

Romidepsin and Tamoxifen Cooperatively Induce Senescence of Pancreatic Cancer Cells Through Downregulation of FOXM1 Expression and Induction of ROS/Lipid Peroxidation

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Abstract

Although progress has been made in chemotherapeutic strategies against pancreatic cancer, overall survival has not significantly improved over the past decade. Thus, the development of better therapeutic regimens remains a high priority. Pancreatic cancer cell lines were treated with romidepsin, an inhibitor of histone deacetylase, and tamoxifen, and their effects on cell growth, signaling and gene expression were determined. Xenografts of human pancreatic cancer CFPAC1 cells were treated with romidepsin and tamoxifen to determine their effects on tumor growth. The inhibition of the growth of pancreatic cancer cells induced by romidepsin and tamoxifen was effectively reduced by N-acetyl cysteine and α -tocopherol, respectively. The combined treatment greatly induced reactive oxygen species production and mitochondrial lipid peroxidation, and these effects were prevented by N-acetyl cysteine and α -tocopherol. Tamoxifen enhanced romidepsin-induced cell senescence. FOXM1 expression was markedly downregulated in pancreatic cancer cells treated with romidepsin, and tamoxifen further reduced FOXM1 expression in cells treated with romidepsin. Siomycin A, an inhibitor of FOXM1, induced senescence in pancreatic cancer cells. Similar results were obtained in knockdown of FOXM1 expression by siRNA. Since FOXM1 is used as a prognostic marker and therapeutic target for pancreatic cancer, a combination of the clinically available drugs romidepsin and tamoxifen might be considered for the treatment of patients with pancreatic cancer.

Introduction

Pancreatic cancer is one of the most aggressive malignancies and the lethality of pancreatic cancer is characterized by poor responses to standard chemotherapy and radiotherapy [1, 2]. The combination of 5-fluorouracil, leucovorin, irinotecan and oxaliplatin (FOLFIRINOX) has recently achieved more effective responses than gemcitabine in patients with advanced pancreatic cancer [3]. However, the use of FOLFIRINOX results in greater toxicity. Therefore, new anticancer drugs have been introduced in the last decade. Despite continuous efforts to develop new agents, none of the drugs have an objective response rate higher than 10% [4–6]. Therefore, the development of new therapeutic strategy is required.

Our recent report indicated that the combination of romidepsin and tamoxifen synergistically induced apoptosis in T-cell malignant cells, suggesting that this combination may be useful for the treatment of T-cell lymphoma patients [7]. Such enhanced activity of drugs used in combination has translated into substantial activity in large clinical trials [8, 9]. Romidepsin is an inhibitor of histone deacetylase and has been approved for the treatment of peripheral T-cell lymphomas. Tamoxifen is a selective estrogen receptor modulator and useful for treatment of estrogen receptor-positive breast cancer. However, anti-tumor effects induced by tamoxifen in estrogen receptor-negative tumors have been reported, suggesting multiple non-estrogen receptor-mediated mechanisms [10, 11].

We examined the combined effects of romidepsin and tamoxifen on pancreatic cancer cells. The effects on pancreatic cancer cells were clearly different from those on T-cell malignant cells. Therefore, in the present investigation, we sought to clarify the synergistic effects of romidepsin and tamoxifen on

pancreatic cancer cells and to examine the therapeutic effects on xenografts of human pancreatic carcinoma cells.

Materials And Methods

Materials

Romidepsin was obtained from Celgene Corporation (Summit, NJ, USA). RPMI 1640 medium, gemcitabine, tamoxifen, paclitaxel, α -tocopherol, TRI reagent, siomycin A, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) and Hoechst 33342 were purchased from Sigma-Aldrich Japan (Tokyo). N-Acetyl-cysteine (NAC) was obtained from Wako Pure Chemical (Tokyo, Japan). Vorinostat (*N*-hydroxy-*N'*-phenyloctanediamide) was purchased from Tokyo Chemical Industry Co. Ltd, Japan. Entinostat and panobinostat were obtained from MedChemExpress (Monmouth Junction, NJ, USA). FOXM1 siRNA and control siRNA were purchased from OriGene Technologies, Inc. (Rockville, MD, USA). MitoPeDPP and a MT-1 MitoMP detection kit were purchased from Dojindo Laboratories (Kumamoto, Japan).

Cells and culture

Human pancreatic cancer cell lines (Panc1 [RRID:CVCL_0480], CFPAC1 [RRID:CVCL_1119], MIAPaCa2 [RRID:CVCL_0428], BxPC3 [CVCL_0186] and Capan2 [RRID:CVCL_0026] were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Sigma-Aldrich Japan) supplemented with 10% fetal bovine serum and 80 μ g/ml gentamicin at 37°C in a humidified atmosphere of 5% CO₂ in air.

Assay of cell growth

Human cancer cells were seeded into 24-well multidishes at 1.0×10^4 cells/ml/well. The cells were cultured with various concentrations of drugs for 4-6 days. After culture with or without drugs, viable cells were examined by the MTT assay [12].

Assay of cell senescence

To examine cell morphology, cells were stained with Giemsa. Senescence-associated β -galactosidase activity was measured using a Senescence detection kit I (PromoKine, Heidelberg, Germany) according to the instructions provided by the manufacturer.

Assay of the cumulative cell number

The cell density of the drug-treated cells was kept at $2-8 \times 10^4$ /ml to maintain the growth phase in a 24-well multidish. The medium of treated cultures was replaced by fresh medium with drugs at least every 4 or 5 days to remove cell debris from dead cells. The viable cell number was measured by the MTT assay. The cumulative cell number was calculated from the MTT values and the dilution used when feeding the culture.

Transplantation Of Human Cancer Cells Into Nude Mice

Four-week-old female athymic nude mice with a BALB/c genetic background were obtained from CLEA Japan (Tokyo) and housed under specific pathogen-free conditions. Mice were subcutaneously inoculated with 2×10^6 CFPAC1 cells (0.1 ml of 2×10^7 cells/ml). Mice were given an intraperitoneal injection of 2 mg/kg romidepsin in 0.1 ml PBS and/or a subcutaneous injection of 15 mg/kg tamoxifen in 0.1 ml corn oil, with the first treatment given 5 days after the inoculation of tumor cells. Treatment was performed three times per week. Tumor volume was measured with vernier calipers. Mice were sacrificed by exposure to CO₂ without removing animals from their home cage with a flow rate displacing 20% of the cage volume per minute. Death was confirmed by observation of pupil dilation as well as ceasing of breath (absence of chest fluctuation) and the heartbeat. Our protocol was approved by the animal ethics committee at Shimane University.

cDNA microarray analysis

Gene expression was measured using the Affymetrix Human Genome U133 Plus 2.0 array and the prescribed procedure outlined in the Affymetrix technical manual. Gene expression levels were normalized using the robust multiarray average method (Takara Bio Inc., Tokyo, Japan). Array data have been deposited in the Gene Expression Omnibus, accession number GSE182526.

Reverse Transcription-polymerase Chain Reaction (Rt-pcr)

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using TRI reagent (Sigma). Total RNA was converted to first-strand cDNA primed with random hexamer in a reaction volume of 20 μ l using an RNA PCR kit (qPCR RT Master Mix, Toyobo Co. LTD, Osaka, Japan), and 2 μ l of this reaction was used as a template in real-time PCR. The qPCR conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. The levels of FoxM1 mRNA were quantified using the $2^{-\Delta\Delta CT}$ method. The threshold cycle values were normalized to the threshold value of glyceraldehyde-3-phosphate dehydrogenase. Primers for the reactions are shown in Table S1.

Western blot

Cells were packed after being washed with cold PBS and then lysed at 1.5×10^7 cells/ml in sample buffer. The resultant lysate was resolved on SDS-polyacrylamide gel, as described elsewhere [7]. Primary antibodies were obtained from the following sources: anti-FOXM1, anti-FOXN3 and anti-FOXO3 antibodies (Abcam, Tokyo, Japan), anti-acetyl histone H3 (Lys9/27) and histone H3 antibodies (Medical & Biological Laboratories, Nagoya, Japan).

SiRNA And Transfection

Panc1 cells were seeded at 5×10^4 cells/well in 24-well plates, and incubated for 4 days in culture medium in the presence of 20 nM siRNA directed against FOXM1. The cells were transfected using Lipofectamine RNAiMAX Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The cells were seeded onto plates and transiently transfected with the desired concentrations of FOXM1 siRNA in Opti-MEM medium (Life Technologies Corp., Grand Island, NY, USA). Sequences of siRNA are shown in Supplementary Table S1. All experiments were conducted in triplicate using independent cultures.

Measurements of intracellular reactive oxygen species (ROS), mitochondrial lipid peroxidation (LPO) and membrane potential

The production of ROS was monitored using dihydroethidium (Muse Oxidative Stress kit, Millipore, Billerica, MA, USA). After incubation, fluorescence images were captured using an automated cellular imaging system (IN Cell Analyzer 2000, Cytiva, Tokyo, Japan) and ROS-positive cells were calculated. LPO was determined using MitoPeDPP [13]. Cells were washed twice with PBS, and then treated with 0.5 μ M MitoPeDPP in Hanks' HEPES buffer for 15 min at 37°C under dark conditions. After being washed once with PBS, the cells were treated with or without romidepsin and/or tamoxifen. After treatment, fluorescence images were captured using an automated cellular imaging system (IN Cell Analyzer 2000) and LPO was evaluated. The mitochondrial membrane potential was measured using an MT-1 MitoMP Detection kit (Dojindo) according to the instructions provided by the manufacturer. The cells were stained for detection of nuclei (Hoechst 33342) immediately before image acquisition. After treatment, fluorescence images were captured using an automated cellular imaging system (IN Cell Analyzer 2000) and mitochondrial membrane potential was evaluated.

Statistical analysis

The results are expressed as means \pm standard deviation (SD). Pairs of data were compared using Student's t-test. Significant differences were considered to exist for probabilities below 5% ($P < 0.05$). For the in vivo experiment, an F-test was performed to demonstrate statistical significance. Significant differences were considered to exist for probabilities below 5%.

Results

Tamoxifen enhanced growth inhibition and cell senescence in pancreatic cancer cells treated with romidepsin

Romidepsin inhibited the growth of pancreatic cancer Panc1 cells and the sensitivity to romidepsin was similar to that of MOLT4 (T-cell malignant) cells (Fig. 1A). When MOLT4 cells were treated with more than 2 nM romidepsin, most of the cells were induced to undergo apoptosis. However, some Panc1 cells were still alive in more than 2 nM romidepsin (Fig. 1B). The treated Panc1 cells were induced to undergo senescence (Fig. 1C and D). Tamoxifen effectively enhanced growth inhibition and β -galactosidase activity in Panc1 cells (Fig. 1E and F). Similar results were obtained when other pancreatic cancer cells were treated with romidepsin and tamoxifen (Fig. 1G). Since romidepsin is an inhibitor of histone deacetylase, we examined the combined effects of other histone deacetylase inhibitors and tamoxifen on the growth of Panc1 cells. As shown in Fig. 2A, the other inhibitors did not effectively cooperate in inhibiting the growth of Panc1 cells, suggesting that the combined effects of romidepsin and tamoxifen are independent of the inhibition of histone deacetylation. A previous report indicated that romidepsin induces apoptosis of HL-60 leukemia cells through the generation of hydrogen peroxide, and this effect is prevented by N-acetyl-cysteine (NAC) [14]. Therefore, we examined the effect of NAC on growth inhibition induced by romidepsin and other histone deacetylase inhibitors. NAC effectively prevented the growth inhibition induced by romidepsin, but not those induced by other inhibitors (Fig. 2B). Since α -tocopherol (a lipophilic antioxidant) effectively inhibited the growth inhibition of pancreatic cancer cells induced by tamoxifen [15], we next examined the effect of α -tocopherol on the growth inhibition induced by tamoxifen or romidepsin. The growth-inhibitory effect of tamoxifen was prevented by α -tocopherol, but not by NAC (Fig. 2C). The growth-inhibitory effect was hardly affected by α -tocopherol (Fig. 2D). The growth-inhibiting effect of romidepsin plus tamoxifen was effectively reduced by a combination of NAC and α -tocopherol (Fig. 2E).

Romidepsin was administered as a 4-hour intravenous infusion, and the mean maximum plasma concentration at the maximum tolerated dose was 472.6 ng/ml (range: 249-577.8 ng/ml) [16]. Therefore, we examined the effect of pulse treatment with high concentrations of romidepsin on the growth of Panc1 cells in the presence or absence of tamoxifen. The cells were treated with 200 or 400 ng/ml (370 or 740 nM) romidepsin for 4 hours, and then washed once with PBS. The washed cells were cultured without romidepsin for 18 days. The pulse treatment with 400 ng/ml romidepsin did not produce 100% killing in Panc1 cells, and the treated cells still proliferated. Tamoxifen significantly inhibited the growth of romidepsin-treated cells. Although the growth of romidepsin-treated Panc1 cells was observed at day 15-18, combined treatment with tamoxifen significantly reduced the viable cell numbers (Fig. 3A).

The *in vitro* studies described above suggested that the combined treatment should be more effective therapeutically than treatment with romidepsin alone. Since CFPAC1 cells were the most resistant to romidepsin in inhibiting cell growth (Fig. 1G), we examined the therapeutic effects of romidepsin and tamoxifen on xenografts of CFPAC1. Although romidepsin alone retarded tumor growth, combined treatment was even more effective (Fig. 3B). Statistical analysis revealed that the difference was significant ($P < 0.05$ at day 28, combined treatment versus control), although the effects of single-agent treatments were not statistically significant (Fig. 3B and C).

The combined effects of romidepsin, tamoxifen and anticancer drugs were examined. A triple combination (romidepsin, tamoxifen and gemcitabine) cooperatively inhibited the growth of Panc1 cells (Fig. 3D). The growth of Panc1 cells was markedly reduced by treatment with 0.6 nM paclitaxel for 4 days. However, 2 weeks after washing out, growing cells reappeared and the proliferation rate of the treated cells became similar to that of untreated cells. Drug-resistant cells were not observed when cells were cultured for 28 days with 1 nM romidepsin plus 3 μ M tamoxifen, and dual combinations (paclitaxel and then romidepsin or paclitaxel and then tamoxifen) were less effective (Fig. 3E).

Gene-expression Profiling Predicts Foxm1 Signaling As A Mediator

To obtain a picture of the putative underlying mechanisms of cell death, gene expression analyses were conducted using a cDNA arrays to determine the profiles in Panc1 cells treated with romidepsin and tamoxifen. We compared gene-expression profiles among untreated control, romidepsin-treated, tamoxifen-treated, and romidepsin plus tamoxifen-treated cells. Many more genes were upregulated and downregulated upon exposure to romidepsin plus tamoxifen than with romidepsin or tamoxifen alone. Whereas romidepsin and tamoxifen individually upregulated 5535 and 2877 genes and downregulated 3258 and 1225 genes, respectively, combined treatment upregulated 7768 and downregulated 4437 genes. Genes that regulate several signaling pathways including FOXO signaling were significantly upregulated and downregulated by the combined treatment (Supplementary Fig. S1). We focused on FOX family genes due to their crucial roles in cell proliferation, senescence, survival and cell cycle control [17–19]. The expression of FOXM1 was the most downregulated by combined treatment among members of the FOX gene family (Supplementary Fig. S1). To confirm the results of the cDNA microarray analysis, the expression of FOXM1, FOXO3 and FOXN3 genes was examined by RT-PCR (Fig. 4A). Since FOXO3 antagonizes FOXM1 function by competing for the same target genes [20, 21], we examined the ratio of FOXO3 to FOXM1 mRNA expression (Fig. 4B). Tamoxifen alone hardly affected this ratio, but greatly enhanced the ratio induced by romidepsin. FOXM1 mRNA expression was downregulated early in cells treated with romidepsin plus tamoxifen (Fig. 4C). Although the upregulation of FOXO3 mRNA expression was confirmed in Panc1 cells, upregulation of FOXO3 and FOXN3 proteins was modest in Panc1 cells (Fig. 4D). On the other hand, FOXM1 protein was greatly downregulated upon exposure to romidepsin plus tamoxifen compared to with romidepsin or tamoxifen alone (Fig. 4D), suggesting that downregulation of FOXM1 is an early and important event in pancreatic cancer cells treated with romidepsin plus tamoxifen.

Cell senescence restricts tumor proliferation, but can also promote cancer progression and metastasis [22–25]. Senescent cells secrete a variety of pro-inflammatory factors in the tumor microenvironment that can support the survival, outgrowth and migration of tumor cells. Thus, we examined the combined effects of romidepsin and tamoxifen on mRNA expression of pro-inflammatory cytokines and chemokines in Panc1 cells (Supplementary Fig. S2A). Although romidepsin induced mRNA of some chemokines, tamoxifen suppressed the expression induced by romidepsin. Guccini et al. indicated that

TIMP1 controls the switch from tumor-controlling to tumor-promoting senescence [26]. Since TIMP1 deletion allows senescence to promote metastasis in prostate cancer cells, we next examined the expression of TIMP1 mRNA in Panc1 cells treated with romidepsin and tamoxifen (Supplementary Fig. S2B). The cells treated with romidepsin plus tamoxifen maintained a high level of expression.

Inhibition of FOXM1 reduces cell proliferation and induces cell senescence

Romidepsin concentration-dependently induced histone acetylation in Panc1 cells, but tamoxifen hardly affected romidepsin-induced histone acetylation (Supplementary Fig. S2C). Activated caspase-3 was hardly observed in the cells treated with romidepsin plus tamoxifen, suggesting that the effects of romidepsin and tamoxifen were not associated with caspase-dependent apoptosis.

Siomycin A is a potent inhibitor of FOXM1 transcriptional inhibitor and reduces the FOXM1 protein level in many cancer cells [27]. Siomycin A concentration-dependently inhibited the growth of MIA-PaCa2 cells (Fig. 4E) and induced cell senescence (Fig. 4F). Cell senescence was confirmed by β -galactosidase staining (Supplementary Fig. S3A). Similar results were obtained in other pancreatic cancer cells (Panc1 and CFPAC1). The growth-inhibitory effects were prevented by treatment with α -tocopherol (Fig. 4E). ROS-positive cells in untreated, siomycin A-treated, α -tocopherol and siomycin A & α -tocopherol-treated cells were 2.3 ± 0.3 , 67.9 ± 7.1 , 1.1 ± 0.1 , and $2. \pm 4$ %, respectively, suggesting that ROS production is associated with the growth inhibition. Siomycin A decreased the level of FOXM1 protein in MIA-PaCa2 cells (Fig. 4G). To determine the role of FOXM1 gene expression in cell survival, siRNAs corresponding to the nucleotide sequences from FOXM1 and a negative siRNA were transiently transfected into Panc1 cells. FOXM1 siRNAs reduced the induction of FOXM1 protein in cells treated with romidepsin plus tamoxifen compared to the control siRNA (Fig. 4H). Although the viability of control siRNA-transfected cells was not significantly different from that of untreated cells, the viability of FOXM1 siRNA-transfected Panc1 cells was significantly lower than that of the control siRNA-transfected cells (Fig. 4I) and the treated cells were induced to undergo senescence (Fig. 4J). Cell senescence was confirmed by β -galactosidase staining (Supplementary Fig. S3B).

Romidepsin and tamoxifen synergistically trigger ROS accumulation, mitochondrial LPO and decrease in mitochondrial membrane potential in pancreatic cancer cells

The growth-inhibitory effects of romidepsin and tamoxifen were reversed by NAC and α -tocopherol, respectively (Fig. 2). Therefore, we measured ROS levels in romidepsin and/or tamoxifen-treated pancreatic cells. Figure 5A shows that combined treatment with romidepsin and tamoxifen results in a marked increase in ROS generation. ROS generation was prevented by treatment with NAC plus α -tocopherol (Fig. 5B). ROS accumulation was observed in cells treated for 9-12 hours (Fig. 5C). LPO in mitochondria was evident in cells treated with romidepsin and tamoxifen for 24 h (Fig. 5D) and LPO was prevented by NAC and α -tocopherol (Fig. 5E). Loss of mitochondrial membrane potential was significantly observed in the cells treated with romidepsin plus tamoxifen (Fig. 5F).

Discussion

A previous report indicated that combined treatment with romidepsin and tamoxifen induces apoptosis in T-cell malignant cells [7]. However, this treatment induced senescence in pancreatic cancer cells, suggesting that it had a different mode of action. Oncogenic Ras stimulates FOXM1 expression by increasing ROS. Elevated FOXM1, in turn, downregulates ROS levels by stimulating the expression of ROS scavenger genes [28]. FOXM1 plays an important role in pancreatic cancer progression. Romidepsin strongly inhibited FOXM1 expression in pancreatic cancer cells, whereas downregulation of FOXM1 expression by romidepsin in T-cell malignant cells was modest. On the other hand, upregulation of FOXO1 expression was evident in T-cell malignant cells treated with romidepsin [7].

FOXM1 plays important roles in the initiation, progression and metastasis of a variety of human tumors including pancreatic cancer [29–32]. Xia et al. demonstrated that FOXM1 mRNA and protein expression was higher in pancreatic tumors than in paired adjacent normal pancreatic tissue specimens [30]. Multivariate analysis revealed that FOXM1 expression is a prognostic marker and therapeutic target for pancreatic cancer [30].

FOXM1 expression is induced by oncogenes and oncogenic signals, and inhibited by tumor suppressor genes. FOXM1 may participate in the integration of various oncogenic and anti-oncogenic signals [32]. Since FOXO3 antagonizes FOXM1 function by competing the same target genes, a loss or gain of the function of FOXM1-FOXO3 axis can alter cell fate [20]. FOXM1 promotes cancer progression while FOXO3 plays opposing roles by modulating cell cycle arrest, cell death, senescence and drug sensitivity. FOXM1 induces its own transcript, and inhibitors of FOXM1 not only inhibit FOXM1 activity as a transcription factor, they also downregulate the mRNA and protein of FOXM1, and it may bind to its own promoter [32]. These data suggest that a positive autoregulatory loop of FOXM1 expression exists in pancreatic cancer cells. FOXM1 is a key regulator of ROS in dividing cells, and controls oxidative stress to escape premature senescence [28]. Depletion of FOXM1 by treatment with siomycin A or siRNA induces cell senescence in pancreatic cancer cells (Fig. 4). Downregulation of FOXM1 by romidepsin plus tamoxifen may be a critical event in inhibiting cell growth and inducing cell senescence of pancreatic cancer cells.

Recent reports have demonstrated that leukemia inhibitory factor (LIF) plays a crucial role in pancreatic cancer progression and LIF blockademay be an attractive approach to improving therapeutic outcomes [33, 34]. LIF production was greatly inhibited by romidepsin treatment (Supplementary Fig. S2A). Moreover, pro-inflammatory cytokines such as IL-1 β and IL-6 were not induced by treatment with romidepsin and/or tamoxifen (Supplementary Fig. S2A). Both senescence-associated phenotype genes and senescence core signature genes are largely regulated by FOXM1, and lack of FOXM1 expression leads to full senescence phenotypes in fibroblasts [35]. The senescence-associated phenotypes produced by romidepsin plus tamoxifen may not be associated with the survival and migration of cancer cells.

Tamoxifen induced cell death in many cancer cells by multiple non-estrogen receptor-mediated mechanisms [10]. To understand the growth-inhibitory effect of tamoxifen on pancreatic cancer cells, we examined the effects of various compounds including estrogen and inhibitors of signaling on the growth

of Panc1 cells. Estrogen did not affect the growth-inhibitory effect of tamoxifen, suggesting a non-estrogen receptor-mediated mechanism. Several inhibitors of protein kinases also had no effect, whereas α -tocopherol effectively reduced tamoxifen-induced growth inhibition, suggesting association with ROS production.

Romidepsin alone might inhibit FOXM1 activity and then downregulate mRNA and protein of FOXM1 by an autoregulatory loop of FOXM1 expression. Tamoxifen may affect FOXM1 expression through induced ROS production. Combined treatment with romidepsin plus tamoxifen rapidly and effectively downregulated mRNA and protein expression of FOXM1 in pancreatic cancer cells. ROS generation was markedly observed at 9-12 hours after treatment. This was followed by LPO in mitochondria and loss of mitochondrial membrane potential (Fig. 5G). Activated caspase-3 was not observed in the cells treated with romidepsin and/or tamoxifen (Supplementary Fig. S2C). The pan-caspase inhibitor Z-VAD-FMK hardly affected the combined effects. These results suggest that caspase-dependent apoptosis is not associated with the combined treatment. Inhibitors of necroptosis (Nec-1) or ferroptosis (Ferr-1) also had no effect on the combined treatment.

FOXM1 increases the cancer stem cell population, drives proliferation, motility and invasiveness, and confers resistance to therapy [29, 31, 32]. Therefore, targeting FOXM1 activity is of great importance and may benefit patients with pancreatic cancer that is activated by oncogenic Ras and has elevated FOXM1 expression. Since both romidepsin and tamoxifen are clinically available, their combined treatment may become a novel therapeutic strategy against pancreatic cancer.

Declarations

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

All authors contributed to the manuscript as follows: Conceptualization, NO, YH, TU and KT; methodology, NO, YH and TU; investigation, NO and YH; writing of original draft, NO and YH; writing, revision and editing, YH, TU and KT; funding and supervision, TU and KT. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of Shimane University (approval no. IZ2-110) and were conducted in accordance with the Japanese laws associated with animal experiments, the institutional regulations of Shimane University and the Science Council of Japan guidelines for proper conduct of animal experiments.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Effect of romidepsin on growth of MOLT4 (T-cell malignant) and Panc1 (pancreatic cancer) cells. Cells were treated with various concentrations of romidepsin for 4 days (A, B). Morphological changes (C) and senescence (D) of romidepsin-treated cells. Panc1 cells were treated with (ii) or without (i) 2 nM romidepsin for 6 days. C, Giemsa staining; D, β -galactase staining. E, Combined effects of romidepsin and tamoxifen on viability of CFPAC1 cells. Cells were treated with various concentrations of romidepsin

in the presence of 0 - 3 μM tamoxifen for 6 days. F, Effect of tamoxifen on romidepsin-induced cell senescence. Panc1 cells were cultured with 1 nM romidepsin (ii, iv) and 4 μM tamoxifen (iii, iv) for 4 days. G, Effect of tamoxifen on IC50 of romidepsin on pancreatic cancer cell lines. Cells were cultured with an IC50 concentration of romidepsin in the presence of 0 - 3 mM tamoxifen for 4 days. Cell viability was assayed by the MTT test. All experiments were performed thrice. Results are presented as the means \pm SD of three separate experiments. * $p < 0.05$, ** $p < 0.01$.

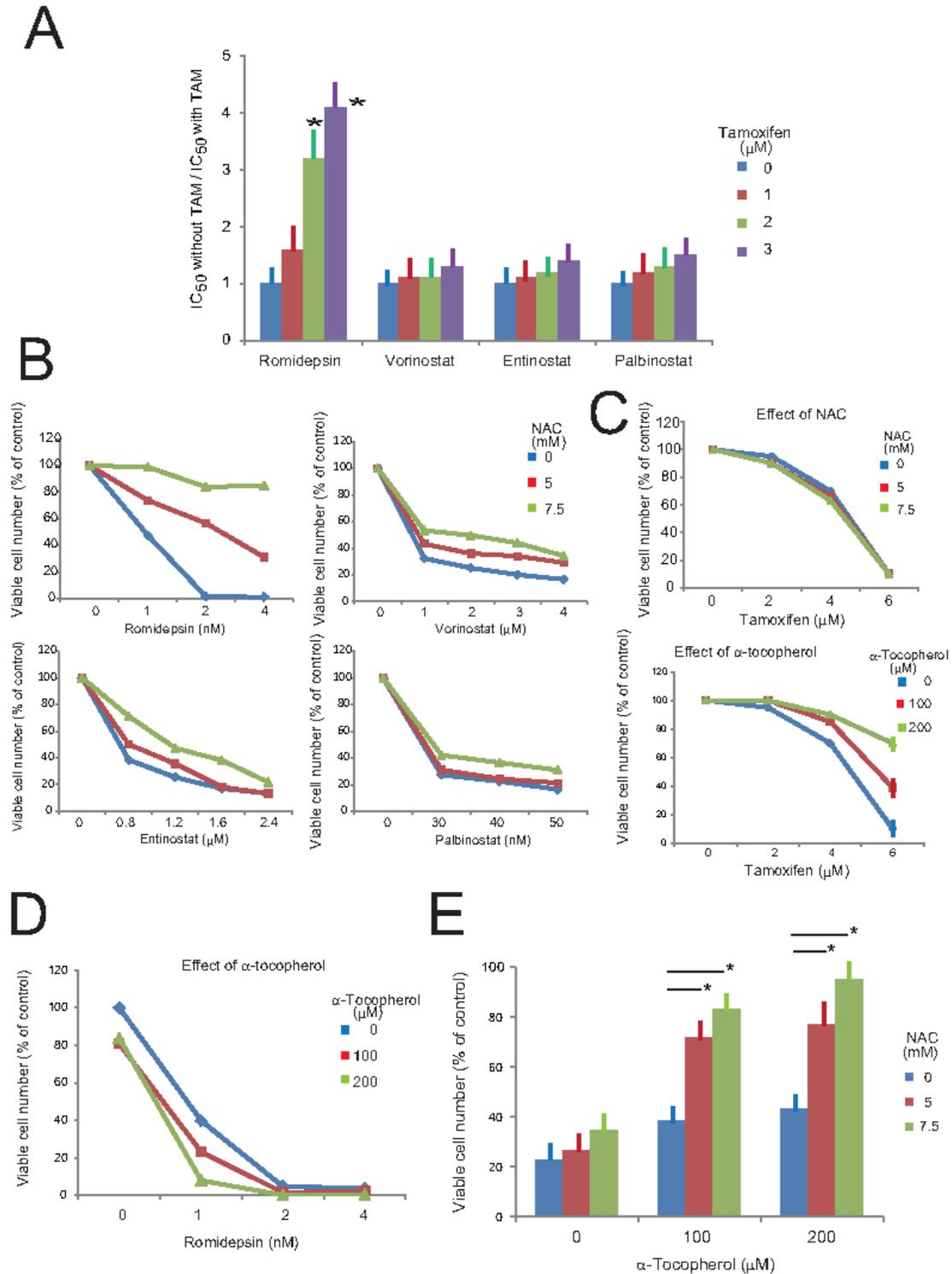


Fig. 2

Figure 2

Effects of tamoxifen on the growth of Panc1 cells in the presence of other histone deacetylase inhibitors, and effects of NAC and α -tocopherol on the growth inhibition by romidepsin and/or tamoxifen. A, Cells were cultured with IC50 concentrations of romidepsin, vorinostat, entinostat or palbinostat in the presence of various concentrations of tamoxifen for 5 days. B, Effect of NAC on the growth inhibition by histone deacetylase inhibitors. Cells were treated with various concentrations of the inhibitors in the presence of 0, 5 or 7.5 mM NAC for 5 days. C, Effect of NAC or α -tocopherol on the growth inhibition by tamoxifen. Panc1 cells were cultured with various concentrations of tamoxifen in the presence of NAC or α -tocopherol for 4 days. D, Effect of α -tocopherol on the growth inhibition by romidepsin. E, NAC and α -tocopherol prevented the growth inhibition by romidepsin plus tamoxifen. Panc1 cells were treated with 4 nM romidepsin plus 1 μ M tamoxifen in the presence of various concentrations of NAC and/or α -tocopherol for 3 days. Results are presented as the means \pm SD of three separate experiments. * $p < 0.05$.

Figure 3

A, Irreversible suppression of proliferation of Panc1 cells by pulse treatment with romidepsin plus continuous treatment with tamoxifen. Panc1 cells were treated with 0, 400, or 800 ng/ml romidepsin for 4 hours and then washed with PBS. The cells were treated with or without 4 mM tamoxifen. The culture medium was replaced by fresh medium with tamoxifen every 5 days and the cell density was kept so as to maintain the growth phase. B, Effect of romidepsin and tamoxifen on the growth of CFPAC1 cells as xenografts. C, Tumor weight at day 28. The values are the means \pm SD of five separate experiments. * $p < 0.05$. D, Combined effects of romidepsin, tamoxifen and gemcitabine on the growth of Panc1 cells. Cells were cultured with various concentrations of gemcitabine plus 1 μ M tamoxifen in presence of 0 (⊗), 0.2 (■), 0.4 (▲) or 0.6 (●) nM romidepsin for 5 days. E, Combined treatment with romidepsin and tamoxifen prevents the appearance of paclitaxel-resistant cells. Panc1 cells were treated with 0.6 nM paclitaxel for 4 days and then washed once with PBS. The treated cells were cultured without or with 1 nM romidepsin and/or 3 μ M tamoxifen. The culture medium was replaced by fresh medium with drugs every 5 days and the cell density was kept so as to maintain the growth phase. Results are presented as the means \pm SD of three separate experiments. * $p < 0.05$.

Figure 4

Downregulation of FOXM1 in pancreatic cancer cells treated with romidepsin plus tamoxifen. A, FOXM1 and FOXO3 mRNA expression in Panc1 cells. B, Ratio of FOXO3 mRNA to FOXM1 mRNA. C, Downregulation of the FOXM1 mRNA level in Panc1 cells by 1 nM romidepsin plus 4 μ M tamoxifen for the indicated periods of time. D, Western blot analyses for FOXM1, FOXO3 and FOXN3 proteins. Panc1 cells were treated with the indicated concentrations of romidepsin and/or tamoxifen for 3 days. E, Effect of siomycin A on the growth of Panc1 cells with or without α -tocopherol. Cells were treated with various concentrations of siomycin A in the absence or presence of 100 μ M α -tocopherol for 3 days. Results are presented as the means \pm SD of three separate experiments. F, Morphological changes in siomycin A-

treated cells (ii). i, untreated cells. G, Western blots for FOXM1 expression of Panc1 cells treated with siomycin A. H, Western blots for FOXM1 knockdown in Panc1 cells. I and J, FOXM1 knockdown reduced cell growth (I) and induced morphological changes to cell senescence (J, treated with control siRNA(i) or siRNAC(ii)). Cells were transiently transfected with a nonspecific siRNA or the FOXM1-specific siRNA. Cells were subsequently cultured for 2 days. All experiments were performed thrice. Results are presented as the means \pm SD of three separate experiments. * $p < 0.05$, ** $p < 0.01$.

Figure 5

Effects of combined treatment with romidepsin and tamoxifen on ROS generation, mitochondrial lipid peroxidation and reduction of mitochondrial membrane potential of pancreatic cancer cells. A, Panc1 cells were treated with romidepsin and/or tamoxifen for 12 hours. ROS production was monitored using dihydroethidium. All experiments were performed thrice. B, Antioxidants prevent ROS generation in Panc1 cells treated with 2 nM romidepsin and/or 2 μ M tamoxifen in the presence or absence of 7.5 mM NAC and 200 μ M α -tocopherol for 12 hours. All experiments were performed thrice. C, Time course of ROS accumulation in MIAPaCa2 and Panc1 cells treated with 2 nM romidepsin and/or 2 μ M tamoxifen. Results are presented as the means \pm SD of three separate experiments. D and E, Mitochondrial lipid peroxides were monitored using a MitoPeDPP kit. Panc1 cells were treated with romidepsin and/or tamoxifen in the presence or absence of 7.5 mM NAC and 200 μ M α -tocopherol for 24 hours. F, Mitochondrial membrane potential was monitored using MT-1 and Hoechst 33342 reagents. Panc1 cells were treated with romidepsin and/or tamoxifen for 24 hours. All experiments were performed thrice. G, Events in pancreatic cancer cells treated with romidepsin plus tamoxifen.

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