 nM, the clinical plasma concentration (PC) observed in patients, and the IC50. Lurbinectedin had a significantly greater inhibitory effect on the colony forming activity of ALDH-high ME180 and CaSki cells (Fig. 3). A similar effect of lurbinectedin was observed in RMG1 cells (Fig. S3). Lurbinectedin completely abolished the colony forming activity in all cell lines at the PC. Collectively,

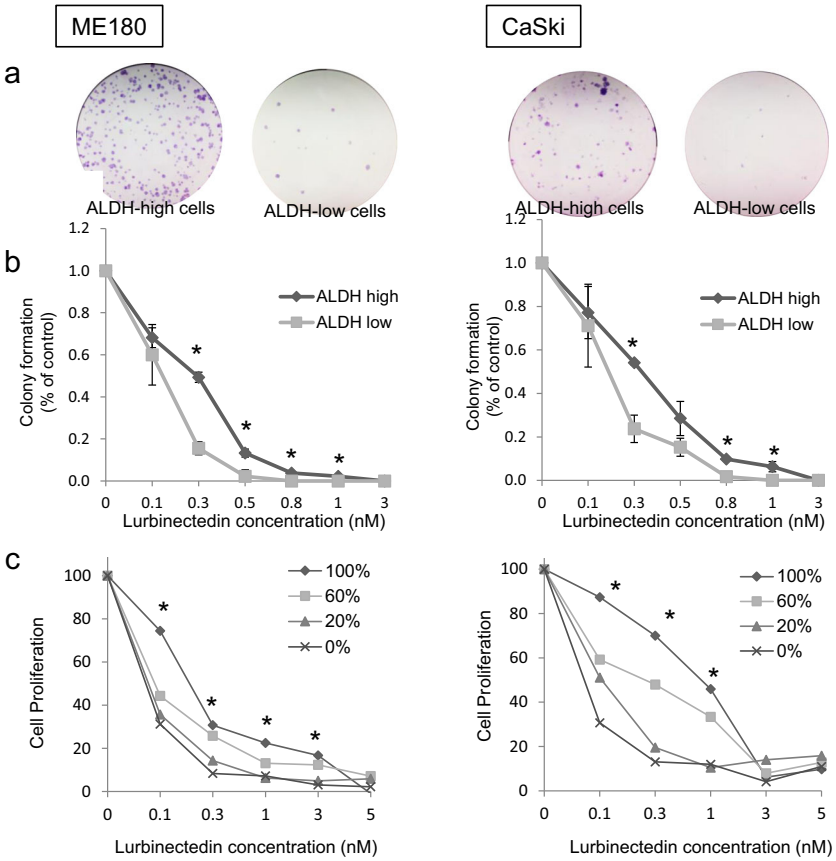

Fig. 2 The effects of lurbinectedin on ALDH-high and ALDH-low cervical cancer cells. ALDH-high and ALDH-low cervical cancer cells (ME180 and CaSki) were plated at low density in 6-well plates (500 cells/well). On the following day, cells were treated with the indicated concentrations of lurbinectedin in the presence of 10% FBS for 24 h. After culturing 1 week for ME180 and 2 weeks for CaSki, the colonies were stained and the colony number was counted. **a** Representative images of untreated cells. **b** Graphs depicting the colony forming ability: 0 nM was set to 1.0. Data points, mean values; bars, SD (*, significantly different from paclitaxel; $P < 0.05$). **c** ALDH-high and ALDH-low cells were mixed together at various ratios and treated with the indicated concentrations of lurbinectedin in the presence of 10% FBS for 72 h. Cell proliferation was assessed using the MTS assay. Data points, mean values; bars, SD (*, significantly different from paclitaxel; $P < 0.05$)

these results indicate that lurbinectedin effectively eliminated both CSCs and non-CSCs of uterine cervical cancer.

In vivo growth-inhibitory effects of lurbinectedin on cervical cancer

To examine the in vivo growth-inhibitory effects of lurbinectedin, we employed an s.c. xenograft model in which athymic mice were s.c. inoculated with ME180 cells. Overall, the drug treatment was well tolerated throughout the study and did not cause any apparent toxicities. As shown (Fig. 4a), no obvious weight loss was observed during the treatments. The mean ME180-derived tumor burden in mice treated with lurbinectedin was 600.0 mm^3 compared with 1131.0 mm^3 in the PBS-treated mice (Fig. 4b). Overall, treatment with lurbinectedin decreased the ME180-derived tumor burden by 47.0% compared with PBS. Next, we investigated the effect of lurbinectedin on the induction of CSCs in vivo. The tumor obtained from the mouse treated with lurbinectedin significantly reduced the frequency of ALDH-high ME180 cells in the tumor (Fig. 4c).

The mechanisms by which lurbinectedin eliminates CSCs

We investigated the mechanisms by which lurbinectedin eliminated CSCs. Because SOX2, Nanog, and Oct4 are master regulators for self-renewal and the maintenance of the stem cell population, we evaluated the effect of lurbinectedin on the expression of these genes. As shown in Fig. 5a, lurbinectedin significantly attenuated the expression of SOX2, Nanog, and Oct4. A similar effect of lurbinectedin was observed in RMG1 cells (Fig. S4a).

Recent studies have indicated that HDAC1-mediated p53 suppression is involved in the induction of CSCs [21], and HDAC inhibition induces growth arrest or apoptosis in CSCs [22–26]. Thus, we investigated the effect of lurbinectedin on the expression of HDAC1 and p53.

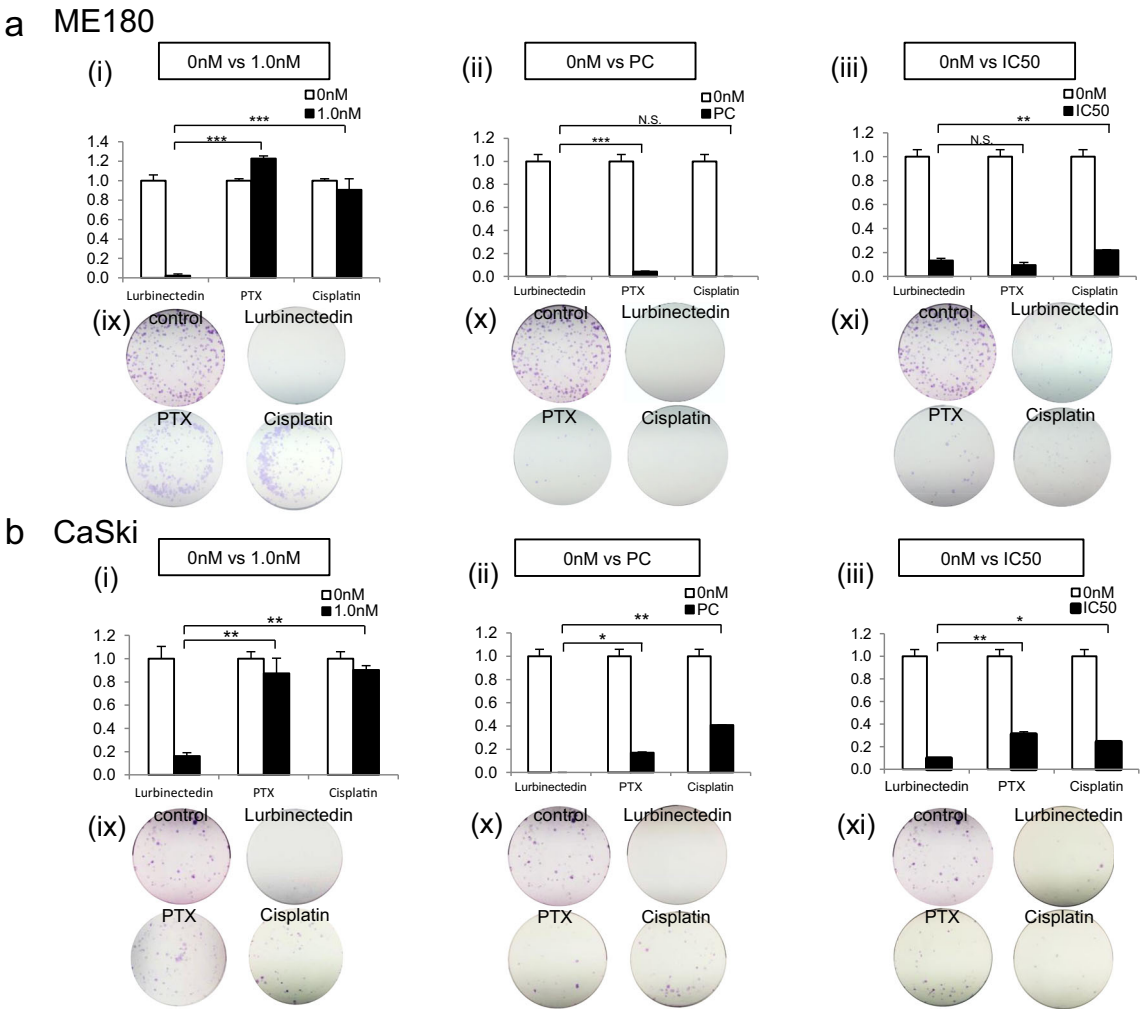

Fig. 3 The effects of various anti-cancer agents on ALDH-high cervical cancer cells. ALDH-high ME180 (**a**) and CaSki (**b**) cells were sorted by flow cytometry and plated at low density (500 cells/well). Cells were treated with the indicated concentrations of lurbinectedin, paclitaxel (PTX), or cisplatin for 24 h. The inhibitory effects of anti-cancer agents were assessed by a colony formation assay. Left: 1.0 nM concentration, center: plasma concentration, right: IC50 concentration. Upper panels: Graphs depicting the colony forming ability (i-iii). Lower panels: Representative images of colonies (ix-xi) where the number in untreated cells (0 nM) was set to 1.0. Plasma concentration (PC); Lurbinectedin, 10 nM, PTX, 120 nM, Cisplatin, 10 μM. Data points, mean values; bars, SD (*, significantly different from paclitaxel; $P < 0.05$). Tukey–Kramer's honestly significant difference test: $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$***

Treatment of ME180 and CaSki cells with lurbinectedin significantly attenuated the expression of HDAC1, whereas the expression of p53 was increased (Fig. 5b). Similar results were obtained from RMG1 cells (Fig. S4b).

Discussion

The lack of effective non-platinum chemotherapy is a major clinical problem in the management of recurrent or advanced cervical cancer. On the basis of preclinical and clinical studies [27, 28], the Gynecologic Oncology Group (GOG) conducted a phase III trial (GOG 240) evaluating the efficacy of bevacizumab and comparing the efficacy of non-platinum doublet topotecan plus paclitaxel with cisplatin plus paclitaxel in recurrent cervical cancer patients. Although 75% of the enrolled patients had been previously treated with platinum-based chemotherapy, topotecan plus paclitaxel was not superior to cisplatin plus paclitaxel in this trial [2]. Thus, an effective non-platinum doublet regimen needs to be developed.

In the current study, we found that lurbinectedin exhibited significant antitumor activity on cervical cancer at low nanomolar concentrations. The in vitro growth-inhibitory effect of lurbinectedin was greater than those of cisplatin or paclitaxel (Fig. 1). These findings indicate that lurbinectedin may have therapeutic efficacy as a single agent in cervical cancer patients.

An additional important finding of our study was the significant antitumor activity of lurbinectedin on CSCs

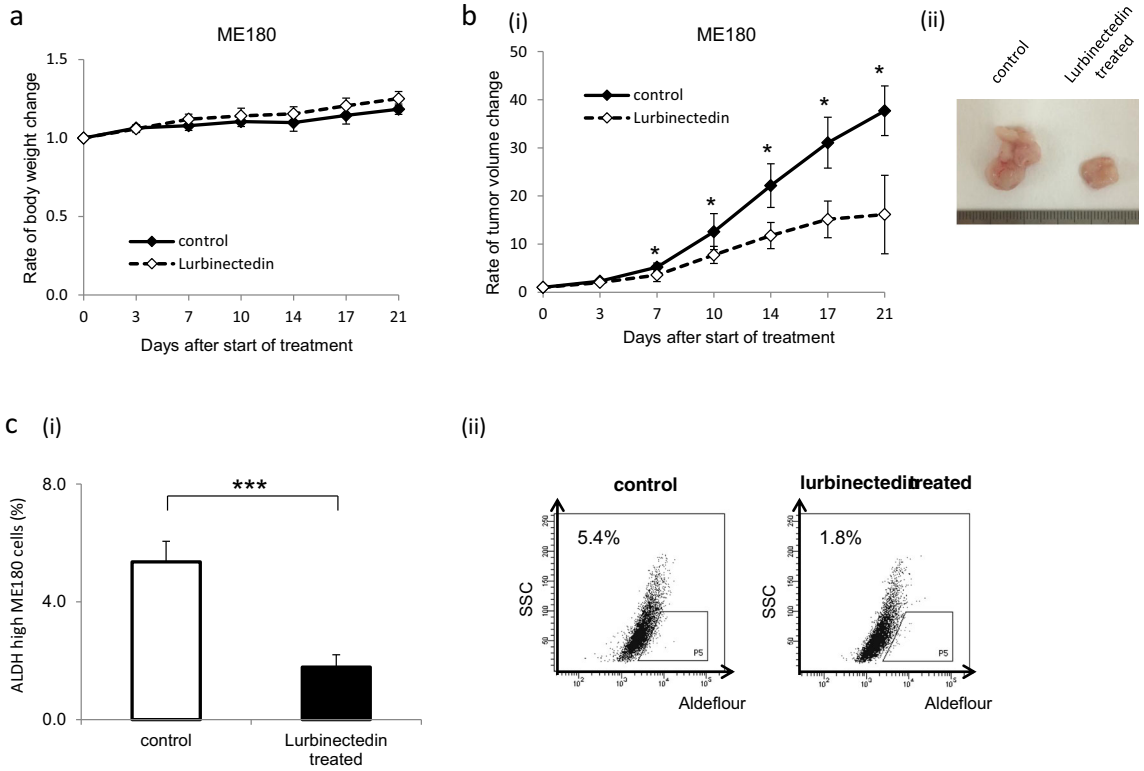

Fig. 4 In vivo inhibitory effects of lurbinectedin. Athymic nude mice were s.c. inoculated with ME180 cells. At one week after the inoculation procedure, the mice were i.v. administered PBS ($n = 5$) or 0.180 mg/kg lurbinectedin (n = 5) each week for 3 weeks. **a** Graphs depicting the changes in mouse body weight seen in each treatment group. The body weight on day 0 is set to 1.0 and the rate of increase was calculated. Data points, mean values; bars, SD (*, significantly different from the control; $P < 0.05$, two-sided Student's t test). **b** (i) Graphs depicting the changes in tumor volume observed in each treatment group. The tumor volume on day 0 is set to 1.0 and the rate of enlargement was calculated. Data points, mean values; bars, SD (*, significantly different from the control; $P < 0.05$, two-sided Student's t test). (ii) Appearance of s.c. tumors are shown. **c** Effect of lurbinectedin on CSCs in tumor. (i) The human EpCam+ mouse CD45- cells in the tumors were gated using flow cytometry and then the percentages of ALDH-high cells were assessed using the Aldefluor assay (Bars SD. n = 5, ***, significantly different from the control; $P < 0.001$, two-sided Student's t test). (ii) Representative dot plots were shown

(Fig. 2) because CSCs are involved in treatment resistance and can be a cause of treatment failure after chemotherapy or radiotherapy [8–10, 29]. To our knowledge, this is the first study to demonstrate that lurbinectedin eliminates CSCs. However, a similar result was also obtained from trabectedin studies: low nanomolar concentrations of trabectedin efficiently inhibited the growth of CSCs and induced apoptosis [30]. Thus, we think that the ability of lurbinectedin to eliminate CSCs is a common aspect of Ecteinascidin.

Lurbinectedin exhibited a significant growth-inhibitory effect on cervical CSCs and non-CSCs at concentrations of 0.5–5 nM (Figs. 1 and 2). According to a previous clinical study, the peak plasma concentration of lurbinectedin is 148.2–153.8 ng/mL (188.8–196.0 nM) [31], which is significantly higher than the concentrations of lurbinectedin used in the current study. Therefore, the use of lurbinectedin in the treatment of recurrent or advanced cervical cancer is reasonable and clinically achievable.

We investigated the mechanism by which lurbinectedin eliminated CSCs (Fig. 5a) and found that lurbinectedin suppressed the expression of SOX2, Nanog, and Oct4, which are master regulators for the self-renewal and maintenance of CSCs [32, 33]. This finding is partially consistent a previous study demonstrating that trabectedin downregulates stem cell-related gene expression [30]. Moreover, lurbinectedin decreased the expression of HDAC1 (Fig. 5b), a dominant subtype of HDAC, and increased p53 expression. This finding is consistent with a recent report showing that HDAC1 suppresses p53 transcriptional activity by enhancing deacetylation and decreasing DNA binding [34]. Our results were also consistent with a recent report demonstrating that HDAC1-mediated inactivation of p53 is involved in the induction of lung cancer stem cells [21]. Another report showed that under stress conditions, p53 is activated by acetylation, blocks Nanog expression by directly binding to its promoter region, and binds the distal regions of genes to suppress them. Activated p53 also suppresses the transcription of SOX2 and Oct4 [35, 36]. Collectively, these results indicate that lurbinectedin eliminates CSCs by inhibiting HDAC1-mediated p53-supression (Fig. S5). However, the stem cell-like properties of CSCs are regulated by not only HDAC1, but

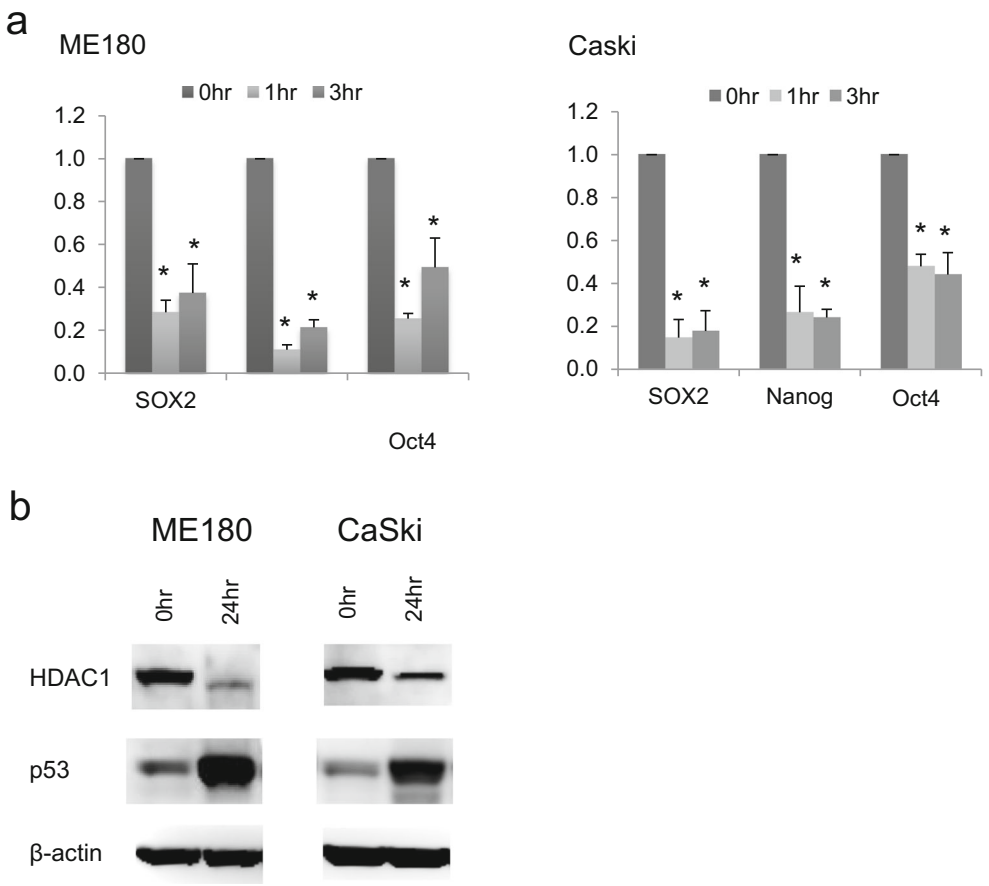

Fig. 5 The mechanisms by which lurbinectedin inhibits cancer stem cells. a The effect of lurbinectedin on the expression of CSC related genes. ME180 and CaSki cells were treated with the indicated concentrations of lurbinectedin for 1 and 3 h. The mRNA expression levels of SOX2, Nanog, and Oct4 were determined by real-time quantitative RT-PCR analysis ($n = 3$). GAPDH was used as internal reference for each sample. *$P < 0.05$, compared with that at 0 nM. b. The effect of lurbinectedin on the HDAC1-p53 pathway. ME180 and CaSki cells were treated with the indicated concentrations of lurbinectedin for 24 h. The expression of HDAC1 and p53 were assessed by western blotting. β-actin was used as an internal reference for each sample

by various signaling pathways such as Notch, Hedgehog, and Wnt [37]. Lurbinectedin may also affect these signaling pathways in our experimental model. Further studies are needed to obtain a deeper understanding of the mechanisms underlying the effects of lurbinectedin on CSCs.

The limitations of our study need to be addressed. We employed, on the basis of previous reports [19, 20], ALDH-activity as a marker of CSCs. However, definitive CSC markers for solid tumors have not been established. In addition, although we evaluated the effects of lurbinectedin in human cervical cancer cell lines, we did not evaluate the inhibitory effects of lurbinectedin in patient-derived tumor samples. Thus, to verify our results, further investigations may be required.

In conclusion, our findings indicate that lurbinectedin is highly effective on uterine cervical cancer. Because lurbinectedin eliminates CSCs, it is a promising agent to overcome the platinum-resistance in cervical cancer. Our preclinical data provide the basis for future clinical trials using lurbinectedin in recurrent or persistent cervical cancer.

Acknowledgements We thank Moe Matsui for her secretarial assistance and Ayako Okamura for her technical assistance.

Funding This study was supported by Grants-in-aid for General Scientific Research no. T17 K16849, A15H025640, and T17 K112760 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Compliance with ethical standards

Conflicts of interest statement All authors have declared that they have no conflict of interests to disclose.

Eriko Yokoi declares that he has no conflict of interest. Seiji Mabuchi declares that he has no conflict of interest. Kotaro Shimura declares that he has no conflict of interest. Naoko Komura declares that he has no conflict of interest. Katsumi Kozasa declares that he has no conflict of interest. Hiromasa Kuroda declares that he has no conflict of interest. Ryoko Takahashi declares that he has no conflict of interest. Tomoyuki Sasano declares that he has no conflict of interest. Mahiru Kawano declares that he has no conflict of interest. Yuri Matsumoto declares that he has no conflict of interest. Michiko Kodama declares that he has no conflict of interest. Kae Hashimoto declares that he has no conflict of interest. Kenjiro Sawada declares that he has no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Informed consent For this type of study, formal consent is not required.

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A (2015) Global cancer statistics, 2012. CA Cancer J Clin 65:87–108. https://doi.org/10.3322/caac.21262
2. Leath CA, Straughn JM (2013) Chemotherapy for advanced and recurrent cervical carcinoma: results from cooperative group trials. Gynecol Oncol 129:251–257. https://doi.org/10.1016/j.ygyno.2012.12.035
3. Tewari KS, Sill MW, Long HJ, Penson RT, Huang H, Ramondetta LM et al (2014) Improved survival with bevacizumab in advanced cervical cancer. N Engl J Med 370:734–743. https://doi.org/10.1056/NEJMoa1309748
4. Moore DH, Tian C, Monk BJ, Long HJ, Omura GA, Bloss JD (2010) Prognostic factors for response to cisplatin-based chemotherapy in advanced cervical carcinoma: a gynecologic oncology group study. Gynecol Oncol 116:44–49. https://doi.org/10.1016/j.ygyno.2009.09.006
5. Hisamatsu T, Mabuchi S, Yoshino K, Fujita M, Enomoto T, Hamasaki T, Kimura T (2012) Prediction of progression-free survival and response to paclitaxel plus carboplatin in patients with recurrent or advanced cervical cancer. Int J Gynecol Cancer 22:623–629. https://doi.org/10.1097/IGC.0b013e3182473277
6. Pattabiraman DR, Weinberg RA (2014) Tackling the cancer stem cells - what challenges do they pose? Nat Rev Drug Discov 13:497–512. https://doi.org/10.1038/nrd4253
7. Bonnet D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3:730–737
8. López J, Poitevin A, Mendoza-Martínez V, Pérez-Plasencia C, García-Carrancá A (2012) Cancer-initiating cells derived from established cervical cell lines exhibit stem-cell markers and increased radioresistance. BMC Cancer 12:48. https://doi.org/10.1186/1471-2407-12-48
9. Kumazawa S, Kajiyama H, Umezu T, Mizuno M, Suzuki S, Yamamoto E, Mitsui H, Sekiya R, Shibata K, Kikkawa F (2014) Possible association between stem-like hallmark and radioresistance in human cervical carcinoma cells. J Obstet Gynaecol Res 40:1389–1398. https://doi.org/10.1111/jog.12357
10. Chhabra R (2015) Cervical cancer stem cells: opportunities and challenges. J Cancer Res Clin Oncol 141:1889–1897. https://doi.org/10.1007/s00432-014-1905-y
11. Leal JF, Martínez-Díez M, García-Hernández V, Moneo V, Domingo A, Bueren-Calabuig JA, Negri A, Gago F, Guillén-Navarro MJ, Avilés P, Cuevas C, García-Fernández LF, Galmarini CM (2010) PM01183, a new DNA minor groove covalent binder with potent in vitro and in vivo anti-tumour activity. Br J Pharmacol 161:1099–1110. https://doi.org/10.1111/j.1476-5381.2010.00945.x
12. Takahashi R, Mabuchi S, Kawano M, Sasano T, Matsumoto Y, Kuroda H, Kozasa K, Hashimoto K, Sawada K, Kimura T (2016) Preclinical investigations of PM01183 (Lurbinectedin) as a single agent or in combination with other anticancer agents for clear cell carcinoma of the ovary. PLoS One 11:e0151050. https://doi.org/10.1371/journal.pone.0151050
13. Kuroda H, Mabuchi S, Kozasa K, Yokoi E, Matsumoto Y, Komura N, Kawano M, Hashimoto K, Sawada K, Kimura T (2017) PM01183 inhibits myeloid-derived suppressor cells in vitro and in vivo. Immunotherapy 9:805–817. https://doi.org/10.2217/imt-2017-0046
14. Poveda A, Del Campo JM, Ray-Coquard I, Alexandre J, Provansal M, Guerra Alía EM et al (2017) Phase II randomized study of PM01183 versus topotecan in patients with platinum-resistant/refractory advanced ovarian cancer. Ann Oncol 28:1280–1287. https://doi.org/10.1093/annonc/mdx111
15. NCT02421588 Clinical Trials. gov. A Service of the U.S. National Institute of Health. Available from: http://www.clinicaltrials.gov (Accessed; August 30, 2015)
16. Nozawa S, Tsukazaki K, Sakayori M, Jeng CH, Iizuka R (1988) Establishment of a human ovarian clear cell carcinoma cell line (RMG-I) and its single cell cloning–with special reference to the stem cell of the tumor. Hum Cell 1:426–435
17. Mabuchi S, Altomare DA, Cheung M, Zhang L, Poulikakos PI, Hensley HH, Schilder RJ, Ozols RF, Testa JR (2007) RAD001 inhibits human ovarian cancer cell proliferation, enhances cisplatin-induced apoptosis, and prolongs survival in an ovarian cancer model. Clin Cancer Res 13:4261–4270. https://doi.org/10.1158/1078-0432.CCR-06-2770
18. Vidal A, Muñoz C, Guillén MJ, Moretó J, Puertas S, Martínez-Iniesta M et al (2012) Lurbinectedin (PM01183), a new DNA minor groove binder, inhibits growth of orthotopic primary graft of cisplatin-resistant epithelial ovarian cancer. Clin Cancer Res 18:5399–5411. https://doi.org/10.1158/1078-0432.CCR-12-1513
19. Liu SY, Zheng PS (2013) High aldehyde dehydrogenase activity identifies cancer stem cells in human cervical cancer. Oncotarget 4:2462–2475. https://doi.org/10.18632/oncotarget.1578
20. Wang L, Guo H, Lin C, Yang L, Wang X (2014) Enrichment and characterization of cancer stem-like cells from a cervical cancer cell line. Mol Med Rep 9:2117–2123. https://doi.org/10.3892/mmr.2014.2063
21. Wang L, Liu X, Ren Y, Zhang J, Chen J, Zhou W, Guo W, Wang X, Chen H, Li M, Yuan X, Zhang X, Yang J, Wu C (2017) Cisplatin-enriching cancer stem cells confer multidrug resistance in non-small cell lung cancer via enhancing TRIB1/HDAC activity. Cell Death Dis 8:e2746. https://doi.org/10.1038/cddis.2016.409
22. Chikamatsu K, Ishii H, Murata T, Sakakura K, Shino M, Toyoda M, Takahashi K, Masuyama K (2013) Alteration of cancer stem cell-like phenotype by histone deacetylase inhibitors in squamous cell carcinoma of the head and neck. Cancer Sci 104:1468–1475. https://doi.org/10.1111/cas.12271
23. Del Bufalo D, Desideri M, De Luca T, Di Martile M, Gabellini C, Monica V et al (2014) Histone deacetylase inhibition synergistically enhances pemetrexed cytotoxicity through induction of apoptosis and autophagy in non-small cell lung cancer. Mol Cancer 13:230. https://doi.org/10.1186/1476-4598-13-230
24. Salvador MA, Wicinski J, Cabaud O, Toiron Y, Finetti P, Josselin E, Lelievre H, Kraus-Berthier L, Depil S, Bertucci F, Collette Y, Birnbaum D, Charafe-Jauffret E, Ginestier C (2013) The histone deacetylase inhibitor abexinostat induces cancer stem cells differentiation in breast cancer with low Xist expression. Clin Cancer Res 19:6520–6531. https://doi.org/10.1158/1078-0432.CCR-13-0877
25. Aztopal N, Erkisa M, Erturk E, Ulukaya E, Tokullugil AH, Ari F (2018) Valproic acid, a histone deacetylase inhibitor, induces apoptosis in breast cancer stem cells. Chem Biol Interact 280:51–58. https://doi.org/10.1016/j.cbi.2017.12.003
26. Di Pompo G, Salerno M, Rotili D, Valente S, Zwergel C, Avnet S et al (2015) Novel histone deacetylase inhibitors induce growth arrest, apoptosis, and differentiation in sarcoma cancer stem cells. J Med Chem 58:4073–4079. https://doi.org/10.1021/acs.jmedchem.5b00126
27. Bahadori HR, Green MR, Catapano CV (2001) Synergistic interaction between topotecan and microtubule-interfering agents. Cancer Chemother Pharmacol 48:188–196

28. Tiersten AD, Selleck MJ, Hershman DL, Smith D, Resnik EE, Troxel AB, Brafman LB, Shriberg L (2004) Phase II study of topotecan and paclitaxel for recurrent, persistent, or metastatic cervical carcinoma. Gynecol Oncol 92:635–638. https://doi.org/10.1016/j.ygyno.2003.11.019
29. Kim BW, Cho H, Choi CH, Ylaya K, Chung JY, Kim JH, Hewitt SM (2015) Clinical significance of OCT4 and SOX2 protein expression in cervical cancer. BMC Cancer 15:1015. https://doi.org/10.1186/s12885-015-2015-1
30. Martinez-Cruzado L, Tornin J, Rodriguez A, Santos L, Allonca E, Fernandez-Garcia MT, Astudillo A, Garcia-Pedrero JM, Rodriguez R (2017) Trabectedin and Campthotecin synergistically eliminate Cancer stem cells in cell-of-origin sarcoma models. Neoplasia 19:460–470. https://doi.org/10.1016/j.neo.2017.03.004
31. Elez ME, Tabernero J, Geary D, Macarulla T, Kang SP, Kahatt C, Pita ASM, Teruel CF, Siguero M, Cullell-Young M, Szyldergemajn S, Ratain MJ (2014) First-in-human phase I study of Lurbinectedin (PM01183) in patients with advanced solid tumors. Clin Cancer Res 20:2205–2214. https://doi.org/10.1158/1078-0432.CCR-13-1880
32. Major AG, Pitty LP, Farah CS (2013) Cancer stem cell markers in head and neck squamous cell carcinoma. Stem Cells Int 2013:319489–319413. https://doi.org/10.1155/2013/319489
33. Rodda DJ, Chew JL, Lim LH, Loh YH, Wang B, Ng HH, Robson P (2005) Transcriptional regulation of nanog by OCT4 and SOX2. J Biol Chem 280:24731–24737. https://doi.org/10.1074/jbc.M502573200
34. Miyajima C, Inoue Y, Hayashi H (2015) Pseudokinase tribbles 1 (TRB1) negatively regulates tumor-suppressor activity of p53 through p53 deacetylation. Biol Pharm Bull 38:618–624. https://doi.org/10.1248/bpb.b15-00003
35. Liu K, Lee J, Kim JY, Wang L, Tian Y, Chan ST, et al. Mitophagy Controls the Activities of Tumor Suppressor p53 to Regulate Hepatic Cancer Stem Cells. Mol Cell. 2017;68:281–292.e5. https://doi.org/10.1016/j.molcel.2017.09.022
36. Li M, He Y, Dubois W, Wu X, Shi J, Huang J (2012) Distinct regulatory mechanisms and functions for p53-activated and p53-repressed DNA damage response genes in embryonic stem cells. Mol Cell 46:30–42. https://doi.org/10.1016/j.molcel.2012.01.020
37. Takebe N, Miele L, Harris PJ, Jeong W, Bando H, Kahn M, Yang SX, Ivy SP (2015) Targeting notch, hedgehog, and Wnt pathways in cancer stem cells: clinical update. Nat Rev Clin Oncol 12:445–464. https://doi.org/10.1038/nrclinonc.2015.61