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Schlafen11 Expression Is Associated With the Antitumor Activity of Trabectedin in Human Sarcoma Cell Lines

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Abstract. *Background/Aim: Trabectedin is a DNA-damaging agent and has been approved for the treatment of patients with advanced soft tissue sarcoma. Schlafen 11 (SLFN11) was identified as a dominant determinant of the response to DNA-damaging agents. The aim of the study was to clarify the association between SLFN11 expression and the antitumor activity of trabectedin. Materials and Methods: The antitumor activity of trabectedin was evaluated under different expression levels of SLFN11 regulated by RNA interference and CRISPR-Cas9 systems, and the combined antitumor activity of ataxia telangiectasia and Rad3-related protein kinase (ATR) inhibitor and trabectedin in sarcoma cell lines using in vitro a cell viability assay and in vivo xenograft models. Results: SLFN11-knockdown cell lines had a lower sensitivity to trabectedin, compared to parental cells. ATR inhibitor enhanced the antitumor activity of trabectedin in SLFN11-knockdown cells and in a SLFN11-knockout xenograft model. Conclusion: SLFN11 expression might be a key factor in the antitumor activity of trabectedin.*

Trabectedin (Yondelis®, ecteinascidin-743, ET-743) is a marine-derived natural product that has been approved for the treatment of patients with advanced soft tissue sarcoma and relapsed platinum-sensitive ovarian cancer in combination with liposomal doxorubicin (1, 2). Ongoing studies suggest that trabectedin is also effective against other solid malignancies, including breast cancer (3).

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Previous studies have shown a peculiar aspect of the mechanism of action of trabectedin. Trabectedin showed a decreased activity (from 2- to 10-fold) in nucleotide excision repair (NER)-deficient cells, compared to NER-proficient cells (4, 5). DNA-bound trabectedin is thought to prevent the correction of DNA lesions through transcription-coupled NER (TC-NER) by creating cytotoxic ternary complexes with DNA-binding proteins of the NER system, such as XPG. On the other hand, NER-deficient cells show an increased sensitivity to platinum drugs, such as cisplatin (4-6). Homologous recombination repair (HRR) has been shown to be important for trabectedin, since HRR-deficient cells were 50- to 100-times more sensitive to trabectedin (7, 8). The lack of HRR has been associated with the persistence of unrepaired DNA double-strand breaks (DSBs) during the S phase of the cell cycle and apoptosis (7). Moreover, the inhibition of the cell-cycle checkpoints that are activated in response to trabectedin might also prove useful to increase drug efficacy (9, 10). In response to replicative damage, ataxia telangiectasia and Rad3-related protein kinase (ATR) plays a major role in coordinating cell-cycle progression and DNA repair (11, 12). The loss of the S-phase checkpoint by ATR inhibitors causes the unscheduled firing of replication origins in S-phase and the induction of DSBs (13, 14). At present, a total of 39 different gene fusions have been identified in 15 different tumor types, accounting for approximately 20% of all soft tissue sarcomas (15). Furthermore, trabectedin interacts with fused genes and inhibits the expressions of oncogenes, which are up-regulated by gene fusions such as EWS-FLI1 and FUS-CHOP (16, 17).

Recently, Schlafen 11 (*SLFN11*) was discovered during bioinformatics analyses of cancer cell databases and was identified as a dominant determinant of cell response to some widely used anticancer drugs, including topoisomerase (Top) 1 inhibitors, Top2 inhibitors, alkylating agents, and DNA synthesis inhibitors (18, 19). The importance of *SLFN11* in drug sensitivity has recently been extended to include

Ewing's sarcoma (20). SLFN11 influences the response of patients with ovarian, non-small cell lung, or colorectal cancers (18, 19), and inhibits checkpoint maintenance and homologous recombination by removing replication protein A (RPA) from single-stranded DNA (21). Recent reports have shown that SLFN11 is recruited to stressed replication forks carrying extended RPA filaments where it blocks replication independently of HRR (22).

In this study, a correlation analysis comparing the antitumor activity of trabectedin and *SLFN11* expression in sarcoma cell lines was performed and demonstrated the importance of *SLFN11* expression as a key factor in the antitumor activity of trabectedin in sarcoma cell lines and xenograft models. Furthermore, the usefulness of combination therapies to overcome the decreased antitumor activity of trabectedin in cells with low *SLFN11* expression levels was evaluated.

Materials and Methods

Cell lines. The human sarcoma cell lines Yamato-SS and Aska-SS were kindly provided by Dr. Norifumi Naka (Osaka International Cancer Institute, Osaka, Japan) and Dr. Kazuyuki Ito (Nozaki Tokushukai Hospital, Osaka, Japan); SYO-1 was kindly provided by Dr. Akira Kawai (National Cancer Center, Tokyo, Japan); HT-1080, KYM-1, NY, RD, SCCH-196, RKN, SKN and HuO9N2 were purchased from the Japanese Collection of Research Bioresources (Osaka, Japan); Hs 925.T, KHOS/NP, KHOS-240S, SK-LMS-1, KHOS-312H, SW 872, SW 982, MES-SA, U-2 OS, SK-ES-1, SJSA-1, SJCRH30 and RD-ES were purchased from ATCC (Manassas, VA, USA); ESS-1 was purchased from DSMZ (Brunswick, Germany); and A673, HOS, MG-63, G-292 clone A141B1 and Saos-2 were purchased from KAC Co., Ltd. (Kyoto, Japan). All the cells were maintained according to the supplier's protocols.

Chemicals. Trabectedin was provided by PharmaMar (Madrid, Spain). VE-821 (http://www.selleckchem.com/products/ve-821.html) was purchased from Selleck Chemicals (Houston, TX, USA). Berzosertib (VE-822; https://www.medchemexpress.com/VE-822.html) was purchased from MedChemExpress (Monmouth Junction, NJ, USA). 5-Aza-2'-deoxycytidine (decitabine) was purchased from Sigma-Aldrich Co., LLC (St. Louis, MO, USA).

Antibodies. Antibodies against SLFN11 (sc-374339), and CHK1 (sc-8408) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against phospho-Ser317-CHK1 (#2344), phospho-Ser345-CHK1 (#2348), phospho-Ser139-H2AX (#9718), and GAPDH (#2118) were obtained from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase-conjugated secondary antibodies to mouse or rabbit IgG were obtained from Cell Signaling Technology.

Cell viability assay. Cell viability was measured using the crystal violet staining method and CellTiter-Glo 2.0 Assay (Promega, Fitchburg, WI, USA). Approximately 2-5×10^3 cells were plated per well on a 96-well plate. The cells were treated with a range of drug concentrations for 72 h. After adding the CellTiter-Glo reagent, luminescence readings were obtained using a VersaMax Absorbance Microplate Reader (Molecular Devices, San Jose, CA, USA) and FlexStation3 (Molecular Devices). The 50% and 75% inhibitory concentrations (IC_{50} and IC_{75}) were calculated using an XLfit (Fit model 205, ITOCHU Techno-Solutions Co., Tokyo, Japan).

Quantitative RT-PCR. For the TaqMan array cards and the TaqMan gene expression assay (Thermo Fisher Scientific, Waltham, MA, USA) experiments, RNA was collected from 30 sarcoma cell lines using an RNeasy kit (Qiagen, Venlo, the Netherlands). Complementary DNA (cDNA) was synthesized using High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific). The reaction was carried out following the methods for use of TaqMan Universal Master Mix II (2×) (Thermo Fisher Scientific), and *ACTB* and *GAPDH* were used as the reference genes.

siRNA transfection. Cells (1-3×10^5) were plated in 6-well plates. The siRNA for human SLFN11 and negative control (Stealth RNAi siRNA, HSS132188 and 12935300) were products of Invitrogen (Carlsbad, CA, USA). siRNA (5 nmol/l) was transfected into each cell with Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions.

Western blotting. Total protein was extracted from whole cells using a cellular protein extraction buffer containing protease and phosphatase inhibitors. The protein contents were measured using a Pierce® BCA Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Protein extracts were separated using SDS-PAGE polyacrylamide gels and electroblotted onto a PVDF membrane (Bio-Rad). The blotted membrane was blocked with Blocking One and Blocking One P (Nacalai Tesque, Kyoto, Japan). The primary antibodies were diluted in Can Get Signal Immunoreaction Enhancer Solution 1 (TOYOBO, Osaka, Japan) at a 1:1,000 dilution for SLFN11, phospho-Ser317-CHK1, phospho-Ser345-CHK1, CHK1, and phospho-Ser139-H2A.X and a 1:5000 dilution for GAPDH. The secondary antibody (Cell Signaling Technology) was diluted in Can Get Signal Immunoreaction Enhancer Solution 2 at a 1:2,000 dilution. Signals were detected by using a ChemiDoc Touch (Bio-Rad).

Establishment of SLFN11-knockout cells. To delete the *SLFN11* gene, we used Edit-R CRISPR RNA (crRNA), which was designed to target the human *SLFN11* gene (CM-01674-01-0002 and CM-01674-02-0002) (Dharmacon, Lafayette, CO, USA). crRNA, Edit-R trans-activating CRISPR RNA, and SMARTCas9 (Puro®) Expression Plasmid were co-transfected into SW 872 cells according to the instruction manual. Approximately 1 week after transfection, the cells were cultured in the presence of puromycin (1.5-2.5 μg/ml). Approximately 3-4 weeks later, puromycin-resistant clones were then selected and allowed to expand. A lack of SLFN11 protein expression in the SW 872 knockout cells was confirmed using western blotting.

In vivo experiments. Five-week-old male BALB/c nude mice were purchased from CLEA Japan, Inc. (Tokyo, Japan) and were housed under specific pathogen-free conditions. Following a quarantine period of about 1 week, tumor fragments (approximately 2 mm diameter) were implanted subcutaneously *via* an implant needle in the vicinity of the right hindmost rib of the nude mice. The length (mm) and width (mm) were measured, and the tumor volume (TV, mm^3) for each fragment was calculated using the following formula: TV=(length) × $(width)^2/2$. When the TV reached 100-200 mm^3, the

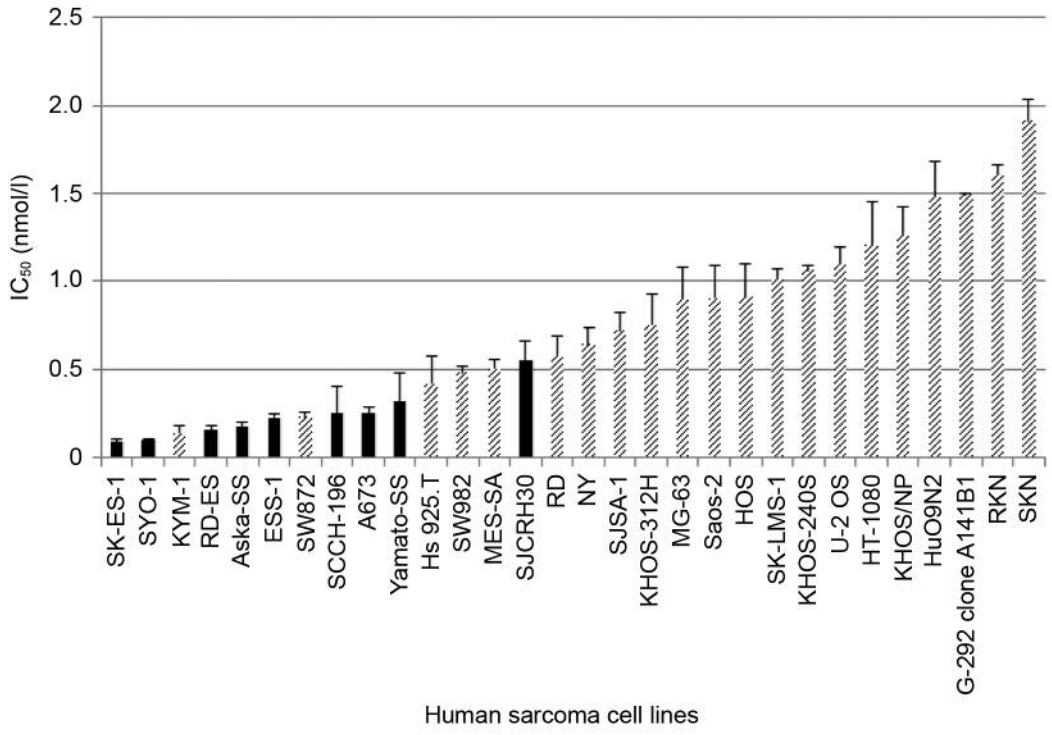

Figure 1. *Trabectedin shows a significant antitumor activity in sarcoma cell lines. Thirty sarcoma cell lines were exposed to trabectedin for 72 h. Nine TRS cell lines (solid black bars) and 21 non-TRS cell lines (hashed bars) were used. The error bars represent the standard deviation (SD, n≥3).*

mice were divided into groups using a stratified randomization method so as to equalize the mean TV in each group. Each group consisted of 6 nude mice. Trabectedin [0.01 mg/mL in a solution of 0.05 mol/l KH_2PO_4(pH4.0)] was administered by intraperitoneal bolus injection on days 1, 5, and 9 at a dose of 0.1 mg/kg, which was the maximum tolerated dose in nude mice. VE-822 [30 mg/kg in a solution of 10% vitamin E tocopheryl polyethylene glycol succinate (VitE TPGS)] was administered by oral gavage on days 1, 2 and 3 of each 4-day cycle. The control group was treated with the vehicle (0.05 mol/l KH2PO4, pH4.0) for trabectedin on days 1, 5, and 9.

The TVs were measured twice a week until day 15. On day 15, the tumor growth inhibition rate (TGI) was calculated using the following formula: TGI=[1 – (mean TV of treated group)/(mean TV of control group)] ×100. The body weight change (BWC, %) was calculated as [(body weight on day 15) – (body weight on day 0)]/(body weight on day 0) ×100.

The animal study was conducted according to institutional guidelines and was approved by the Institutional Animal Care and Use Committee of Taiho Pharmaceutical Co., Ltd.

Statistical analysis. The correlation analysis for the antitumor activity of trabectedin and the gene expression of *SLFN11* was performed using JMP (version 9) software (SAS Institute Inc., Cary, NC, USA). The significance of the difference in the mean *SLFN11* expression levels between the 2 groups (TRS *vs*. non-TRS) was analyzed using the Aspin-Welch two-tailed *t*-test. Differences were considered significant when $p<0.05$. The analysis was performed using JMP (version 9) software.

The significance of the difference in the mean *SLFN11* expression levels according to decitabine treatment at each timepoint was analyzed using the Dunnett test. The significance of the difference in the mean TV between the 2 groups on day 15 was analyzed using the Aspin-Welch two-tailed *t*-test. Differences were considered significant when $p<0.05$. The analyses were performed using EXSUS version 8.0 software (CAC Croit Corp., Tokyo, Japan).

Results

Antitumor activity of trabectedin in 30 human sarcoma cell lines. To evaluate the antitumor activity of trabectedin in sarcoma cell lines, the IC_{50} of trabectedin was determined in both translocation related sarcoma (TRS) cell lines, which contain gene fusions, and in non-TRS cell lines using an *in vitro* cell viability assay. Trabectedin showed nanomolar ranges of IC_{50} values in both TRS and non-TRS cell lines, and the ranges of all the IC_{50} values were extremely narrow (Figure 1).

Expression level of SLFN11 was positively correlated with the antitumor activity of trabectedin. To determine the relationship between the *SLFN11* expression levels and the sensitivity to trabectedin in 30 sarcoma cell lines, the

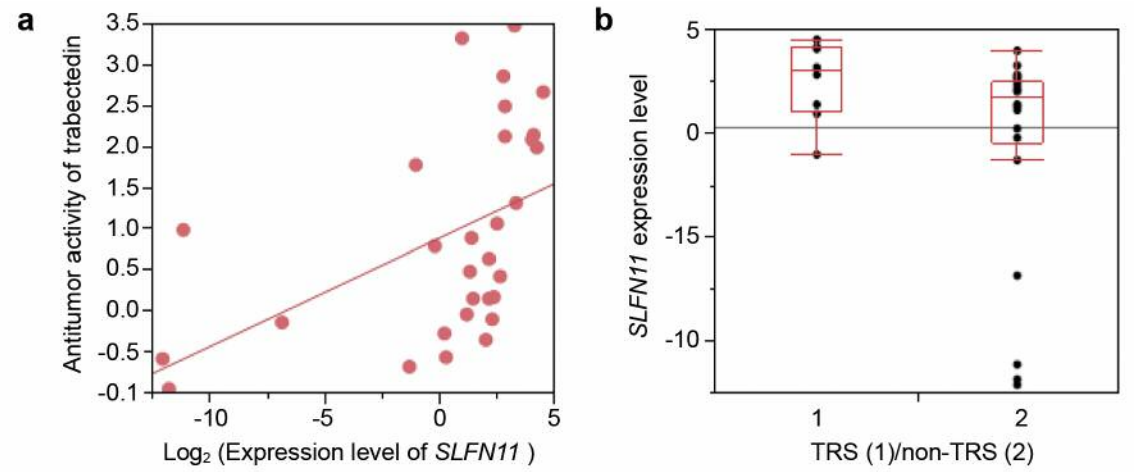

Figure 2. *Antitumor activity of trabectedin was correlated with SLFN11 expression levels. (a) Correlation between SLFN11 expression (mRNA expression level of SLFN11=[2$^{-\Delta Ct}$] ×1000, ΔCt=[Ct of SLFN11] – [mean of Cts of ACTB and GAPDH]) and $-log_2IC_{50}$ of trabectedin across sarcoma cell lines. Correlation coefficients: r=0.479, p<0.01. (b) No significant difference in SLFN11 expression was seen between the TRS and non-TRS cell lines. p=0.1203.*

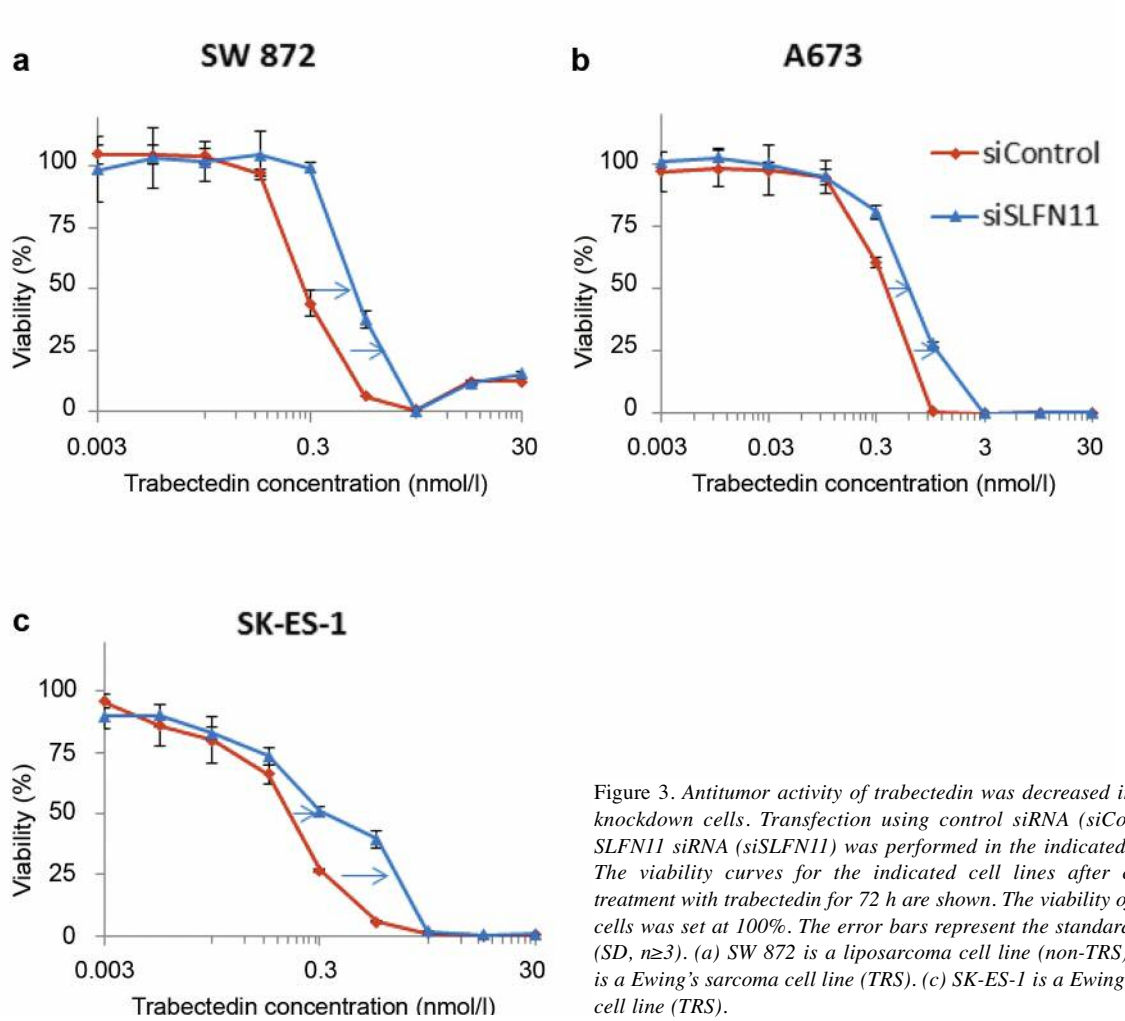

Figure 3. *Antitumor activity of trabectedin was decreased in SLFN11-knockdown cells. Transfection using control siRNA (siControl) and SLFN11 siRNA (siSLFN11) was performed in the indicated cell lines. The viability curves for the indicated cell lines after continuous treatment with trabectedin for 72 h are shown. The viability of untreated cells was set at 100%. The error bars represent the standard deviation (SD, n≥3). (a) SW 872 is a liposarcoma cell line (non-TRS). (b) A673 is a Ewing's sarcoma cell line (TRS). (c) SK-ES-1 is a Ewing's sarcoma cell line (TRS).*

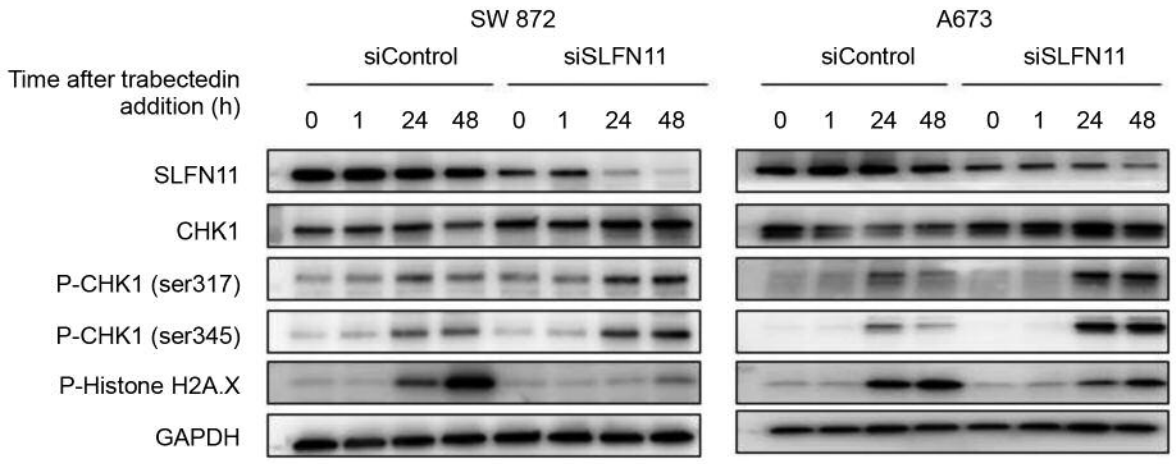

Figure 4. *DNA damage checkpoint was activated by trabectedin in SLFN11-knockdown cells. SW 872 and A673 cells, which were transfected with siControl and siSLFN11, were exposed for 1 h with trabectedin (20 nmol/l) followed by 24 and 48 h post-incubation in drug-free media, as indicated. The phosphorylation of CHK1 was upregulated in all cell lines, and CHK1 was degradated in the control cells.*

correlation between the antitumor activity ($-\log_2 IC_{50}$) of trabectedin and the *SLFN11* expression levels was determined using quantitative PCR in each cell line. The expression level of *SLFN11* was positively correlated with the antitumor activity of trabectedin, and cell lines with high levels of *SLFN11* expression were more sensitive to trabectedin (correlation coefficients: r=0.479, $p<0.01$) (Figure 2a). Furthermore, no significant difference was seen in the gene expression levels of TRS and non-TRS cell lines (Figure 2b).

Differences in antitumor activity of trabectedin between high and low SLFN11 expression cells. To determine the causal relation of SLFN11 and trabectedin sensitivity, *SLFN11* was knocked down using siRNA in three high *SLFN11* expressing cell lines (liposarcoma SW 872, Ewing's sarcoma A673 and SK-ES-1) and determined the IC_{50} and IC_{75} values using cell viability assays. All three *SLFN11*-knockdown cell lines had a lower sensitivity to trabectedin than the control cells, which were treated with negative control siRNA (siControl). The IC_{50} and IC_{75} values of the knockdown cells were 3.4 and 2.8 fold (SW 872), 1.8 and 2.2-fold (A673), and 2.6 and 3.6-fold (SK-ES-1) higher than those of each control group, respectively (Figure 3a, b, c).

Furthermore, the SW 872 and A673 cell lines, which were transfected with siSLFN11 and siControl cells, were exposed to 20 nmol/l of trabectedin for 1 h, followed by 48 h post-incubation in drug-free media. The phosphorylation of checkpoint kinase 1 (CHK1) on Ser317 and Ser345 residues after trabectedin exposure were observed in both cells independently of *SLFN11* expression levels. On the other hand, CHK1 expression was reduced in the high *SLFN11* expression cells. Additionaly, the phosphorylation of Histone H2A.X after trabectedin exposure was lower in the *SLFN11*-knockdown cells, compared with high *SLFN11* expression cells (Figure 4).

Quantitative PCR analysis indicated that the U-2 OS (osteosarcoma) cell line expressed minimal levels of *SLFN11*. In public data from the CellMiner portal site (https://discover.nci.nih.gov/cellminercdb/), the *SLFN11* gene was hypermethylated in the U-2 OS cell line, compared with other high *SLFN11* expression sarcoma cell lines (Figure 5a). Furthermore, treatment of the U-2 OS cell line with 1 μmol/l decitabine restored the SLFN11 expression levels (Figure 5b). To determine whether a DNA methyltransferase inhibitor enhances the trabectedin antitumor activity in *SLFN11*-hypermethylated cells, the U-2 OS cell line was exposed to 1 μmol/l of decitabine for 24 h before adding trabectedin for 72 h and assaying cell viability. The IC_{50} value of decitabine in combination with trabectedin was approximately 2-fold lower than that of trabectedin alone (Figure 5c).

ATR inhibitor enhanced the antitumor activity of trabectedin in SLFN11-knockdown cells. Because *SLFN11* blocks replication regardless of ATR-CHK1 activity (22), in low SLFN11 expression cells, DNA damage response might be dependent on ATR. Therefore, the combined antitumor activity of an ATR inhibitor (VE-821) and trabectedin was evaluated. SW 872 and A673 cell lines, which were transfected with siSLFN11 or siControl, were exposed to 1 μmol/l of VE-821 and trabectedin for 72 h (Figure 6a and b). As a result, VE-821 enhanced the antitumor activities of trabectedin by 2-fold in the two *SLFN11*-knockdown cell lines.

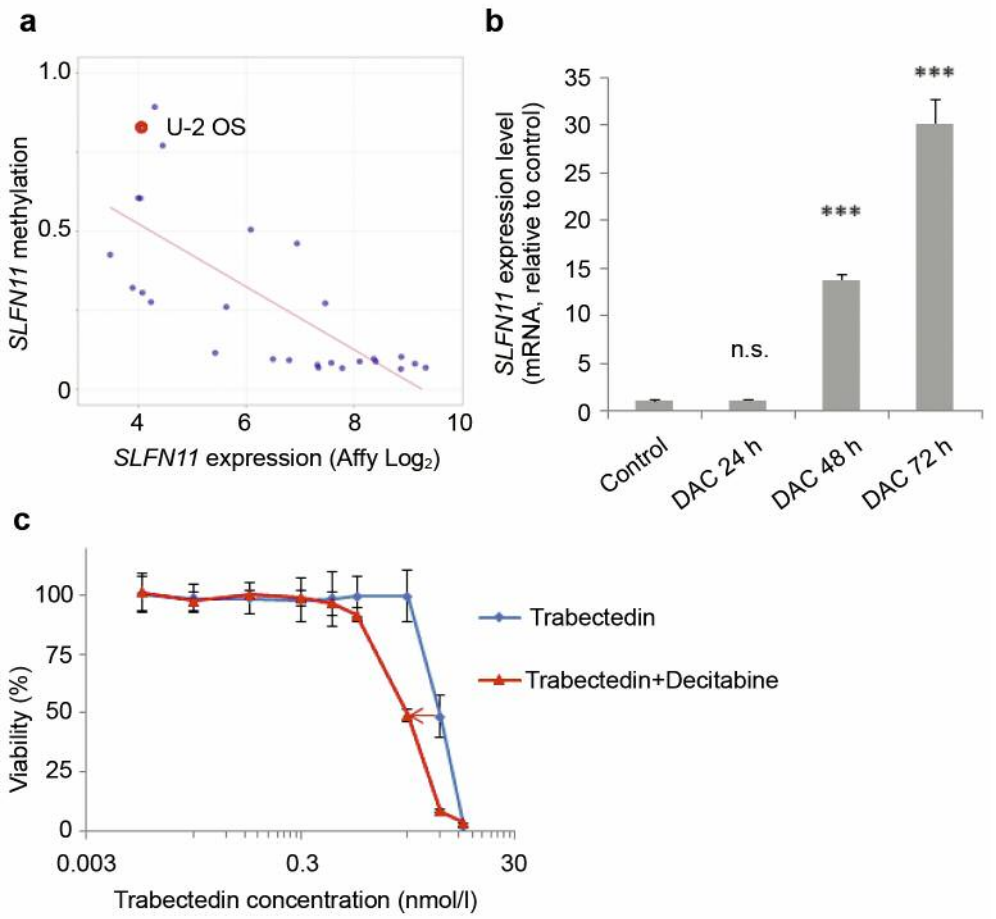

Figure 5. *Demethylation of SLFN11 enhanced the antitumor activity of trabectedin. (a) SLFN11 expression in the cancer cell lines of the CCLE collection matched with promoter methylation determined from data obtained from the GDSC database for individual common cell lines across the two databases (blue dots) obtained using CellMiner (http://discover.nci.nih.gov/cellminercdb/). The U-2 OS cell line does not express SLFN11 and exhibits promoter methylation; the U-2 OS cell line is marked in red. (b) The expression of the SLFN11 RNA transcript was restored in the methylated U-2 OS cell line by treatment with the demethylating drug 5-aza-2'-deoxycytidine (DAC, decitabine). n.s: Not significant, ***p<0.001. (c) Viability curves of the indicated cell lines after continuous treatment for 72 h with trabectedin alone (blue) and after exposure to 1 μmol/l of decitabine for 24 h before the addition of trabectedin (red). The viability of untreated cells was set at 100%. The error bars represent the standard deviation (SD, n≥3).*

ATR inhibitor enhanced the antitumor activity of trabectedin in a SLFN11-knockout SW 872 xenograft model. Next, the combined antitumor activity of trabectedin and ATR inhibitor (VE-822) was examined in xenograft nude mice models of SW 872 and *SLFN11*-knockout SW 872 (Figure 7a and b). The SLFN11 protein expression levels in each SW 872 tumor fragment used in this study were examined by western blotting (Figure 7c). Mice were treated with trabectedin at 0.1 mg/kg/day (dosed by intraperitoneal bolus injection on days 1, 5, and 9) and VE-822 at 30 mg/kg/day (dosed by oral gavage on days 1, 2, and 3 of each 4-day cycle) or a combination of both agents together. Trabectedin monotherapy induced significant antitumor activity (83% TGI) in the high *SLFN11* expression SW 872 xenograft model on Day 15. Although trabectedin monotherapy had marginal antitumor activity (37% TGI) in the *SLFN11*-knockout SW 872 xenograft model, the combination with VE-822 induced a higher antitumor activity, compared with trabectedin monotherapy. The combination was well tolerated, and no increase in body weight loss was observed compared with trabectedin monotherapy.

Discussion

To clarify the role of SLFN11 in the antitumor mechanism of trabectedin, the relation between *SLFN11* expression levels and the antitumor activities of trabectedin were evaluated. We demonstrated, for the first time, the role of SLFN11 in cellular responses to trabectedin. Our study

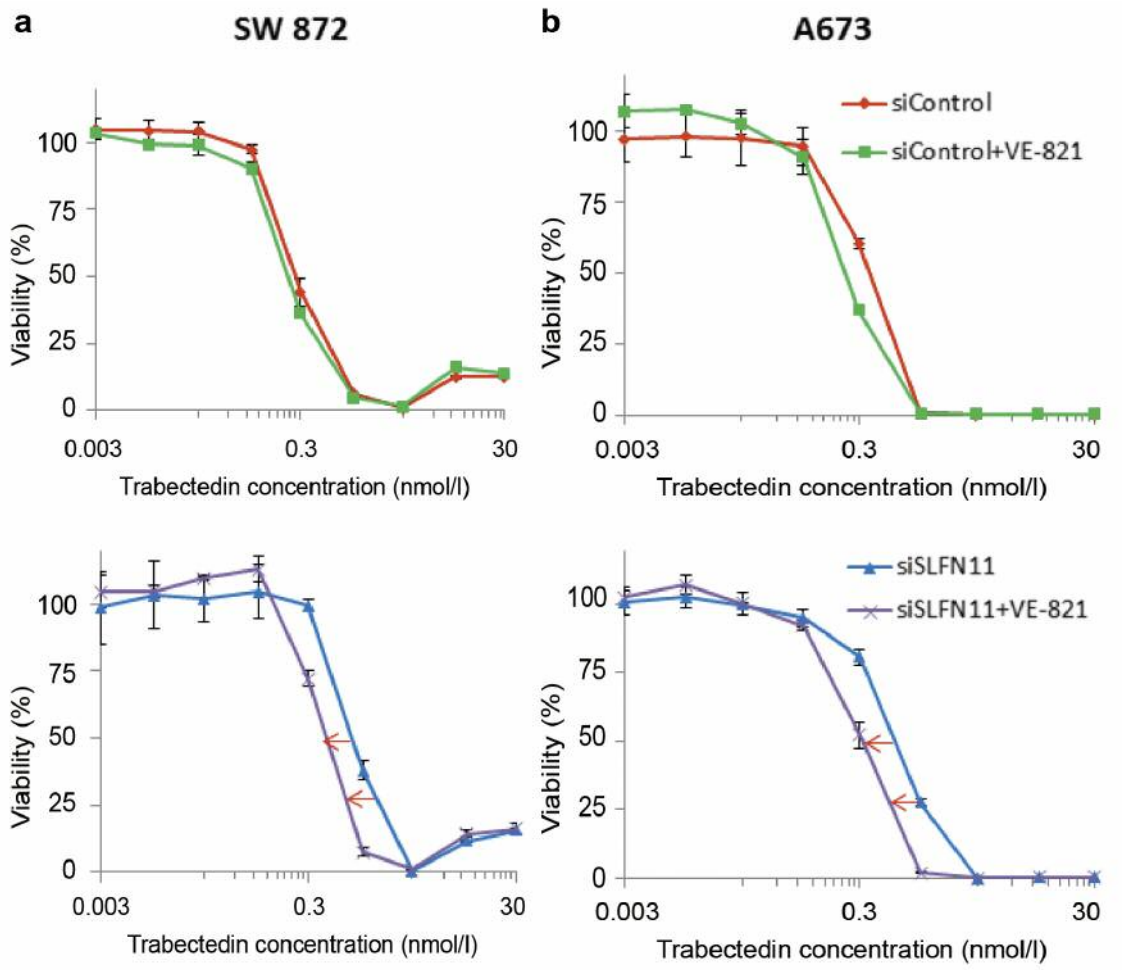

Figure 6. *ATR inhibitor enhanced the antitumor activity of trabectedin in SLFN11-knockdown sarcoma cells in vitro. High SLFN11 expression (a) SW 872 and (b) A673 cell lines transfected with siControl (upper) or siSLFN11 (lower) were exposed to trabectedin alone or a combination of 1 μmol/l of VE-821 and trabectedin for 72 h. The viability of untreated cells was set at 100%. Error bars represent the standard deviation (SD, n≥3).*

showed that trabectedin had an IC_{50} in the nanomolar range in both TRS and non-TRS cell lines, and *SLFN11* expression was correlated with sensitivity to trabectedin. The antitumor mechanism of trabectedin through the function of SLFN11 seems to act independently of fusion genes. Therefore, we focused on the role of SLFN11 in the antitumor activities of trabectedin.

In this study, *SLFN11*-knockdown cell lines and a xenograft model of *SLFN11*-knockout cells showed a lower sensitivity to trabectedin, compared to their parental high *SLFN11* expression cells, *in vitro* and *in vivo*, respectively. In aggremment, the sensitivities to other DNA-damaging anticancer drugs such as topotecan, irinotecan, and cisplatin are also reportedly linked to *SLFN11* expression (19). On the other hand, *SLFN11* expression did not exhibit any association with the response to drugs targeting other components, such as paclitaxel (data not shown). Since the enhancement of the antitumor activities through the modification of SLFN11 expression levels seems to be specific to DNA-damaging anticancer drugs (19), we hypothesized that SLFN11 might be involved in DNA repair. Although one group has reported a significant combined antitumor activities of trabectedin and ATR inhibitors in ovarian cancer cell lines (23), our results demonstrated that an ATR inhibitor enhanced the antitumor activity of trabectedin in *SLFN11*-knockdown cell lines *in vitro* and in *SLFN11*-knockout tumors *in vivo*. The above study showed that the ATR/CHK1 and ATM/CHK2 pathways were activated in response to trabectedin, and that the dual inhibition of ATR and ATM increased the antitumor activity of trabectedin. Furthermore, lack of *SLFN11* expression is a major cause of resistance to PARP inhibitors, and addition

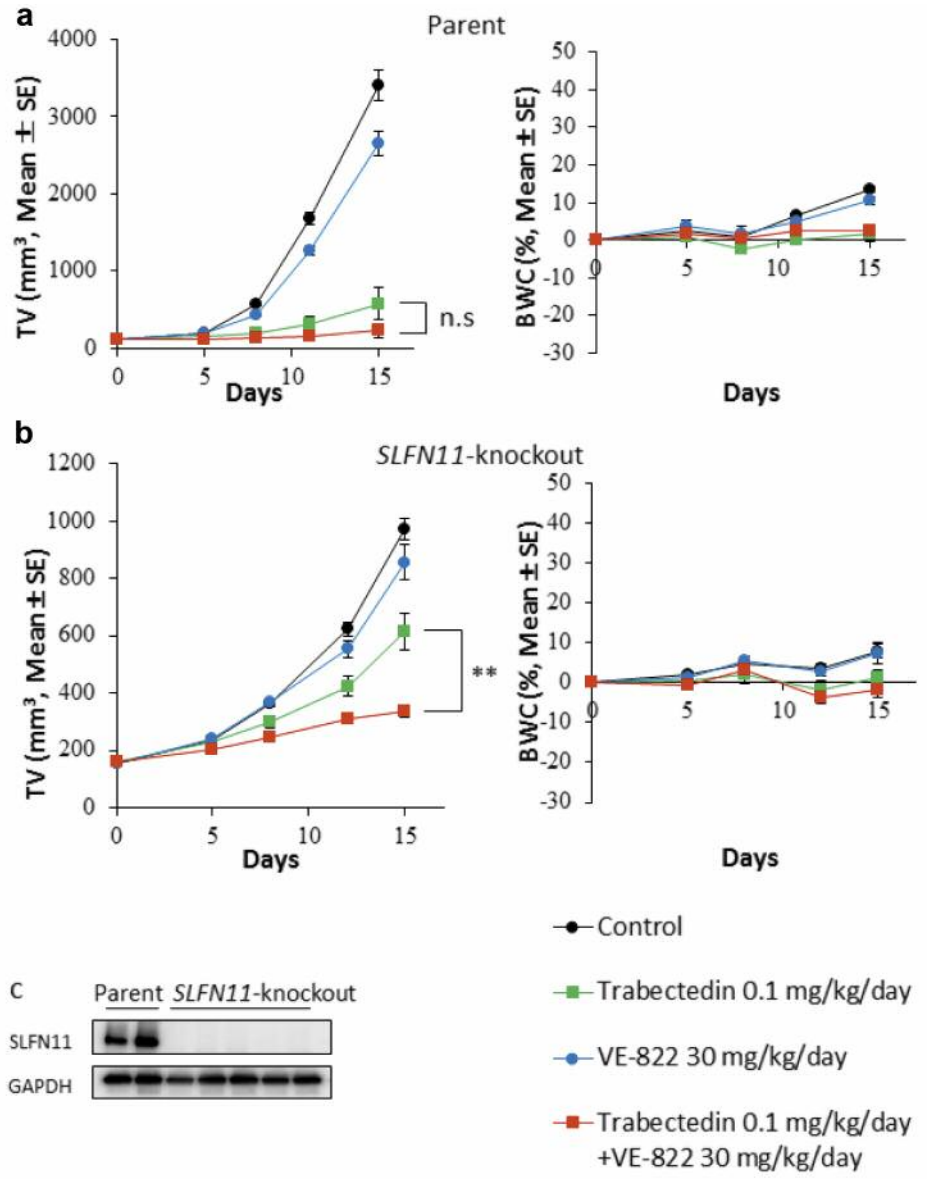

Figure 7. *ATR inhibitor enhanced the antitumor activity of trabectedin in SLFN11-knockout sarcoma xenografts in vivo. A mouse xenograft experiment using (a) SW 872 (high SLFN11 expression) and (b) SLFN11-knockout SW 872 cell lines was performed. Mice bearing tumors were treated with the vehicle (dosed on days 1, 5, and 9), trabectedin (dosed on days 1, 5, and 9), VE-822 (dosed on days 1, 2, and 3 of each 4-day cycle), or a combination of both drugs. The tumor volume (TV, left) and the relative body weight change (BWC, right) were plotted. The error bars represent the SE (n=6). n.s: Not significant, **p<0.01. (c) The lack of SLFN11 protein expression in the SLFN11-knockout SW 872 tumor fragments used in this study was examined using western blotting.*

of an ATR inhibitor overcomes such resistance (24). On the other hand, the antitumor mechanism of trabectedin does not necessarily involve only DNA-damaging effects, but other effects such as the suppression of transcription factors, cell-cycle regulation, or tumor microenvironment regulation might also be involved (10, 16, 25). Therefore, *SLFN11*-knockdown cell lines did not show resistance to trabectedin, but rather exhibited a lower sensitivity to trabectedin, unlike other DNA-damaging anticancer drugs.

In addition, a previously reported analysis of DNA methylation using the cancer cell line panel NCI60 has identified *SLFN11* CpG promoter island hypermethylation as a predictive biomarker of platinum resistance (18, 26). Some studies have revealed that the DNA methyltransferase

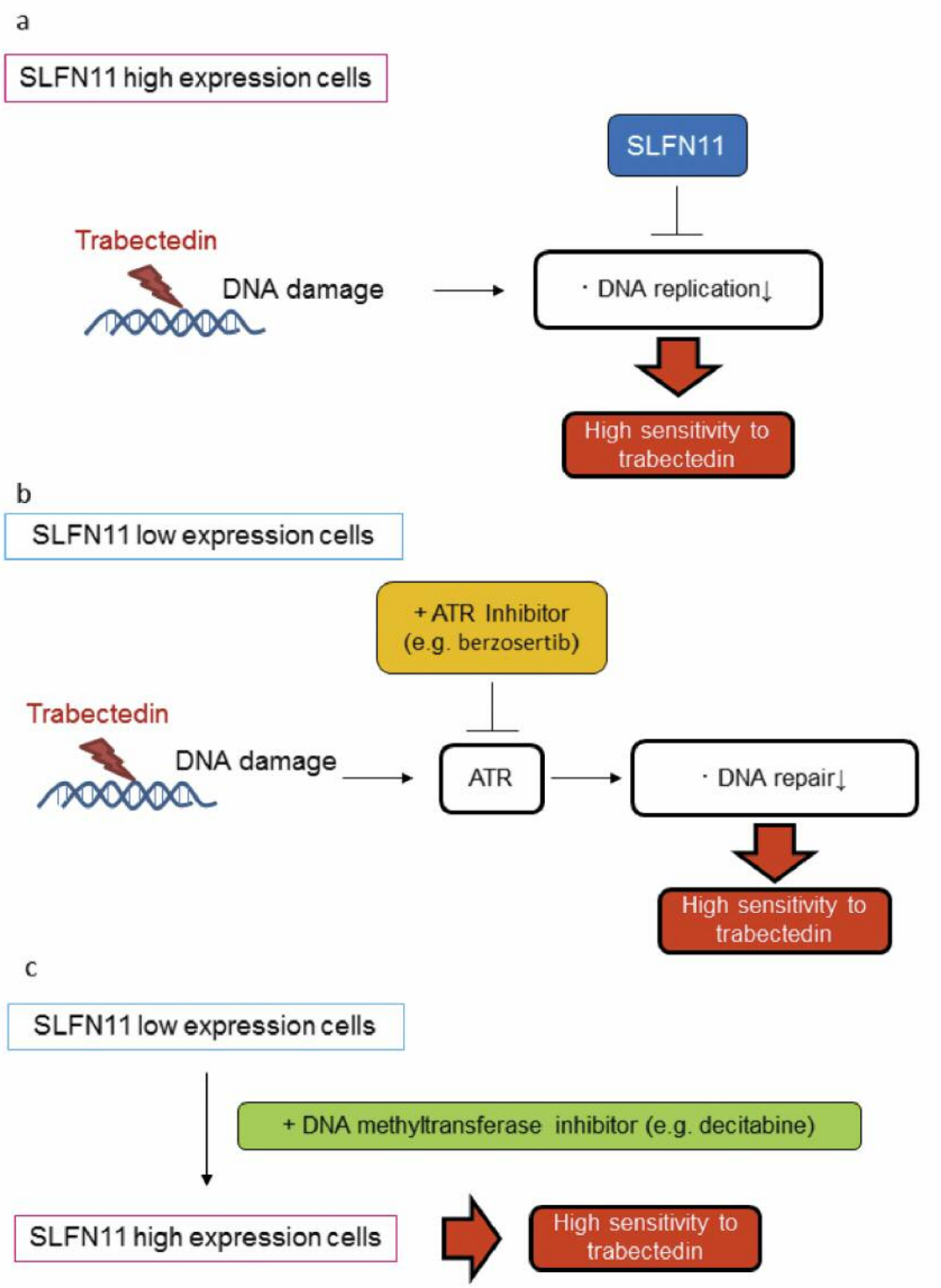

Figure 8. *Speculated mechanism of the combined effect of trabectedin and an ATR inhibitor, berzosertib, or a DNA methyltransferase inhibitor, decitabine, in low-SLFN11 expression cells. (a) In high-SLFN11 expression cells, when trabectedin is added to the cells, SLFN11 might block replication (22). Therefore, trabectedin has a high antitumor activity in these cells. (b) On the other hand, DNA damage response might be dependented on ATR in low-SLFN11 expression cells, the inhibition of the DNA repair system by the ATR inhibitor berzosertib leads to enhancement of the antitumor activity of trabectedin. (c) The restoration of SLFN11 expression by the DNA methyltransferase inhibitor decitabine also leads to enhancement of the antitumor activity of trabectedin. Therefore, a combination therapy of trabectedin and berzosertib or decitabine is promising against low-SLFN11 expression tumors.*

inhibitor and the EZH2 inhibitor induce *SLFN11* expression in several cancer cell lines (18, 27). Induction of *SLFN11* expression by decitabine in a sarcoma cell line and the enhancement of the antitumor activity of trabectedin by combination with decitabine were observed in the present study. These results suggest that combination treatment consisting of DNA methyltransferase inhibitor and DNA-damaging anticancer drugs, including trabectedin, could be a promising treatment against *SLFN11*-hypermethylated cells (Figure 8).

Recently, Murai *et al*. have shown that SLFN11 is recruited to stressed replication forks carrying extended RPA filaments where it blocks replication by changing the chromatin structure across replication sites independently of HRR (22). This might be the reason why the antitumor activity of trabectedin in high *SLFN11* expression cells shows a higher sensitivity to trabectedin, compared with *SLFN11*-deficient cells. Furthermore, the report suggests that in a DNA repair system, such as HRR, DNA damage response might be dependented on ATR in low SLFN11 expression cells, and the combination of DNA-damaging anticancer drugs including trabectedin and ATR inhibitors seems promising against *SLFN11*-deficient cells (Figure 8).

The present study suggests the importance of *SLFN11* expression as a key factor in the antitumor activity of trabectedin. Furthermore, the combination of trabectedin and an ATR inhibitor or DNA methyltransferase inhibitor was useful against *SLFN11*-deficient cells, in which trabectedin has a low antitumor activity.

Conflicts of Interest

Junya Iwasaki, Toshiharu Komori, Fumio Nakagawa, Hideki Nagase, Junji Uchida, and Kenichi Matsuo are employees of Taiho Pharmaceutical Co., Ltd. Yoshihiro Uto has no potential conflicts of interest to report.

Authors' Contributions

JI and TK designed the study. JI, TK and FN performed experiments. JI analyzed the data and draft the manuscript. HN, JU, KM and YU supervised the work and helped draft and revise the manuscript. All Authors read and approved the final manuscript.

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