

The heat shock protein 90 inhibitor SNX5422 has a synergistic activity with histone deacetylase inhibitors in induction of death of anaplastic thyroid carcinoma cells

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Abstract The influence of the heat shock protein 90 (hsp90) inhibitor SNX5422 alone or in combination with the histone deacetylase (HDAC) inhibitors PXD101, suberoylanilide hydroxamic acid (SAHA), and trichostatin A (TSA) on survival of anaplastic thyroid carcinoma (ATC) cells was investigated. In 8505C and CAL62 cells, SNX5422 caused cell death with concomitant changes in the expression of hsp90 client proteins. After treatment of both SNX5422 and PXD101, SAHA and TSA, compared with treatment of SNX5422 alone, cell viability was diminished, whereas inhibition rate and cytotoxic activity were enhanced. All of the combination index values were lower than 1.0, suggesting the synergism between SNX5422 and PXD101, SAHA and TSA in induction of cell death. In cells treated with both SNX5422 and PXD101, SAHA and TSA, compared with cells treated with SNX5422 alone, the protein levels of Akt, phospho-4EBP1, phospho-S6 K, and survivin were diminished, while those of γ H2AX, acetyl. histone H3, acetyl. histone H4, cleaved PARP, and cleaved caspase-3 were enhanced. In conclusion, these results demonstrate that SNX5422 has a cytotoxic activity in conjunction with alterations in the expression of hsp90 client proteins in ATC cells. Moreover, SNX5422 synergizes with HDAC inhibitors in induction of cytotoxicity accompanied by the suppression of PI3K/Akt/mTOR signaling and survivin, and the over-expression of DNA damage-related proteins in ATC cells.

Keywords Anaplastic thyroid carcinoma · SNX5422 · HDAC inhibitor · Synergism · Akt

Abbreviations

17-AAG	17-allylamino-17-demethoxygeldanamycin
ATC	Anaplastic thyroid carcinoma
CI	Combination index
cIAP	Cellular inhibitor of apoptosis protein
DMSO	Dimethylsulfoxide
ED ₅₀	The concentrations of each drug required for 50 % inhibition
FTC	Follicular thyroid carcinoma
HDAC	Histone deacetylase
Hsp70	Heat shock protein 70
Hsp90	Heat shock protein 90
IAP	Inhibitor of apoptosis protein
PARP	Poly(ADP-ribose) polymerase
PTC	Papillary thyroid carcinoma
γ H2AX	Phospho-histone H2A.X
SAHA	Suberoylanilide hydroxamic acid
TSA	Trichostatin A
xIAP	X-linked inhibitor of apoptosis protein

Introduction

Anaplastic thyroid carcinoma (ATC) is the most lethal thyroid malignancy because of extrathyroidal invasion, distant metastasis, and resistance to conventional therapies [1]. Clinically significant improvement of survival rate in ATC has not been achieved despite multimodal strategies, and thus new therapeutic agents are under investigation [1].

Heat shock protein 90 (hsp90) plays essential roles in conformation, stabilization, activation, and localization of

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oncogenic client proteins such as Akt and survivin regulating survival, growth and invasion in malignant cells [2]. Several hsp90 inhibitors as single agents or in combination therapy are undergoing clinical trials in cancer patients [3]. In this regard, we previously reported that the hsp90 inhibitors 17-allylamino-17-demethoxygeldanamycin (17-AAG), NVP-AUY922, and radicicol alone or in combination with chemotherapeutic agents induced cytotoxicity in ATC cells [4–6].

SNX5422 (PF-04929113), prodrug of SNX2112 (PF-04928473), is a potent, highly selective small molecule inhibitor of hsp90, and binds to the NH₂-terminal nucleotide site of hsp90 [7–9]. In phase I trials, SNX5422 exhibited significant responses with tolerable toxicity in refractory solid tumors and hematologic malignancies [10–12]. However, the effect of SNX5422 alone or in combination with chemotherapeutic agents on survival of ATC cells has not been evaluated.

Histone deacetylase (HDAC) results in the deacetylation of target proteins [13]. HDAC inhibitors cause the acetylation of histone proteins, and enhance DNA damage response [13]. The HDAC inhibitors PXD101, suberoylanilide hydroxamic acid (SAHA), and valproic acid in combination with chemotherapeutic agents have a cytotoxic activity in thyroid cancer cells [5, 14–17]. Considering that HDAC inhibitors mitigate the chaperone function of hsp90 [18], combination of hsp90 inhibitors with HDAC inhibitors is expected to improve the therapeutic efficiency achieved by each drug. Although our previous study showed that NVP-AUY922 synergized with PXD101 in induction of death of ATC cells [5], the impact of SNX5422 in combination with HDAC inhibitors on survival of ATC cells has not been identified.

The aim of the present study was to elucidate the effect of SNX5422 alone or in combination with HDAC inhibitors on survival of ATC cells. Our results clarify that SNX5422 induces cytotoxicity accompanied by modulation of hsp90 client proteins, and has a synergistic activity with HDAC inhibitors in induction of cytotoxicity in ATC cells.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and streptomycin/penicillin were obtained from Life Technologies (Carlsbad, CA, USA). The hsp90 inhibitors 17-AAG and SNX5422 were purchased from Selleck Chemicals (Houston, TX, USA), while the HDAC inhibitors PXD101, SAHA, and trichostatin A (TSA) were obtained from Sigma (St. Louis, MO,

USA). These were dissolved in dimethylsulfoxide (DMSO), which was provided to the control within permissible concentrations. The final concentration of the vehicle DMSO in the control did not exceed 0.1 % in all treatments. The primary antibodies raised against hsp90, hsp70, Raf-1, GSK3 β , phospho-4EBP1 (Ser65), phospho-S6 K (Thr389), survivin, γ H2AX, acetyl. histone H3, acetyl. histone H4, cleaved poly(ADP-ribose) polymerase (PARP), and cleaved caspase-3 were purchased from Cell Signaling Biotechnology (Danvers, MA, USA). The primary antibodies raised against total and phospho-Akt (Ser473) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the primary antibody raised against β -actin from Sigma were obtained. All other reagents were purchased from Sigma unless otherwise stated.

Cell culture

For experiments, human ATC cell lines of 8505C and CAL62 cells were used. 8505C and CAL62 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ GmbH, Braunschweig, Germany), and grown in DMEM supplemented with 10 % heat-inactivated FBS and 1 % streptomycin/penicillin. Cells received fresh medium at regular intervals. Treatments and experiments were performed using cells that were 70 % confluent.

Cell viability assay

Cell viability was determined by the CCK-8 Assay Kit (Dojindo laboratories, Kumamoto, Japan). Cells (5×10^3 /100 μ l) in each well on 96-well plates were incubated overnight, and treated with the drugs for an additional 4 h at 37 °C. Absorbance was measured at 450 nm using a spectrophotometer (Molecular Devices, Palo Alto, CA, USA). All experiments were performed in triplicate.

FACS analysis

The dead cells were analyzed by the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences Pharmingen, San Diego, CA, USA). Cells (1×10^5 /ml) in each well on 6-well plates were incubated, harvested, washed, and fixed according to manufacturer's protocol. FITC annexin V and/or propidium iodide (PI) in 1 \times binding buffer was added for 15 min at room temperature, and analysis was made using a FACSort flow cytometry (Becton–Dickinson, San Jose, CA, USA) and CellQuest software program (Becton–Dickinson, San Jose, CA, USA). The percentage of dead cells was calculated according to the following equation: dead cells (%) = [(annexin V-stained cells + PI-stained

cells + annexin V- and PI-stained cells)/all cells \times 100]. All experiments were performed in triplicate.

Cytotoxicity assay

Cytotoxic activity was measured by the LDH Cytotoxicity Assay Kit (BioVision, Linda, CA, USA). Cells (5×10^3 /100 μ l) in each well on 96-well plates were incubated, and centrifuged at 250 g for 10 min. Supernatant of 100 μ l was transferred in clear 96-well plates. After addition of reaction mixture (2.5 μ l Catalyst solution in 112.5 μ l Dye solution), cells were incubated for 30 min at room temperature. Absorbance was measured at 495 nm using a spectrophotometer. All experiments were performed in triplicate.

Drug combination analysis

Combination index (CI) and isobologram were calculated by CalcuSyn program version 2.11 (Biosoft, Great Shelford, Cambridge, UK), and the effect of drug interactions was quantitatively determined. CI values less than 1.0, 1.0, and greater than 1.0 indicate synergism, additivity, and antagonism, respectively. The isobologram is formed by plotting the doses of each drug required for 50 % inhibition (ED_{50}) on the x- and y-axis, and connecting them to draw a line segment, which is ED_{50} isobologram. Combination data points that fall on below and above the line segment demonstrate additivity, synergism, and antagonism, respectively. All reactions were performed in triplicate.

Western blotting

Cells were lysed in RIPA buffer (Sigma) containing 1 \times protease inhibitor cocktail and 1 \times phosphatase inhibitor cocktail set V (Calbiochem, La Jolla, CA, USA). Protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL, USA). Equivalent amounts of protein (50 μ g) were separated by 10 % SDS-PAGE, and transferred to Immobilon-P Membrane (Millipore, Bedford, MA, USA). Western blotting was performed using specific primary antibodies and horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies. Bands were detected using ECL Plus Western Blotting Detection System (Thermo Fisher Scientific, Rockford, IL, USA). All reactions were performed in triplicate.

Statistical analysis

All data are expressed as mean \pm standard error (S.E.). Data were analyzed by unpaired Student's *t* test or ANOVA as appropriate. A *p* value less than 0.05 was

considered to be statistically significant. All analyses were performed using SPSS program version 21.0 (SPSS, Chicago, IL, USA).

Results

SNX5422 induces cell death with concomitant regulation of hsp90 client proteins in ATC cells

In our previous studies, the hsp90 inhibitors 17-AAG, NVP-AUY922, and radicicol caused cell death in conjunction with modulation of hsp90 client proteins in ATC cells [4–6]. In this study, we investigated the influence of SNX5422 on cell survival and the expression of hsp90 client proteins in ATC cells.

First, cells were treated with 17-AAG and SNX5422 at 0.5, 1, 2, 5, and 10 μ M for 72 h, and cell viability and the percentage of dead cells were measured using CCK-8 assay and FACS analysis, respectively. After treatment, SNX5422, compared with 17-AAG, decreased cell viability (Fig. 1a), and increased the percentage of dead cells (Fig. 1b) in a dose-dependent manner.

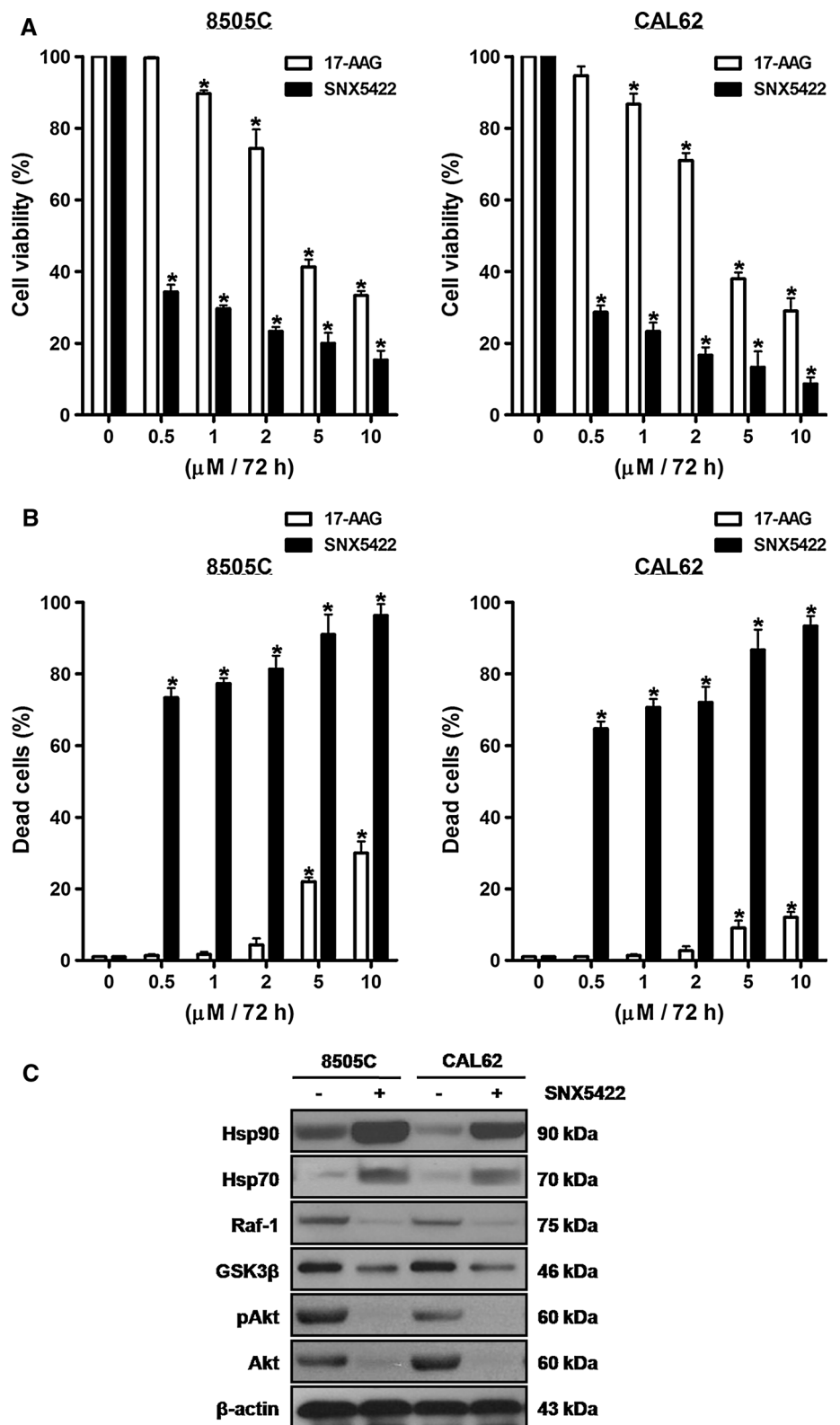
Next, when cells were treated with SNX5422 at 5 μ M for 72 h, the protein levels of hsp90 and hsp70 increased, whereas those of Raf-1, GSK3 β , and total and phospho-Akt decreased.

SNX5422 in combination with HDAC inhibitors synergistically induces cell death by suppression of PI3K/Akt/mTOR signaling in ATC cells

We previously reported that the hsp90 inhibitor NVP-AUY922 in combination with the HDAC inhibitor PXD101 synergistically resulted in death of ATC cells [5]. In this study, SNX5422 led to death of ATC cells, and thus the combined effect of SNX5422 with the HDAC inhibitors PXD101, SAHA, and TSA on survival of ATC cells was evaluated.

Cells were simultaneously treated with both SNX5422 and the HDAC inhibitors, and the interactions between SNX5422 and the HDAC inhibitors were analyzed. After cotreatment, cell number was counted using a hemacytometer, and inhibition rate was calculated as follows: inhibition rate (%) = (cell number before treatment—cell number after treatment)/cell number before treatment \times 100. All of the CI values were lower than 1.0 (Table 1 and 2), with averages at ED_{50} of 0.53 (PXD101), 0.52 (SAHA), and 0.44 (TSA) in 8505C cells (Fig. 2a), and of 0.34 (PXD101), 0.49 (SAHA), and 0.21 (TSA) in CAL62 cells (Fig. 2b). The combination data

Fig. 1 The influence of SNX5422 on cell survival and the expression of hsp90 client proteins in ATC cells. **a, b** 8505C and CAL62 cells were treated with 17-AAG and SNX5422 at 0.5, 1, 2, 5, and 10 μ M for 72 h, and cell viability and the percentage of dead cells were measured using CCK-8 assay and FACS analysis, respectively. **c** 8505C and CAL62 cells were treated with SNX5422 at 5 μ M for 72 h, and the protein levels of hsp90, hsp70, Raf-1, GSK3 β , and total and phospho-Akt were measured. All experiments were performed in triplicate. The blots are representative of independent experiments. Data are expressed as mean \pm SE. * p < 0.05 versus each matched control



points in the isobologram were all located below the isobologram line at ED₅₀, suggesting the synergism between SNX5422 and the HDAC inhibitors in induction of death of ATC cells.

To obtain additional evidences for the synergism, cells were treated with both SNX5422 and the HDAC inhibitors at 5 μ M for 24 h, and cell viability and cytotoxic activity were measured using CCK-8 assay and cytotoxicity assay,

Table 1 CI values at combined concentrations determined by the median effect analysis method in 8505C cells

PXD101, SAHA, TSA (μM)	SNX5422 (μM)	PXD101 + SNX5422	SAHA + SNX5422	TSA + SNX5422
1	1	0.607	0.616	0.554
3	3	0.776	0.788	0.676
5	5	0.454	0.524	0.352
7	7	0.319	0.330	0.166

CI combination index

Table 2 CI values at combined concentrations determined by the median effect analysis method in CAL62 cells

PXD101, SAHA, TSA (μM)	SNX5422 (μM)	PXD101 + SNX5422	SAHA + SNX5422	TSA + SNX5422
1	1	0.229	0.275	0.191
3	3	0.615	0.593	0.471
5	5	0.798	0.764	0.586
7	7	0.883	0.895	0.578

CI combination index

respectively. After cotreatment of both drugs, compared with treatment of each drug, cell viability was reduced (Fig. 3a), and cytotoxic activity was elevated (Fig. 3b).

In our previous study, synergistic activity between NVP-AUY922 and PXD101 was mediated by inactivation of PI3K/Akt signaling [5], and thus whether the synergism between SNX5422 and the HDAC inhibitors is related to PI3K/Akt signaling was examined.

In treatment of both SNX5422 and the HDAC inhibitors at 5 μM for 24 h, compared with treatment of SNX5422 at 5 μM for 24 h alone, the protein levels of phospho-4EBP1 and phospho-S6 K as well as total and phospho-Akt were reduced (Fig. 3c), implying that SNX5422 has a synergistic activity with the HDAC inhibitors by repression of PI3K/Akt/mTOR signaling in ATC cells.

The synergism between SNX5422 and HDAC inhibitors goes with the inactivation of survivin and the activation of DNA damage-related proteins in ATC cells.

Hsp90 inhibitors induce the activation of γH2AX , a marker of double-stranded DNA breaks during DNA damage response, and HDAC inhibitors induce the activation of DNA damage response as well as the acetylation of histone H3 and histone H4 [13, 19–21]. Meanwhile, survivin, a client protein of hsp90, inhibits the caspase-dependent apoptotic pathway, and is considered as a resistance factor to chemotherapeutic agents [22–25].

In this study, after treatment of SNX5422 at 5 μM for 24, 48, and 72 h, and at 1, 5, and 10 μM for 48 h, the protein levels of survivin and γH2AX were enhanced (Fig. 4a). In cells treated with both SNX5422 and the HDAC inhibitors at 5 μM for 24 h, compared with cells

treated with SNX5422 alone at 5 μM for 24 h, survivin protein levels were diminished, whereas the protein levels of γH2AX , acetyl. histone H3, acetyl. histone H4, cleaved PARP, and cleaved caspase-3 were enhanced (Fig. 4b).

Discussion

Hsp90 inhibitors disrupt multiple targets on signal transduction pathway, and thereby induce cytotoxicity in cancer cells [26]. Among hsp90 inhibitors, SNX5422 poses a potent cytotoxic activity in various malignancies [7–12]. In this regard, we previously reported that 17-AAG, NVP-AUY922, and radicicol induced cytotoxicity with concomitant regulation of hsp90 client proteins in ATC cells [4–6]. However, the influence of SNX5422 on cell survival and the expression of hsp90 client proteins have not been investigated in ATC cells. In the present study, SNX5422, compared with 17-AAG, potentially resulted in cell death with changes in the expression of hsp90 client proteins including Akt in ATC cells. Our data demonstrate for the first time that SNX5422 induces cell death with modulation of hsp90 client proteins in ATC cells. Furthermore, these results suggest that SNX5422 may have clinical implications for the treatment of human ATC, which is resistant to conventional therapies.

The combined regimens consisted of HDAC inhibitors and chemotherapeutic agents lead to cytotoxicity in thyroid cancer cells [5, 14–17]. The combination of PXD101 with docetaxel, paclitaxel, and carboplatin has a cytotoxic activity in thyroid cancer cells, and the combined effect is

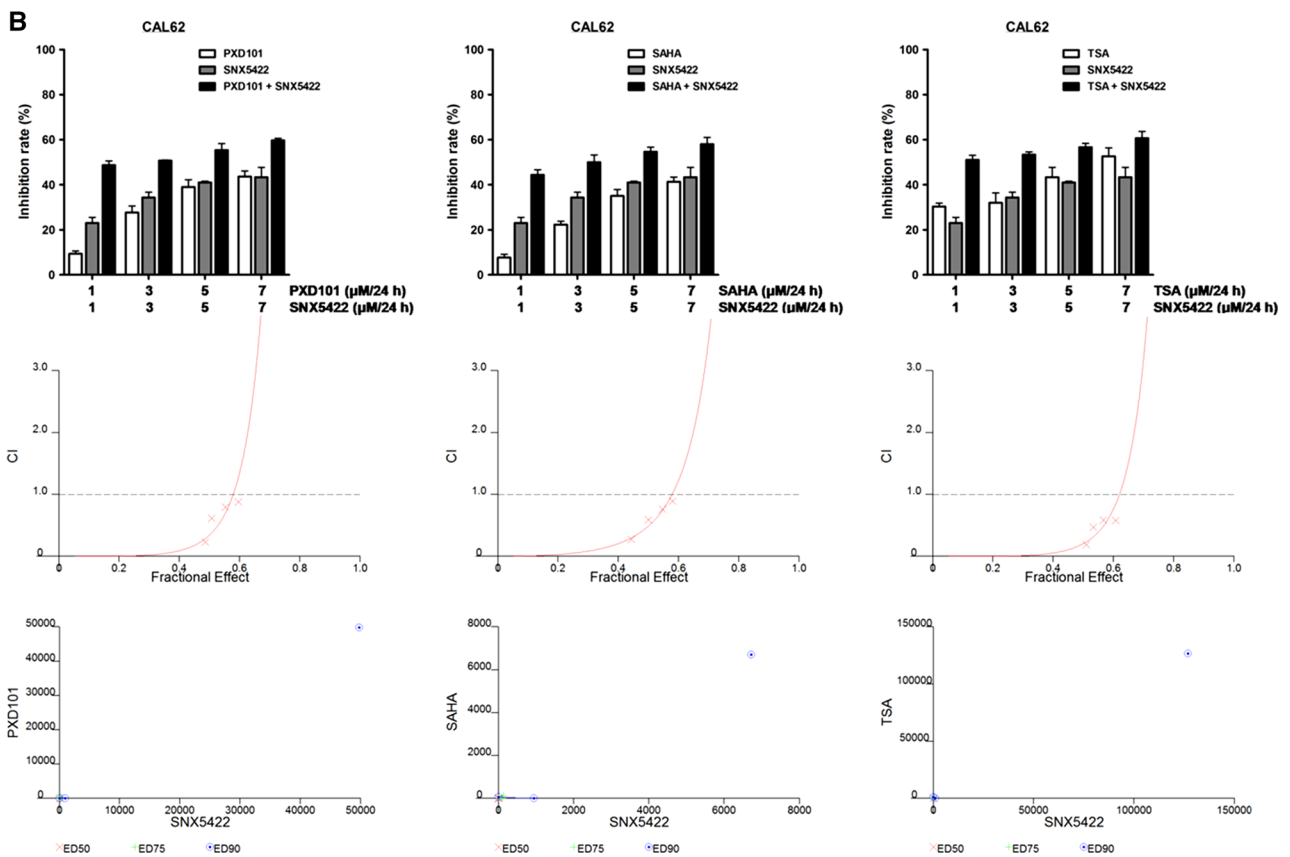
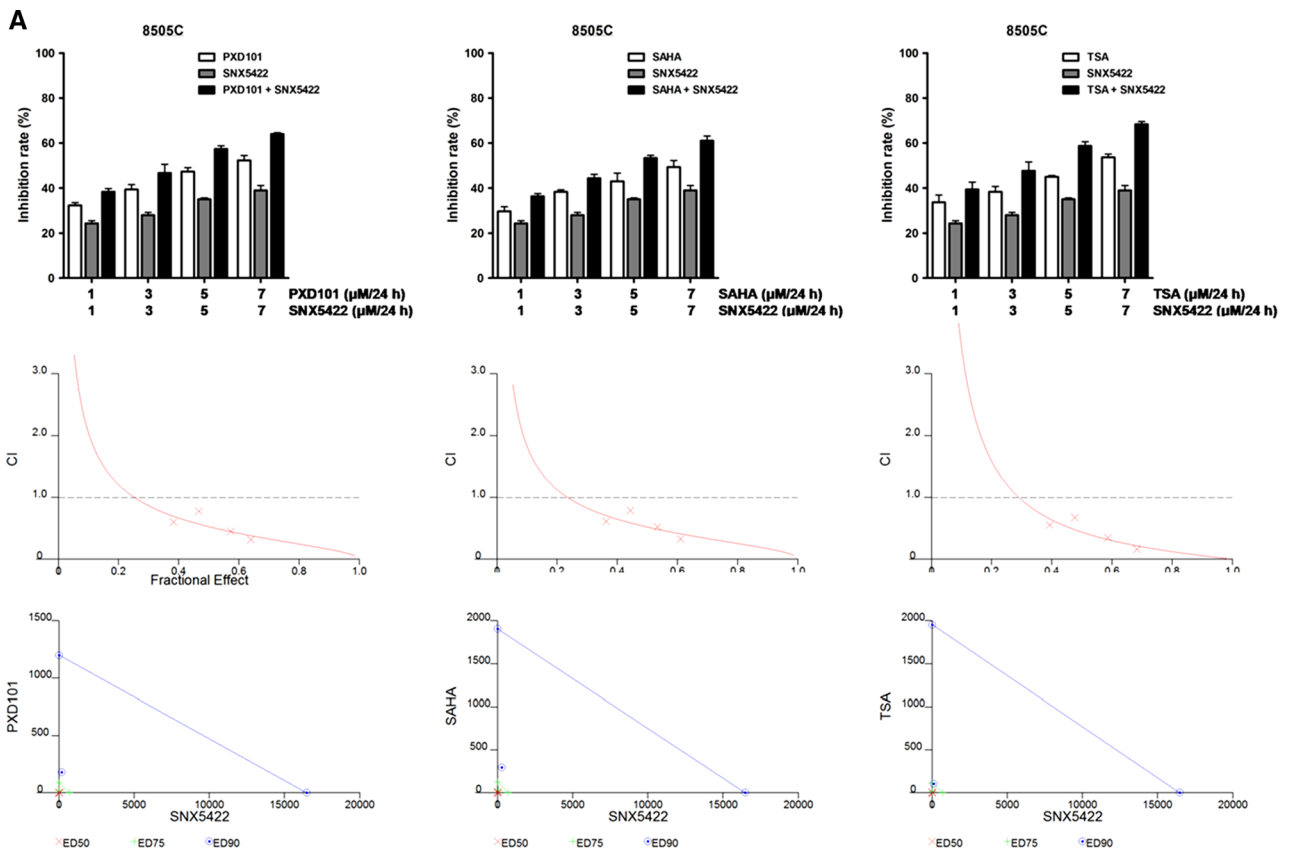


Fig. 2 The combined effect of SNX5422 with PXD101, SAHA, and TSA on survival of ATC cells. **a, b** 8505C and CAL62 cells were treated with both SNX5422 and PXD101, SAHA and TSA at 1, 3, 5, and 7 μ M for 24 h. Cell number was counted using a hemacytometer, and inhibition rate was calculated as follows: inhibition rate (%) = (cell number before treatment – cell number after treatment)/cell number before treatment \times 100. CI and isobologram were calculated. The horizontal dash lines at CI = 1.0 are drawn. All experiments were performed in triplicate. Data are expressed as mean \pm SE. CI combination index

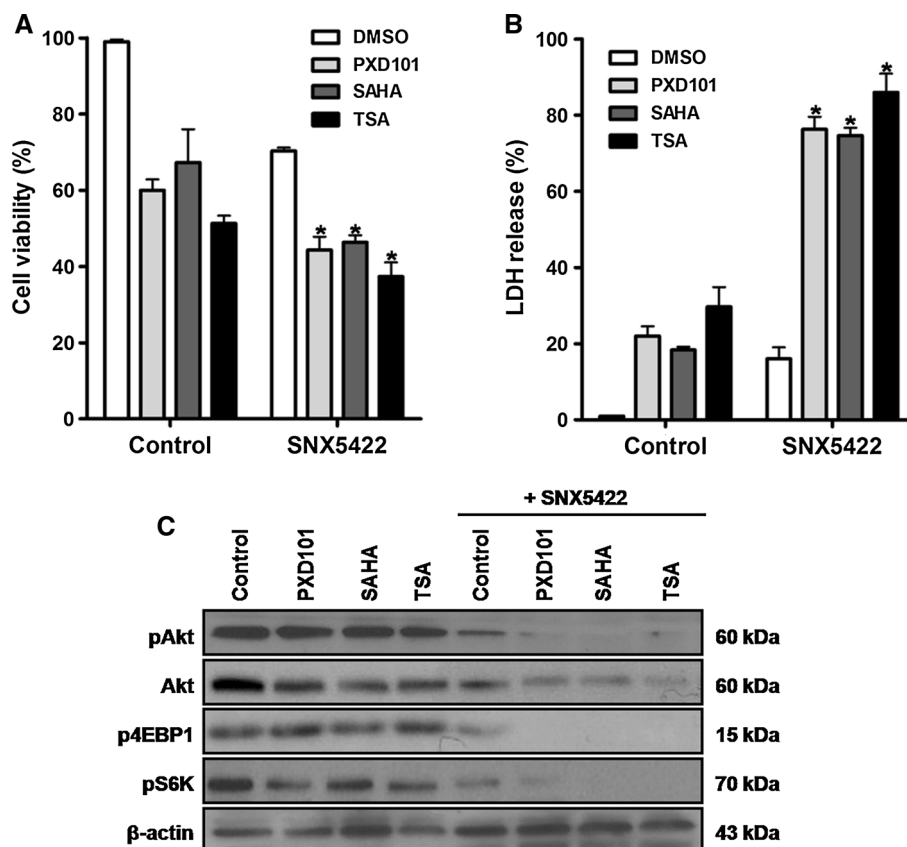
higher in ATC cells than PTC and FTC cells [14]. In addition, SAHA and valproic acid in combination with chemotherapeutic agents improve therapeutic efficacy in ATC cells [15–17]. With regard to the combined effect of hsp90 inhibitors with HDAC inhibitors, our previous study showed that NVP-AUY922 had a synergistic activity with PXD101 in induction of cytotoxicity of ATC cells [5]. In the present study, treatment of both SNX5422 and PXD101, SAHA and TSA, compared with treatment of SNX5422 alone, augmented cell death with the increment of cytotoxic activity in ATC cells. In drug combination analysis, all of the CI values were lower than 1.0, suggesting the synergism between SNX5422 and PXD101, SAHA and TSA in induction of death of ATC cells. These results imply that SNX5422 in combination with HDAC

inhibitors may be an excellent therapeutic challenge in human ATC.

PI3K/Akt/mTOR signaling plays a critical role in survival of thyroid cancer cells [4–6, 14, 27]. In the previous studies reported by us and Lin et al., 17-AAG, NVP-AUY922, and radicicol inhibited PI3K/Akt signaling in ATC cells, and PXD101 suppressed PI3K/Akt/mTOR signaling in thyroid cancer cells [4–6, 14]. In the present study, cotreatment of SNX5422 and PXD101, SAHA and TSA, compared with single treatment of SNX5422, decreased the expression of Akt as well as the phosphorylation of 4EBP1 and S6 K by mTORC1 in ATC cells. These results connote that the synergism between SNX5422 and HDAC inhibitors is mediated by repression of PI3K/Akt/mTOR signaling in ATC cells.

Hsp90 inhibitors as well as HDAC inhibitors increase the expression of DNA damage-related proteins [13, 19–21]. Meanwhile, survivin, a resistance factor to chemotherapeutic agents, is overexpressed in ATC compared with well differentiated thyroid carcinoma [28]. In our previous study, the synergism between NVP-AUY922 and PXD101 was associated with the inactivation of survivin and the activation of DNA damage-related proteins in ATC cells [5]. In the present study, treatment of both SNX5422 and PXD101, SAHA and TSA, compared with

Fig. 3 The impact of SNX5422 in combination with PXD101, SAHA, and TSA on cell survival and PI3K/Akt/mTOR signaling in ATC cells. **a, b** 8505C cells were treated with both SNX5422 and PXD101, SAHA and TSA at 5 μ M for 24 h, and cell viability and cytotoxic activity were measured using CCK-8 assay and cytotoxicity assay, respectively. **c** 8505C cells were treated with both SNX5422 and PXD101, SAHA and TSA at 5 μ M for 24 h, and the protein levels of total and phospho-Akt, phospho-4EBP1, and phospho-S6 K were measured. All experiments were performed in triplicate. The blots are representative of independent experiments. Data are expressed as mean \pm SE. * p < 0.05 versus cells treated with SNX5422 alone



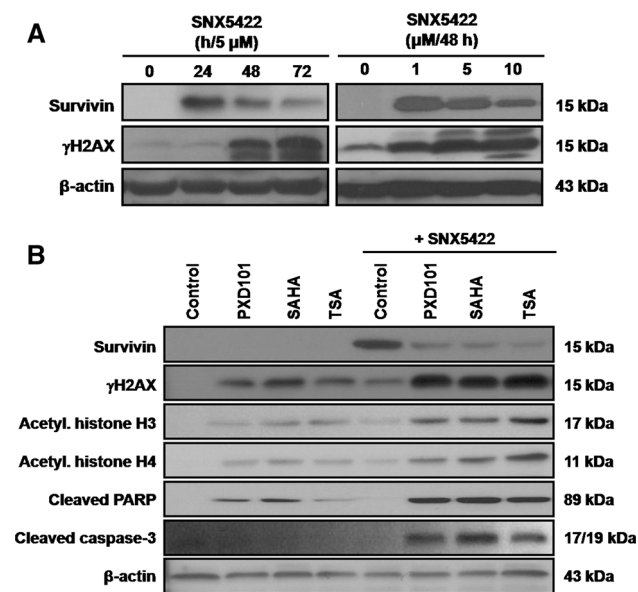


Fig. 4 The combined effect of SNX5422 with PXD101, SAHA, and TSA on the expression of survivin and DNA damage-related proteins in ATC cells. **a** 8505C cells were treated with SNX5422 at 5 μM for 24, 48, and 72 h, and at 1, 5, and 10 μM for 48 h. The protein levels of survivin and γH2AX were measured. **b** 8505C cells were treated with both SNX5422 and PXD101, SAHA and TSA at 5 μM for 24 h. The protein levels of survivin, γH2AX, acetyl. histone H3, acetyl. histone H4, cleaved PARP, and cleaved caspase-3 were measured. All experiments were performed in triplicate. The blots are representative of independent experiments

treatment of SNX5422 alone, increased the expression of γH2AX, acetyl. histone H3 and acetyl. histone H4, whereas it decreased the expression of survivin in ATC cells. These results denote that alterations in DNA damage-related proteins and survivin may be a possible mechanism elucidating the synergism between SNX5422 and HDAC inhibitors in ATC cells. Moreover, the data showing induction of cleaved caspase-3 and cleaved PARP after cotreatment of both drugs provide additional evidences for the synergism between SNX5422 and HDAC inhibitors in ATC cells.

In conclusion, our results are the first to demonstrate that SNX5422 has a cytotoxic activity in conjunction with changes in the expression of hsp90 client proteins in ATC cells. Furthermore, SNX5422 synergizes with HDAC inhibitors in induction of cytotoxicity accompanied by the suppression of PI3K/Akt/mTOR signaling and survivin, and the overexpression of DNA damage-related proteins in ATC cells. This study will provide the therapeutic implications of SNX5422 in combination with HDAC inhibitors in human ATC refractory to conventional therapies.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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