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Disulfiram potentiates the anticancer effect of cisplatin in atypical teratoid/rhabdoid tumors (AT/RT)

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Running Title: Combination effect of disulfiram and cisplatin in AT/RT

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Abstract

Atypical teratoid/rhabdoid tumor (AT/RT) is the most malignant tumor of the central nervous system that generally occurs in young children. Despite the use of intensive multimodal therapy for AT/RT, the prognosis is still poor. The brain tumor initiating cells in AT/RT cells has been suggested as one of the challenges in AT/RT treatment. These cells have high expression of aldehyde dehydrogenase (ALDH). We investigated the combination effect of the ALDH inhibitor, disulfiram and cisplatin in the treatment of AT/RT cells. Isobologram analysis revealed that the combination therapy synergistically increases AT/RT cell death. The enzyme activity of ALDH AT/RT cells was effectively reduced by the combination therapy. We proposed that the synergistic augmentation occurs, at least partially through an increase in cleaved Poly (ADP-ribose) polymerase (PARP)-dependent apoptosis mediated by activating transcription factor 3 (ATF3). In AT/RT mouse model, the combination therapy decreased tumor volume and prolonged the survival. Immunofluorescence assay in mouse brain tissues were consistent with expression of ATF3 and cleaved PARP. Our study demonstrates enhanced anti-cancer effect of combination therapy of disulfiram and cisplatin. This combination might provide a viable therapeutic strategy for AT/RT patients.

Keyword: AT/RT, ALDH, Disulfiram, Cisplatin, Combination therapy, ATF3

Introduction

Atypical teratoid/rhabdoid tumor (AT/RT) represents one of the most malignant brain tumors in children under three years of age [1]. Because AT/RT is very aggressive, most patients are treated with intensive multimodal therapy [2, 3]. Despite countless advances in therapeutic

agents, the survival rate of AT/RT remains < 30 % and is even lower for patients under the age of 3 [4, 5] .

Distinct subpopulations of undifferentiated cells, termed cancer stem cells (CSCs) or tumor-initiating cells (TICs), have been suggested to be responsible for drug resistance and cancer recurrence [6-8]. These cells exhibit high expression levels of aldehyde dehydrogenase (ALDH) in AT/RT cells [9]. In our previous study, we confirmed that disulfiram, an ALDH inhibitor approved by the U.S. Food and Drug Administration (FDA), modulates stemness and has anticancer effects in the AT/RT experimental model [10, 11].

Cisplatin (*cis*-diamminedichloroplatinum; CDDP) is one of the most extensively used platinum-based compounds, and it exerts clinical activity against a wide spectrum of solid tumors, including AT/RT [12, 13]. Cisplatin often leads to initial therapeutic success associated with partial response or disease stabilization [14, 15]. However, the limitation of the clinical utility of cisplatin as an anticancer agent is resistance in some tumors [16] and dose-dependent nephrotoxicity [17-19]. Increasing evidence has suggested that cisplatin resistance is associated with CSCs [20-22]. Notably, breast cancer-derived ALDH-positive stem-like cells have been reported to play an important role in cisplatin resistance, and treatment with disulfiram increases the cisplatin sensitivity of ALDH-positive cells [23].

Our aim was to assess the effectiveness of combination therapy of disulfiram and cisplatin in AT/RT cells *in vitro* and *in vivo*. Furthermore, we investigated the potential signaling pathway affected by this combination that leads to anticancer effects.

Materials and methods

Cell cultures

AT/RT tumor tissues (N = 4, Supplementary Table S1) were collected from patients who underwent initial surgery at Seoul National University Children's Hospital upon receipt of the appropriate written consent approved by the Institutional Review Board (IRB #1801-117-917). After confirmation of the pathologic diagnosis of AT/RT, the cells were isolated and cultured as described previously [21]. AT/RT subgroups were identified by immunohistochemistry in patient tissues (Supplementary Fig. S1). The established AT/RT cell lines BT12 (high Tyr and c-Myc, low n-Myc and Gli2) and BT16 (high Gli2, c-Myc but low MITF) were received from Nationwide Children's Hospital. Human neural stem cell line, HB1.F3 was used as a normal control for cell viability analysis. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM, WelGENE, Daegu, Korea) containing 10 % fetal bovine serum (FBS; Gibco, Grand Island, NY) and 1× antibiotic-antimycotic. The primary cultured cells were used under passage number 7. The cells were incubated at 37°C and 5 % CO_2 in an incubator.

Cell growth inhibition by disulfiram and anticancer drugs

Disulfiram, cisplatin, and etoposide were purchased from Selleckchem (Houston, TX), and 4-hydroperoxycyclophosphamide (4-HC, an activated form of cyclophosphamide) was purchased from Cayman Chemicals (Ann Arbor, MI). All drugs were diluted in dimethyl sulfoxide (DMSO, Sigma Aldrich, St Louis, MO) except cisplatin, which was prepared in DMEM, and aliquots prepared were stored at −80°C. The primary AT/RT cells (N = 4), established AT/RT cell lines (N = 2) and HB.F3 cells (N = 1) were cultured and seeded 24 h prior to drug treatment. Cells were exposed to drugs (0, 0.1, 1, 5, 10, 20, 50 and 100 μM) for 72 h. Cells grown in media containing an equivalent amount of DMSO without any drug were used as a control. Cell viability was assessed by the EZ-Cytox assay (Daeil Lab, Seoul, Korea) according to the

manufacturer's protocol. The median inhibitory concentration (IC_{50}) values of each drug were determined and calculated by nonlinear regression analysis using Prism software (La Jolla, CA). The results are shown as a percentage of cell viability in treated cells compared with untreated control. For further experiments after the IC_{50} analysis, two primary cultured AT/RT cells (SNUH.AT/RT09 and SNUH.AT/RT11) and two established AT/RT cell lines (BT12 and BT16) were used.

Isobologram analysis

To evaluate the dose-response of disulfiram-based combination therapy (disulfiram with cisplatin, etoposide or 4-HC), the corresponding combination was prepared proportionally at a 1:1 ratio (0, 0.1, 1, 5, 10, 20, 50 and 100 μM). The synergy, additivity or antagonism in the different combinations was calculated on the basis of the multiple drug effect equation and quantitated by the combination index (CI) with fraction affected (Fa) > 0.5 according to the Chou-Talalay algorithm utilizing the CompuSyn software (Paramus, NJ, www.combosyn.com) [24, 25]. The CI values were interpreted as synergistic (CI < 1), additive (CI = 1) and antagonistic (CI > 1). Fa < 0.5 was considered irrelevant because it indicates lower growth inhibition, and a large fraction of the cell population indicated growth.

Aldefluor assay

An Aldefluor assay (STEMCELL Technologies, WA) was conducted as described previously [26]. Cells were stained with the ALDH inhibitor diethylaminobenzaldehyde (DEAB) as a negative control. Flow cytometry was used to acquire data by using a FACS caliber cytometer (BD Biosciences, San Jose, CA). Aldefluor fluorescence was calculated at 488 nm, and

fluorescence emission was detected using a standard fluorescein isothiocyanate (FITC) 530/30-nm bandpass filter.

ALDH activity assay

The ALDH enzymatic activity assay was performed by using a colorimetric ALDH activity assay kit (Abcam, Cambridge, MA) following the manufacturer's instructions. Briefly, the cells (2×10^6) were harvested after disulfiram and/or cisplatin treatment. The NADH standard was treated into a 96-well plate, and 50 µl/well of ALDH assay buffer was added. The cells were mixed and ALDH assay buffer and incubated for 20-60 min at RT, and the absorbance of the end products was measured at 450 nm. The ALDH activity was expressed in nmol/min/ml.

Western blot analysis

After drug treatment, proteins were lysed using radioimmunoprecipitation assay buffer (RIPA, GenDEPOT, Texas), and western blotting was performed as previously reported [21]. Primary antibodies against the following targets were used: pDNA-PKcs (1:1,000, Abcam), γ-H2AX (1:2,000, Abcam), phospho-p53 (p-p53, 1:1,000, Abcam), phospho-C-Jun (p-C-Jun, 1:1,000, Abcam), C-Jun (1:1,000, Abcam), P38 (1:1,000, Abcam), phospho-p38 (p-p38, 1:1,000, Abcam), activating transcription factor 3 (ATF3, 1:200, Cell Signaling), cleaved poly(ADP-ribose) polymerase (PARP, 1:1,000, Abcam), Survivin (1:5,000, Abcam) and β-actin (1:5,000, Sigma-Aldrich). Band density was analyzed via Image J software. Data were normalized according to the corresponding β-actin levels.

Orthotopic AT/RT xenograft mouse model and combination therapy with disulfiram and cisplatin

The mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC number 18-0020-C1A0) at the Seoul National University Hospital. Seven-week-old female BALB/c-nude mice were anesthetized by intraperitoneal (i.p.) injection of 30 mg/kg Zoletil and 10 mg/kg xylazine. BT16 cells expressing luciferase (BT16-effluc) were used for bioluminescence imaging as described previously [21]. BT16-effluc cells (1.2×10^6 in 3 µl of PBS) were injected via a stereotaxic device into the brains using a Hamilton syringe at an injection rate of 1 µl/min. Stereotaxic coordinates were chosen as 1 mm anterior and 2 mm lateral to the bregma and at 3 mm depth from the dura.

Seven days post injection of BT16-effluc cells, the mice were randomly sorted into four groups: saline-treated group (control), disulfiram-treated group (disulfiram), cisplatin-treated group (cisplatin), and disulfiram and cisplatin combination-treated group (disulfiram+cisplatin). The mice were i.p. injected with 25 mg/kg disulfiram [9, 10, 21] for 5 consecutive days and 5 mg/kg cisplatin twice for a period of two weeks, as indicated in the treatment scheme (Supplementary Fig. S2). On the first day, post-disulfiram treatment in the left flank, cisplatin treatment was given in the right flank, and injections of both drugs were given to the combination-treated group, one after the other.

For long-term survival analysis, the survival endpoint was 120 days, and the number of mice used per group was as follows: control (N = 12), disulfiram-treated group (N = 13), cisplatin-treated group (N = 10), and disulfiram and cisplatin combination-treated group (N = 14). Four mice in each group were used for short-term tumor volume analysis with an endpoint of 54 days.

Bioluminescence imaging and survival analysis

Brain tumor growth was monitored via bioluminescence imaging using the Xenogen IVIS®-100 Imaging System by imaging every 7 days until day 63 post injection. For detection of *in vivo* live imaging, the mice received i.p. administration of 150 mg/kg D-Luciferin (Caliper Life Sciences, Hopkinton, MA). After anesthetizing the mice with 2 % isoflurane (Piramal Healthcare, Bethlehem, PA) in 100 % $O_{2,}$ images were acquired by recording the bioluminescent signal for 1-3 mins. The signals were analyzed and quantified by calculating the luminescence intensity in the region of interest (ROI) [21].

Tumor volume and immunofluorescence analysis

The mice were sacrificed for histological analysis 63 days after BT16-effluc cell injection. A cardiac perfusion was performed, and frozen blocks were prepared for sectioning as previously reported [21]. The tissues were stained with hematoxylin and eosin to assess the tumor volume. Immunofluorescence was performed using primary antibodies for the following targets: ALDH1 (1:100, Abcam), pDNA-PKcs (1:1000, Abcam), γH2AX (1:1000, Abcam), ATF3 (1:50, Santa Cruz Biotechnology, TX) and cleaved PARP (1:400, Abcam). The secondary antibody used was Alexa Fluor 594- or 488-conjugated anti-rabbit IgG or 594-conjugated anti-goat IgG (1:500; Invitrogen, Carlsbad, CA), and the sections were mounted with antifading solution containing 4'-6'-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA). Fluorescent images were obtained using fluorescence microscopy (Leica, DMi8, Wetzlar, Germany). Quantification of positively stained cells was performed from a minimum of three randomly stained slides.

Statistical analysis

Data were calculated as the mean ± standard deviation (SD) or percentage of controls ± SD. Differences between two groups were analyzed using Student's t-test, and multiple groups were assessed using one-way analysis of variance (ANOVA) followed by a post hoc test. The survival data were presented by Kaplan-Meier survival graphs and analyzed by the log-rank test. The statistical analyses were conducted using GraphPad Prism 5 software (La Jolla, CA), and each experiment was performed independently at least 3 times. Differences with values of $P < 0.05$ were considered statistically significant.

Results

Combination therapy with disulfiram and cisplatin has synergistic effects

We first measured IC_{50} values of each drug alone (disulfiram, cisplatin, etoposide, and 4-HC) in four primary AT/RT cell cultures (SNUH.AT/RT05, SNUH.AT/RT08, SNUH.AT/RT09 and SNUH.AT/RT11), two established AT/RT cell lines (BT12 and BT16) and HB1.F3 cells. The mean IC_{50} values the drugs were 12.0 ± 10.8 μM for disulfiram, 34.6 ± 17.0 μM for cisplatin, 16.5 ± 3.8 μM for etoposide and 33.1 ± 30.8 μM for 4-HC in AT/RT cells (Fig. 1A, Supplementary Table S2). Compared to AT/RT cells, HB1.F3 had higher IC_{50} values for drugs except 4-HC (33.1 ± 8.9 μM for disulfiram, 59.0 ± 9.3 μM for cisplatin, 80.0 ± 9.4 μM for etoposide and 36.7 ± 9.4 μM for 4-HC).

To qualitatively evaluate whether the combination of disulfiram with cisplatin or etoposide generates a synergistic cytotoxic effect, the CI and Fa were calculated. The combination therapy showed a synergistic effect in AT/RT11, BT12 and BT16 cells and an additive effect in AT/RT09 cells (Supplementary Fig. S3). At the optimal concentration of the selected

combination, the CI and Fa values of disulfiram and cisplatin were 1.07 and 0.89, respectively, in SNUH.AT/RT09; 0.57 and 0.86 in SNUH.AT/RT11; 0.45 and 0.80 in BT12; and 0.19 and 0.75 in BT16 (Fig. 1B and Table 1). In the combination of disulfiram and cisplatin, an additive effect was observed in SNUH.AT/RT09 cells, but in the combination of disulfiram and etoposide, one of the primary cell cultures (SNUH.AT/RT09) was identified to have an antagonistic effect at all dose combinations. Therefore, cisplatin was chosen as the drug to be combined with disulfiram. Drug doses with the most synergistic effect and overall disulfiram+cisplatin combination doses less than the IC_{50} values were chosen for further experiments (20 μM of disulfiram and 20 μM of cisplatin for SNUH.AT/RT09 and SNUH.AT/RT11 and 5 μM of disulfiram and 5 μM of cisplatin for BT12 and BT16 cells, Fig. 1C).

Combination therapy with disulfiram and cisplatin inhibits ALDH activity

We observed that approximately 2.43 ± 5.52 % ALDH-positive cells in AT/RT cells (3.49 ± 1.84 % in SNUH.AT/RT09, 5.52 ± 2.06 % in SNUH.AT/RT11, 2.43 ± 1.10 % in BT12 and 2.96 ± 1.32 % in BT16 cells; Fig. 2A). Next, we quantitatively analyzed ALDH activity in response to disulfiram or disulfiram and cisplatin combination therapy. As expected, disulfiram single treatment reduced ALDH activity 2-3-fold in SNUH.AT/RT09, SNUH.AT/RT11 and BT16 cells compared to control cells (Fig. 2B). Unlike the results seen in other cells groups, ALDH was not significantly decreased by disulfiram single treatment in BT12 cells. Notably, compared to the control treatment, combination therapy inhibited ALDH activity more effectively in all primary cells cultures and cell lines by approximately 8-fold and 10-fold, respectively (all $p<0.001$, Supplementary Table S3).

Combination therapy with disulfiram and cisplatin induces AT/RT cell death through ATF3 regulation

To understand the molecular mechanism associated with these synergistic effects, we first monitored the alterations of the protein expression involved in several signaling pathways leading to cell death in response to disulfiram or cisplatin [21]. In the majority of AT/RT cells, the combination of disulfiram and cisplatin resulted in a synergistic increase of pDNA-PKcs, γ-H2AX, ATF3 and cleaved PARP expression compared with control and single treatment (Fig. 3A and Supplementary Fig. S4). Survivin was decreased in disulfiram or cisplatin single treatment and no additional change was observed with combination treatment. Compared to the control treatment, disulfiram increased ATF3 protein expression.

On the other hand, cisplatin increased the expression of DNA damage response-related proteins such as DNA-PKcs and γ-H2AX. This change induces the phosphorylation of c-Jun and p53, which increases the protein expression of ATF3. In combination therapy with disulfiram and cisplatin, increased expression of cleaved PARP and ATF3 was suggested to lead to the synergistic effect on apoptosis (Fig. 3B).

Combination therapy with disulfiram and cisplatin increases the survival rate of the AT/RT mouse model

Bioluminescence imaging was used to observe changes in tumor cell growth *in vivo* by measuring the intensity in ROI. The intensity in the ROI of the combination-treated group was significantly decreased over time compared with that in the control, disulfiram or cisplatin single treatment group (Fig. 4A and 4B, Supplementary Table S4 and S5). The analysis of long-term median survival revealed that the combination-treated group (79.5 days) lived significantly

longer than the control (58.5 days, $P < 0.0001$), disulfiram (69 days, $P = 0.027$) and cisplatin (37 days, $P = 0.0002$)-treated groups (Fig. 4C). The long-term survival results demonstrated that combination therapy with disulfiram and cisplatin resulted not only in decreased bioluminescence signal but also longer survival in comparison to those in the single drug treatment groups. In the combination-treated group, mice lost less weight, which indicates that the combination of disulfiram and cisplatin is less toxic than cisplatin treatment alone (Supplementary Fig. S5).

Combination therapy with disulfiram and cisplatin reduces histological tumor volume in the AT/RT animal model

Mouse brain tissues were used for the histopathological and immunofluorescence analyses. The tumor volume of the combination-treated group (3.68 ± 4.39 mm^3) was significantly smaller than that of the control (81.65 ± 3.21 mm^3, $P < 0.001$), disulfiram (47.09 ± 30.84 mm^3, $P < 0.05$) or cisplatin (40.40 ± 16.92 mm^3, $P < 0.05$)-treated groups (Fig. 5A).

Immunofluorescence assays of mouse brain tumor tissues revealed that the expression of ALDH1 was decreased by the combination-treated group and the disulfiram-treated group and that pDNA-PKcs, γH2AX, ATF3, and cleaved PARP expression was significantly increased compared to the control- and single agent-treated groups (Fig. 5B). These results are consistent with protein expression changes observed by western blotting *in vitro*.

Discussion

In this study, we demonstrated that combination therapy with disulfiram and cisplatin had a synergistic anticancer effect on AT/RT. The underlying mechanism of action was assumed to be

increased expression of cleaved PARP with activation of ATF3. Furthermore, the combination therapy effectively reduced tumor volume and increased the overall survival compared to the control and single drug-treated groups *in vivo*.

The poor prognosis of AT/RT is due to the fact that AT/RT develops at a young age, making radiation therapy difficult. Hence, various chemotherapy-based strategies have been tested [24]. Chemotherapies for AT/RT that are currently used include cisplatin, cyclophosphamide, vincristine and etoposide [1]. However, the prognosis of AT/RT remains dismal [4].

ALDH has been described as a CSC marker in various kinds of tumors including breast cancer [25], lung cancer [26], colorectal cancer [27], glioblastoma [28], and AT/RT [9]. In addition, ALDH has shown to be associated with resistance to chemotherapeutic agents, such as cisplatin [29, 30]. We further studied ALDH and its inhibitor, disulfiram, in AT/RT. First, we confirmed the presence of 23.5 % ALDH-positive cells in AT/RT cells cultured in serum-free stem cell culture conditions and also confirmed a reduction in stem cell capabilities by knockdown of ALDH [9]. Second, we verified that the ALDH inhibitor, disulfiram, has anticancer effects, as demonstrated by its modulation of the stemness and metabolism of brain tumor-initiating cells in AT/RT [10]. Third, we proved that disulfiram has a radiosensitizing effect on AT/RT cells [21]. Although combination therapy with disulfiram and radiation showed additive effects, the use of radiation therapy is limited in children under 3 years of age. Therefore, we explored the combination effect of disulfiram and conventional anticancer drugs available to patients with AT/RT.

Cisplatin, etoposide, and cyclophosphamide are commonly applied cytotoxic drugs in AT/RT. Among them, cisplatin was chosen as the most potent combination with disulfiram for the following reasons. (1) The combination of disulfiram with cisplatin was more effective than the

combination with etoposide, at least in the AT/RT cells we used. In the cisplatin combination at a total dose of 40 μM or less, a synergistic effect was observed in three AT/RT cells models and an additive effect was observed in one AT/RT cell model. However, etoposide combination showed a higher CI value than cisplatin at the same dose and an antagonistic effect at all doses in one primary cell culture. (2) There is a possibility that disulfiram can inhibit anticancer drug resistance caused by the ALDH-positive status of cells [30]. (3) Disulfiram may reduce cisplatin-induced acute nephrotoxicity [31]. 4-HC was excluded from further experiments because the IC_{50} of the single treatment was relatively high compared to that of other drugs, and the therapeutic effect of combination therapy with disulfiram was negligible in our previous animal study [10].

Cisplatin is an important component of pediatric chemotherapy regimens used to treat neuroblastoma, osteosarcoma, germ cell tumors, hepatoblastoma, brain tumors, and retinoblastoma [31]. However, the dose-dependent toxicity of cisplatin and its limitations on dose escalation are very well known. Our animal study showed the very intriguing results that mice receiving combination therapy (79.5 days) had higher median survival and significantly smaller tumor volumes, whereas mice receiving the cisplatin single treatment (37 days) had faster weight loss and shorter survival compared to the mice in the control group (58.5 days). Our observation suggests that disulfiram might attenuate the toxicity of cisplatin and should be thoroughly investigated with further experiments.

The mechanism of cisplatin treatment is known to involve the induction of ATF3; ATF3 has a proapoptotic effect through the MAPK pathway, and suppression of ATF3 leads to cisplatin resistance [32-35]. Disulfiram is also reported to be an inducer of ATF3 [36]. Our results

indicate that combination therapy with disulfiram and cisplatin may induce cell death via increased expression of cleaved PARP and activation of ATF3.

To date, phase 2 and phase 3 clinical trials of disulfiram single treatment on other types of cancers [37-39] including glioblastoma[42] and one clinical trial of disulfiram and cisplatin combination therapy in germ cell tumors [40] are ongoing. Although pediatric trials have not yet started, our results demonstrate that disulfiram is a viable option for AT/RT treatment, especially when used with cisplatin. This provides a rationale for further evaluation of disulfiram-based combination therapy for children with AT/RT.

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Conflict of interest statement

None declared.

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Figure Legends

Fig. 1. Combination therapy with disulfiram and cisplatin has a synergistic effect on atypical teratoid/rhabdoid tumor (AT/RT) cells. (A) Cells were treated with increasing concentrations of disulfiram, cisplatin, etoposide, or 4-hydroperoxycyclophosphamide (4-HC). Cell viability was decreased in a dose-dependent manner. (B) The synergistic effects of disulfiram and were are calculated via isobologram analysis. Isobologram analysis representing the individual doses of drugs used to achieve 90 % growth inhibition (Fa = 0.9, green), 75 % growth inhibition (Fa = 0.75, red), and 50 % growth inhibition (Fa = 0.5, blue). In a standard plot, the diagonal line indicates an additive effect, any point below the line shows synergism, and any point above it indicates antagonism. (C) The graphs show that the combination treatment is effective for inhibiting the viability of the majority of AT/RT cells. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

Fig. 2. Combination therapy with disulfiram and cisplatin inhibits aldehyde dehydrogenase (ALDH) activity. (A) ALDH positivity was evaluated by Aldefluor assay. The proportion of primary cultured cells expressing ALDH ranged from 3.49 ± 1.84 % to 5.52 ± 2.06 %, while cell lines showed ALDH positivity ranging from 2.43 ± 1.10 % to 2.96 ± 1.32 %. (B) ALDH enzyme activity was calculated by using an ALDH activity assay kit. Combination therapy with disulfiram and cisplatin effectively inhibits ALDH activity in AT/RT cells. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

Fig. 3. Protein expression after combination therapy with disulfiram and cisplatin. (A) Disulfiram single treatment induces increased expression of the pDNA-PKcs, γ-H2AX and

ATF3. Cisplatin single treatment induces increased expression of the pDNA-PKcs, γ-H2AX, p-p53 and p-c-Jun. Combination therapy with disulfiram and cisplatin resulted in an increase in pDNA-PKcs, γ-H2AX, ATF3 and cleaved PARP compared to control or single treatment. (B) Schematic diagram of the signaling pathways involved in the synergistic effect of combination therapy with disulfiram and cisplatin. Disulfiram and cisplatin can pass through cell membranes using passive diffusion and/or transporters. Disulfiram enters the cell, inhibiting ALDH enzyme activity and increasing ATF3, therefore activating cleaved PARP. Disulfiram can also increase the phosphorylation of DNA-PKcs and γ -H2AX. Cisplatin increases DNA-PKcs and γ-H2AX, thus activating c-Jun and p-53 phosphorylation, which not only increases ATF3 but also decreases survivin by increasing p-p53, thereby resulting in the activation of PARP. In summary, the action of each of these drugs increases ATF3 and synergistically increases cleaved PARP, leading to the apoptosis of AT/RT cells.

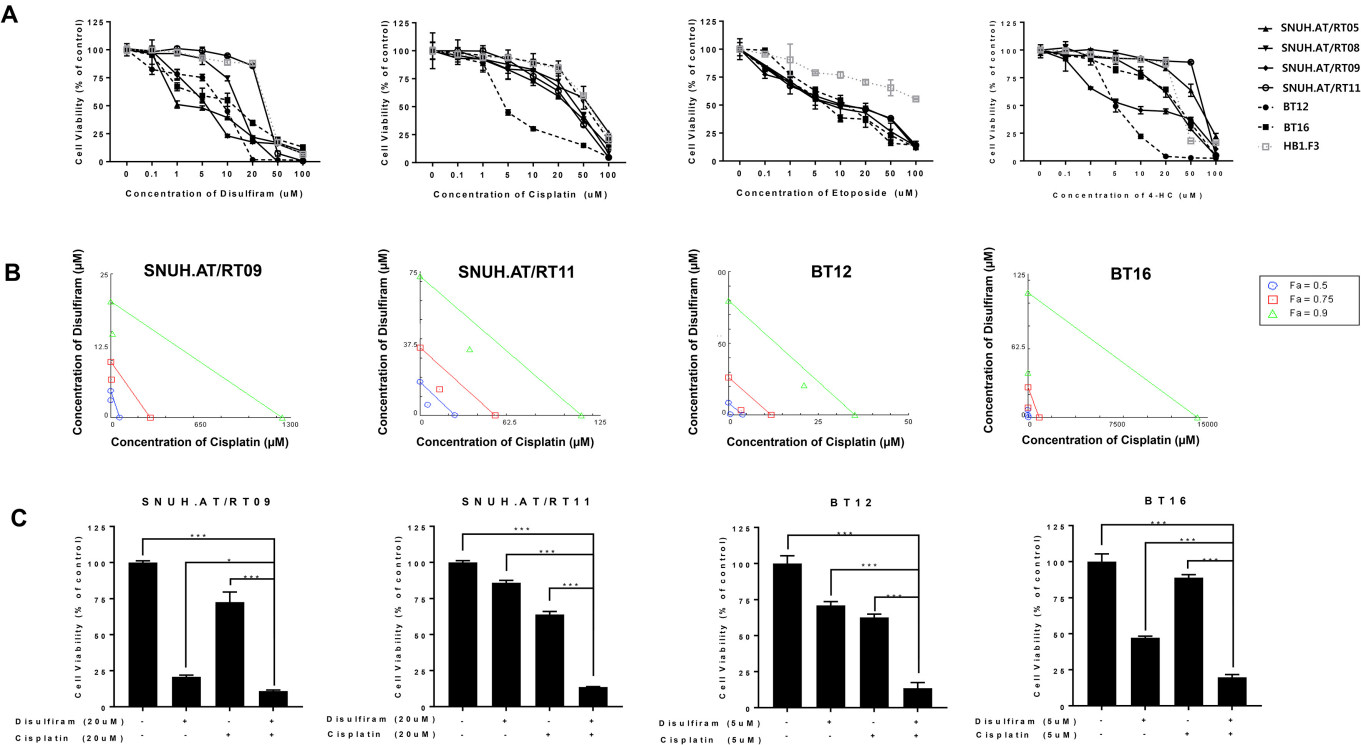
Fig. 4. Long-term therapeutic efficacy of combination therapy with disulfiram and cisplatin in the AT/RT mouse model. (A) Representative bioluminescence imaging (BLI) results obtained at various time points using an IVIS-100 imaging system. (B) Quantification of serial BLI of tumor-occupied areas from *in vivo* live imaging. ***$p < 0.001$. (C) The median survival of the groups is illustrated by Kaplan-Meier survival curves and compared using the log-rank test. The combination-treated group (disulfiram + cisplatin) showed a decreased bioluminescence signal and survival benefit compared with those of the other groups.

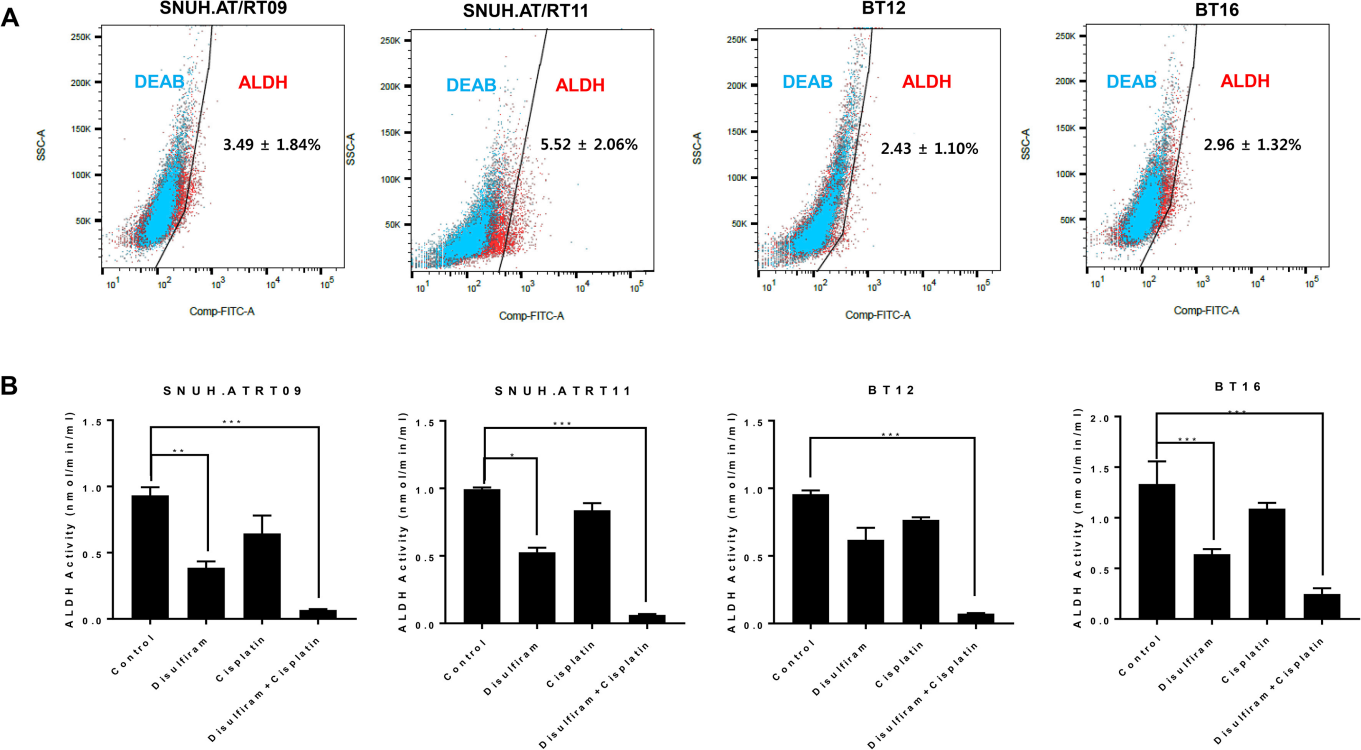
Fig. 5. Short-term therapeutic efficacy of combination therapy with disulfiram and cisplatin in the AT/RT mouse model. (A) Representative histological images and the graph of

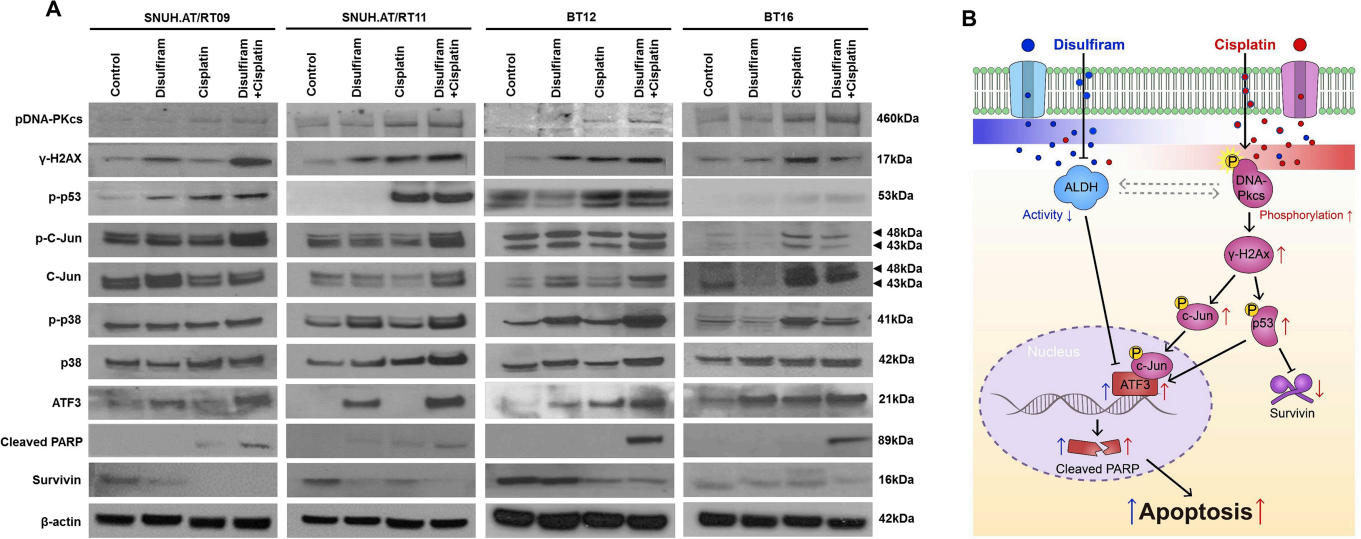
tumor volume. The combination-treated group (disulfiram + cisplatin) showed a significantly smaller tumor volume compared to the other groups. Hematoxylin and eosin (H&E) staining, ×1.25. The outlines indicate tumor margins. (B) Representative immunofluorescence images and quantitative graphs. The combination-treated group (disulfiram + cisplatin) had significantly decreased expression of ALDH1 (green) and increased expression of pDNA-PKcs (red), γH2AX (red), ATF3 (green), and cleaved PARP (red) compared to the other groups. Scale bars: 100 μm. Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (blue). *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

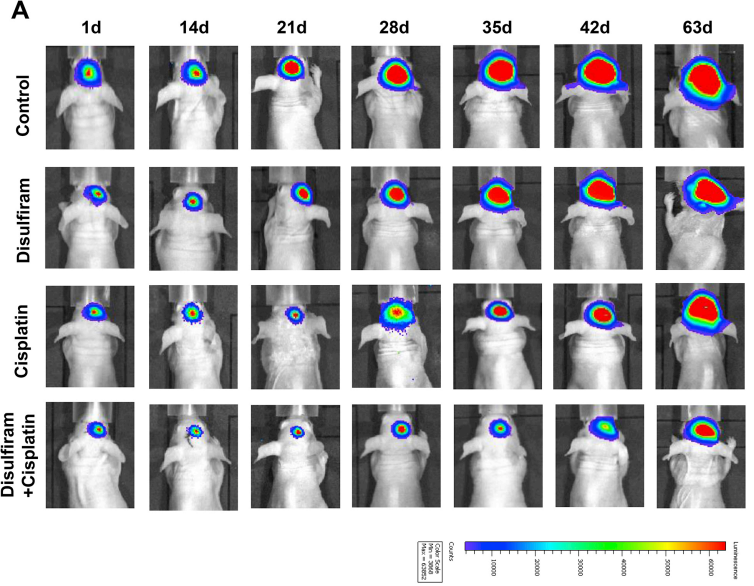
Table 1. Combination index (CI) value in combination therapy with disulfiram and cisplatin.

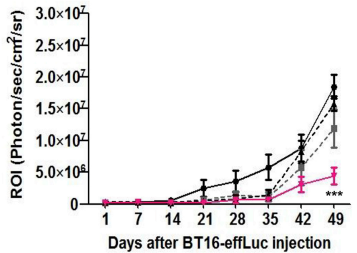
Cells	Disulfiram (μM)	Cisplatin (μM)	Total Dose (μM)	CI value	Fa value	Interpretation
SNUH.AT/RT09	0.1	0.1	0.2	0.36	0.03	Synergistic
	1	1	2	2.04	0.06	Antagonistic
	5	5	10	1.53	0.40	Antagonistic
	10	10	20	1.58	0.63	Antagonistic
	20	20	40	1.07	0.89	Additive
	50	50	100	0.23	1.00	Synergistic
	100	100	200	0.97	0.99	Synergistic
SNUH.AT/RT11	0.1	0.1	0.2	0.10	0.03	Synergistic
	1	1	2	0.62	0.06	Synergistic
	5	5	10	1.62	0.14	Antagonistic
	10	10	20	1.16	0.44	Antagonistic
	20	20	40	0.57	0.86	Synergistic
	50	50	100	0.45	0.97	Synergistic
	100	100	200	0.60	0.99	Synergistic
BT12	0.1	0.1	0.2	0.08	0.31	Synergistic
	1	1	2	0.68	0.35	Synergistic
	5	5	10	0.45	0.80	Synergistic
	10	10	20	0.29	0.93	Synergistic
	20	20	40	0.58	0.93	Synergistic
	50	50	100	1.35	0.93	Antagonistic
	100	100	200	2.47	0.94	Antagonistic
BT16	0.1	0.1	0.2	4.04	0.04	Antagonistic
	1	1	2	0.07	0.65	Synergistic
	5	5	10	0.19	0.75	Synergistic
	10	10	20	0.31	0.78	Synergistic
	20	20	40	0.32	0.86	Synergistic
	50	50	100	0.50	0.89	Synergistic
	100	100	200	0.75	0.91	Synergistic

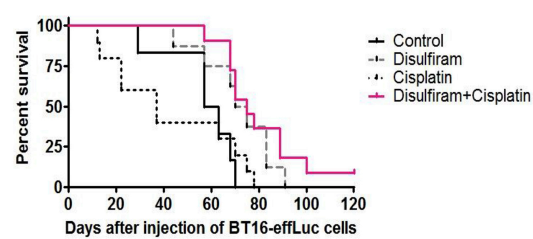

A
Cell Viability (% of control)
Concentration of Disulfiram (uM)
Concentration of Cisplatin (uM)
Concentration of Etoposide (uM)
Concentration of 4-HC (uM)
SNUH.AT/RT05
SNUH.AT/RT08
SNUH.AT/RT09
SNUH.AT/RT11
BT12
BT16
HB1.F3
B
SNUH.AT/RT09
SNUH.AT/RT11
BT12
BT16
Concentration of Disulfiram (μM)
Concentration of Cisplatin (μM)
Fa = 0.5
Fa = 0.75
Fa = 0.9
C
SNUH.AT/RT09
SNUH.AT/RT11
BT12
BT16
Cell Viability (% of control)
Disulfiram (20uM)
Cisplatin (20uM)
Disulfiram (5uM)
Cisplatin (5uM)

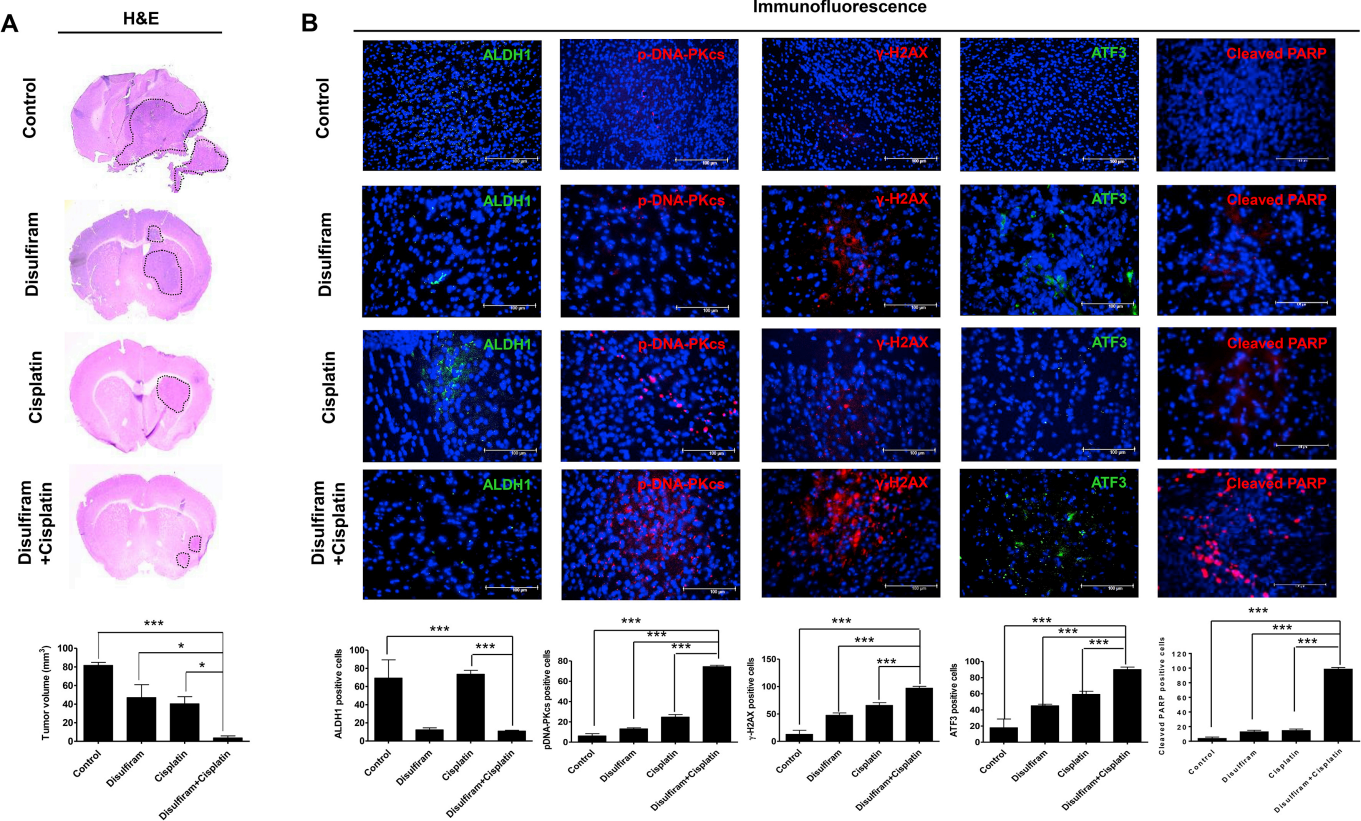

A
SNUH.AT/RT09
DEAB
ALDH
3.49 ± 1.84%
SNUH.AT/RT11
5.52 ± 2.06%
BT12
2.43 ± 1.10%
BT16
2.96 ± 1.32%
SSC-A
Comp-FITC-A
B
SNUH.ATRT09
SNUH.ATRT11
BT12
BT16
ALDH Activity (nmol/min/ml)
Control
Disulfiram
Cisplatin
Disulfiram+Cisplatin


A
SNUH.AT/RT09
SNUH.AT/RT11
BT12
BT16
Control
Disulfiram
Cisplatin
Disulfiram +Cisplatin
pDNA-PKcs
γ-H2AX
p-p53
p-C-Jun
C-Jun
p-p38
p38
ATF3
Cleaved PARP
Survivin
β-actin
460kDa
17kDa
53kDa
48kDa
43kDa
48kDa
43kDa
41kDa
42kDa
21kDa
89kDa
16kDa
42kDa
B
Disulfiram
Cisplatin
ALDH
Activity ↓
DNA-Pkcs
Phosphorylation ↑
γ-H2Ax ↑
c-Jun ↑
p53 ↑
Nucleus
c-Jun
ATF3 ↑
Survivin
Cleaved PARP
↑Apoptosis↑


A
1d
14d
21d
28d
35d
42d
63d
Control
Disulfiram
Cisplatin
Disulfiram +Cisplatin


B
ROI (Photon/sec/cm²/sr)
3.0×10⁷
2.5×10⁷
2.0×10⁷
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
1
7
14
21
28
35
42
49
***
Days after BT16-effLuc injection
Control
Disulfiram
Cisplatin
Disulfiram+Cisplatin


C
Percent survival
100
75
50
25
0
0
20
40
60
80
100
120
Days after injection of BT16-effLuc cells
Control
Disulfiram
Cisplatin
Disulfiram+Cisplatin


A
H&E
Control
Disulfiram
Cisplatin
Disulfiram +Cisplatin
B
Immunofluorescence
ALDH1
p-DNA-PKcs
γ-H2AX
ATF3
Cleaved PARP
Tumor volume (mm3)
ALDH1 positive cells
pDNA-PKcs positive cells
γ-H2AX positive cells
ATF3 positive cells
Cleaved PARP positive cells
Control
Disulfiram
Cisplatin
Disulfiram+Cisplatin

Highlights

- Isobologram analysis reveals that the combination therapy of aldehyde dehydrogenase (ALDH) inhibitor, disulfiram and cisplatin synergistically increases atypical teratoid/rhabdoid tumor (AT/RT) cell death.
- Combination therapy with disulfiram and cisplatin inhibits ALDH activity and induces apoptosis in AT/RT *in vitro* and *in vivo*.
- The underlying mechanism of action is assumed to be increased expression of cleaved PARP with activation of ATF3.

Conflict of interest

The authors declare that there are no conflicts of interest in regard to this study.