Endocrine (2019) 65:110–120
https://doi.org/10.1007/s12020-019-01885-1

ORIGINAL ARTICLE

Evodiamine in combination with histone deacetylase inhibitors has synergistic cytotoxicity in thyroid carcinoma cells

Si Hyoung Kim[1] · Jun Goo Kang[1] · Chul Sik Kim[1] · Sung-Hee Ihm[1] · Moon Gi Choi[1] · Seong Jin Lee[1]

Received: 8 December 2018 / Accepted: 25 February 2019 / Published online: 17 May 2019
© Springer Science+Business Media, LLC, part of Springer Nature 2019

Abstract

Purpose The impact of evodiamine in combination with histone deacetylase (HDAC) inhibitors on survival of thyroid carcinoma cells was identified.

Methods TPC-1 and SW1736 human thyroid carcinoma cells were used.

Results After treatment with evodiamine and PXD101, cell viability, the percentage of viable cells and Bcl2 protein levels decreased, whereas cytotoxic activity, the percentage of apoptotic cells, the protein levels of γH2AX, acetyl. histone H3 and cleaved PARP, and reactive oxygen species (ROS) production increased. In cells treated with both evodiamine and PXD101, compared with PXD101 alone, decrement of cell viability, the percentage of viable cells, and Bcl2 protein levels as well as increment of cytotoxic activity, the percentage of apoptotic cells, the protein levels of γH2AX and cleaved PARP, and ROS production were significant, causing decrement of Bcl2/Bax ratio. Furthermore, all of the combination index values were <1.0, suggesting synergistic cytotoxicity of two agents. Wortmannin decreased cell viability and the percentage of viable cells, whereas it increased cytotoxic activity and the percentage of apoptotic cells without alteration in ROS production. The changes in cells treated with both evodiamine and suberoylanilide hydroxamic acid or trichostatin A were similar to those in cells treated with both evodiamine and PXD101.

Conclusions Our results demonstrate that evodiamine synergizes with HDAC inhibitors in inducing cytotoxic activities by involving survival-related proteins and ROS in thyroid carcinoma cells. Moreover, repression of PI3K/Akt signaling synergistically reinforces cytotoxicity of evodiamine combined with HDAC inhibitors in thyroid carcinoma cells.

Keywords Thyroid carcinoma · Evodiamine · HDAC inhibitor · Synergism · Akt

Abbreviations

ATC	anaplastic thyroid carcinoma
CI	combination index
DMSO	dimethylsulfoxide
ED_{50}	the concentrations of each drug required for 50% inhibition
FTC	follicular thyroid cancer
HDAC	histone deacetylase
PARP	poly (ADP-ribose) polymerase
PTC	papillary thyroid cancer
γH2AX	phospho-histone H2A.X
ROS	reactive oxygen species
SAHA	suberoylanilide hydroxamic acid
TSA	trichostatin A.

Supplementary information The online version of this article (https://doi.org/10.1007/s12020-019-01885-1) contains supplementary material, which is available to authorized users.

✉ Seong Jin Lee
leesj@hallym.ac.kr

[1] Division of Endocrinology and Metabolism, Department of Internal Medicine, College of Medicine, Hallym University, Chuncheon, Republic of Korea

Introduction

In follicular cell-derived thyroid cancer, well-differentiated thyroid cancer (WDTC) including papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) is most commonly diagnosed with excellent prognosis, while it is unfavorable to radioactive iodine (RAI) therapy in about two thirds of patients with metastatic lesion [1–3]. In contrast, undifferentiated thyroid cancer (UDTC) including anaplastic thyroid cancer (ATC) is rarely diagnosed and has fatal prognosis due to local extension, extrathyroidal invasion, distant metastasis, and rapid progression [1–3]. Since patients with RAI therapy-refractory WDTC and UDTC are hardly responsive to standard treatment modalities, new

paradigm to enhance therapeutic efficacy against cancer cells is under consideration [1–3].

Evodiamine is a natural indole alkaloid extracted from the fruit of *Evodia rutaecarpa* and a multitarget compound having a wide spectrum of biological activities [4]. Evodiamine has been used for management of vomiting, diarrhea, abdominal pain, headache, and postpartum bleeding in oriental herbal medicine [4]. Evodiamine poses favorable properties in thermoregulation, nociception, inflammation, obesity, cardiovascular disease, infectious disease, and Alzheimer's disease and possesses antioncogenic actions in various cancer cells [4, 5]. As one of the plausible mechanisms for antimitogenic activity, it was suggested that evodiamine resulted in cell death via regulation of survival-related proteins including Bcl2 family proteins, phosphohistone H2A.X (γH2AX) and acetyl. histone H3 as well as reactive oxygen species (ROS) and via modulation of multiple signal pathways including phosphoinositide-3 kinase (PI3K)/Akt signaling in cancer cells [6–14]. In view of the effect of evodiamine combined with chemotherapeutic agents, evodiamine exerts beneficial properties in breast and ovary cancer models resistant to chemotherapeutic agents [15–17]. In this regard, we recently reported that evodiamine alone or in combination with chemotherapeutic agents had cytotoxicity by regulating survival-related proteins and PI3K/Akt signaling in thyroid carcinoma cells [18].

Histone deacetylase (HDAC) inhibitors such as PXD101, suberoylanilide hydroxamic acid (SAHA), and trichostatin A (TSA) activate DNA damage response and have a negative influence on cell survival through modulation of ROS and PI3K/Akt signaling in cancer cells [19–23]. In thyroid carcinoma cells, PXD101 (belinostat), a pan-HDAC inhibitor, as single and combined regimens exhibits antitumor actions via repression of PI3K/Akt signaling [21–23]. Moreover, SAHA alone or in combination with chemotherapeutic agents induces a cytotoxic activity and increases the antiproliferative property of vitamin D analog [24, 25]. In addition, TSA augments the expression of thyroid-specific genes relevant to iodide handling [26]. In this respect, our previous studies showed that HDAC inhibitors had synergistic actions with heat shock protein 90 (hsp90) inhibitors in inducing cytotoxicity in ATC cells [22, 23]. With regard to combined effect of evodiamine with HDAC inhibitors, a synthetic chemical, composed of evodiamine derivative and SAHA, attenuates survival and proliferation of cancer cells [27]. Furthermore, evodiamine accelerates SAHA-induced cell death by downregulating hypoxia-inducible factor (HIF)-1α in hypoxic hepatoma cells [28]. However, influence of evodiamine in combination with HDAC inhibitors on survival of thyroid carcinoma cells has not been investigated.

The aim of the present study was to evaluate the effect of evodiamine combined with HDAC inhibitors on survival of thyroid carcinoma cells. Our results demonstrate that evodiamine synergistically produces cytotoxic activities with HDAC inhibitors through participation of survival-related proteins and ROS, and suppression of PI3K/Akt signaling stimulates the synergism between evodiamine and HDAC inhibitors in induction of cytotoxicity in thyroid carcinoma cells.

Materials and methods

Materials

RPMI1640, fetal bovine serum (FBS), L-glutamine, and streptomycin/penicillin were purchased from Life Technologies (Carlsbad, CA, USA). Evodiamine and the HDAC inhibitors PXD101, SAHA, and TSA were obtained from BioVision (Linda, CA, USA) and 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 experiments. The primary antibodies raised against Bcl2, Bax, γH2AX, acetyl. histone H3, and cleaved poly (ADP-ribose) polymerase (PARP) 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 (St. Louis, MO, USA) were obtained. All other reagents were purchased from Sigma unless otherwise stated.

Cell culture

For experiments, TPC-1 human PTC cells and SW1736 human ATC cells were used. TPC-1 cells were obtained from Professor Young Joo Park (Division of Endocrinology and Metabolism, Seoul National University, Republic of Korea) and grown in RPMI1640 supplemented with 10% heat-inactivated FBS and 1% streptomycin/penicillin. SW1736 cells were purchased from Cell Lines Service (CLS GmbH, Eppelheim, Germany), and grown in RPMI1640 supplemented with 2 mM L-glutamine, 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.

CCK-8 assay

Cell viability was determined by the CCK-8 Assay Kit (Dojindo laboratories, Kumamoto, Japan). Cells (5×10^3/

100 µl) in each well on 96-well plates were incubated overnight and treated with agents for an additional 4 h at 37 °C. Absorbance was measured using Glomax™ Discover System GM3000 (Promega, Madison, WI, USA). All experiments were performed in triplicate.

Multiplexed cytotoxicity assay

Cells (5×10^3/100 µl) were seeded in 96-well plates, and reagents of the Multitox-Glo Multiplex Cytotoxicity Assay Kit (Promega) were added to cells after treatments as stated in the manufacturer's protocol. Fluorescent and luminescent values were measured using Glomax™ Discover System GM3000 (Promega). Viability was computed as a ratio of live/dead cells and expressed as percentage of untreated cells. 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 µl) in each well on 96-well plates were incubated and centrifuged at $250 \times g$ for 10 min. Supernatant of 100 µl was transferred in clear 96-well plates. After addition of reaction mixture (2.5 µl Catalyst solution in 112.5 µl Dye solution), cells were incubated for 30 min at room temperature. Absorbance was measured using Glomax™ Discover System GM3000 (Promega). All experiments were performed in triplicate.

FACS analysis

Apoptotic cells were analyzed by the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences Pharminogen, San Diego, CA, USA). Cells (1×10^5/ml) in each well on 6-well plates were incubated, and harvested, and fixed according to manufacturer's protocol. FITC annexin V and/or propidium iodide (PI) in 1 x binding buffer was added for 15 min at room temperature, and analysis was made using a CytoFLEX™ Flow Cytometer (Beckman Coulter Inc., Brea, CA, USA) and CytExpert Software (Beckman Coulter Inc., Brea, CA, USA). All experiments were performed in triplicate.

Measurement of ROS production

ROS production was measured by the ROS-Glo™ H_2O_2 Assay Kit (Promega). Cells (1×10^4/ml) in each well on 96-well plates were incubated and treated with H_2O_2 Substrate solution (25 µM/well) and incubated at 37 °C. After addition of ROS-Glo™ Detection solution (100 µl/well), cells were incubated for 20 min at room temperature. Absorbance was measured using Glomax™ Discover System GM3000 (Promega). All experiments were performed in triplicate.

Western blotting

The total protein was extracted by RIPA buffer (Sigma) containing 1× protease inhibitor cocktail and 1× phophatase inhibitor cocktail set V (Calbiochem, La Jolla, CA, 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). The protein levels were quantified by densitometry using the ImageJ software (NIH) and normalized to β-actin levels. The relative levels of protein to β-actin were obtained. 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 documented. CI values <1.0, 1.0, and >1.0 reveal synergism, additivity, and antagonism, respectively. The isobologram is formed by plotting the doses of each agent 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 reveal additivity, synergism, and antagonism, respectively. All combinations were performed in triplicate.

Statistical analysis

All data are expressed as mean ± standard error (S.E.). Data were analyzed by unpaired Student's t test or analysis of variance as appropriate. A p value <0.05 was considered to be statistically significant. All analyses were performed using SPSS program version 24.0 (SPSS, Chicago, IL, USA).

Results

Evodiamine exerts synergistic cytotoxicity with PXD101 in thyroid carcinoma cells

In TPC-1 and SW1736 cells, to investigate the influence of evodiamine in combination with the HDAC inhibitor PXD101, cells were simultaneously treated with both evodiamine and PXD101, and then the interactions were interpreted by obtaining CI using Chou–Talalay equation, where $CI < 1.0$ manifests synergism, $CI = 1.0$ manifests additivity, and $CI > 1.0$ manifests antagonism (Fig. 1a, b, Table 1). Cell viability was measured using CCK-8 assay,

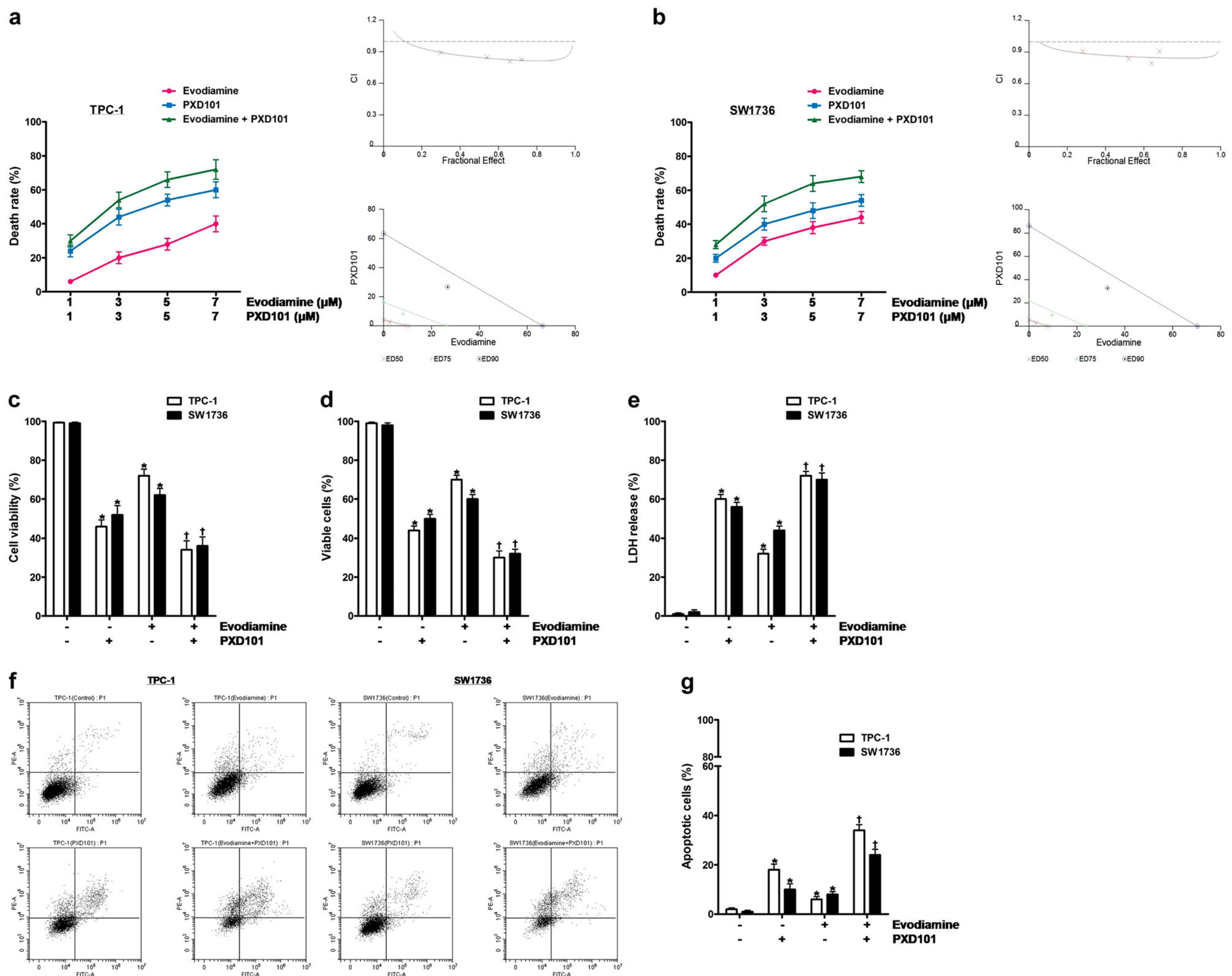

Fig. 1 The impact of evodiamine combined with PXD101 on survival of thyroid carcinoma cells. **a**, **b** TPC-1 and SW1736 cells were simultaneously treated with both evodiamine and PXD101 at 1, 3, 5 and 7 μM for 24 h. Cell viability was measured using CCK-8 assay, and death rate was calculated as 100–cell viability (%). Combination index (CI) and isobologram were obtained. The horizontal dash lines at CI = 1.0 are drawn. **c**–**g** TPC-1 and SW1736 cells were treated with both evodiamine and PXD101 at 5 μM for 24 h, and then cell viability, the percentage of viable cells, cytotoxic activity, and the percentage of apoptotic cells were measured. All experiments were performed in triplicate. Data are expressed as mean ± S.E. *$p < 0.05$ vs. each matched control. †$p < 0.05$ vs. cells treated with PXD101 alone

and death rate was calculated as 100–cell viability (%). As a result of cotreatment, all of the CI values were <1.0, and the combination data points were all located below the isobologram line at ED_{50}, implying that evodiamine in combination with PXD101 synergistically induces death of thyroid carcinoma cells.

Next, to further identify the synergistic activity of evodiamine with PXD101 in inducing cytotoxicity, cells were treated with both evodiamine and PXD101 at 5 μM for 24 h after which cell viability (Fig. 1c), the percentage of viable cells using multiplexed cytotoxicity assay (Fig. 1d), cytotoxic activity using cytotoxicity assay (Fig. 1e), and the percentage of apoptotic cells using FACS analysis (Fig. 1f, g) were measured. After single treatment of evodiamine and PXD101, cell viability and the percentage of viable cells were diminished, and cytotoxic activity and the percentage of apoptotic cells were enhanced. In cells treated with both evodiamine and PXD101, compared with PXD101 alone, diminution of cell viability and the percentage of viable cells as well as enhancement of cytotoxic activity and the percentage of apoptotic cells were significant.

The synergism between evodiamine and PXD101 in leading to a cytotoxic activity is involved in survival-related proteins in thyroid carcinoma cells

To evaluate the effect of evodiamine in combination with PXD101 on expression of survival-related proteins, cells were treated with evodiamine and PXD101 at 5 μM for 24 h, and then the protein levels of Bcl2, Bax, γH2AX,

Table 1 CI values at combined doses determined by the median effect analysis method in thyroid carcinoma cells treated with both evodiamine and PXD101

Cells	EVO (μM)	PXD101 (μM)	CI EVO+PXD101
TPC-1	1	1	0.892
	3	3	0.849
	5	5	0.807
	7	7	0.828
SW1736	1	1	0.910
	3	3	0.838
	5	5	0.794
	7	7	0.908

CI values <1.0, 1.0, and >1.0 indicate synergism, additivity, and antagonism, respectively

CI combination index, *EVO* evodiamine

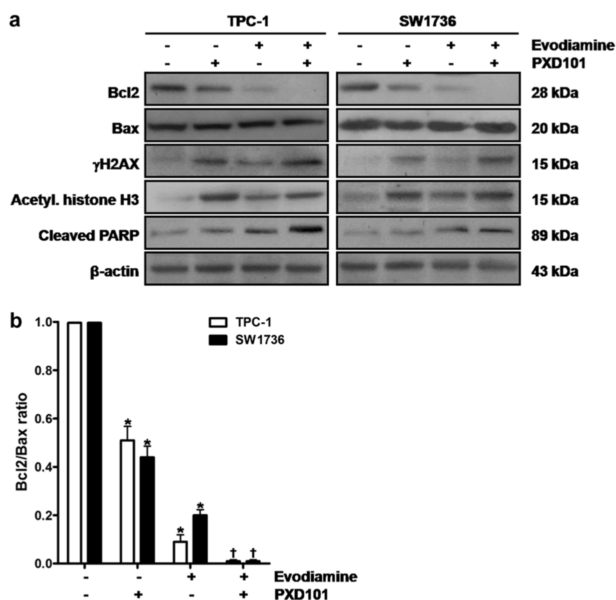

Fig. 2 The influence of evodiamine in combination with PXD101 on expression of survival-related proteins in thyroid carcinoma cells. **a**, **b** TPC-1 and SW1736 cells were treated with evodiamine and PXD101 at 5 μM for 24 h, after which the protein levels of Bcl2, Bax, γH2AX, acetyl. histone H3, and cleaved poly (ADP-ribose) polymerase were measured. The protein levels of Bcl2 and Bax were quantified by densitometry, and Bcl2/Bax ratio was estimated. All experiments were performed in triplicate. The blots are representative of independent experiments. Data are expressed as mean ± S.E. *$p<0.05$ vs. each matched control. †$p<0.05$ vs. cells treated with PXD101 alone

acetyl. histone H3, and cleaved PARP were measured (Fig. 2a). Under single treatment of evodiamine and PXD101, the protein levels of γH2AX, acetyl. histone H3, and cleaved PARP were elevated, and Bcl2 protein levels were reduced without change in Bax protein levels. In cells treated with both evodiamine and PXD101, compared with PXD101 alone, the protein levels of γH2AX and cleaved PARP were elevated, and Bcl2 protein levels were reduced without alteration in those of Bax and acetyl. histone H3. Moreover, Bcl2/Bax ratio was reduced in single treatment of evodiamine and PXD101 and became further evident in combination of evodiamine with PXD101 (Fig. 2b).

Evodiamine synergizes with PXD101-induced cytotoxicity in conjunction with ROS production in thyroid carcinoma cells

To explore whether synergistic combination of evodiamine with PXD101 was relevant to ROS production, cells were treated with both evodiamine and PXD101 at 5 μM for 24 h, and then ROS production was measured (Fig. 3). ROS production increased as a result of single treatment of evodiamine and PXD101, and increment of ROS production was augmented by the combination of two agents.

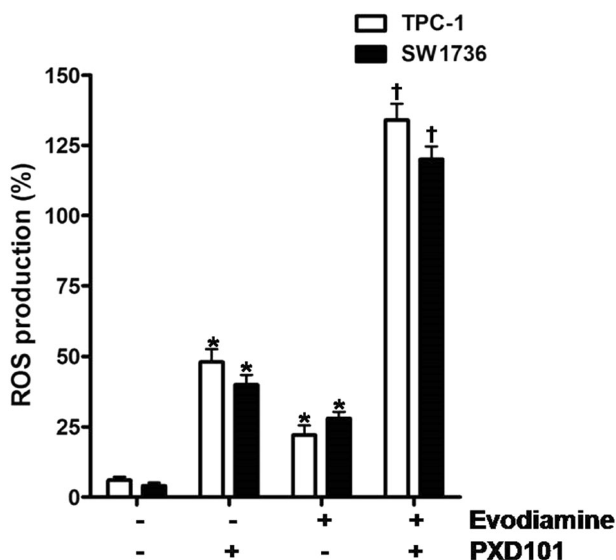

Fig. 3 The relation of reactive oxygen species (ROS) production to the combined effect of evodiamine with PXD101 in thyroid carcinoma cells. TPC-1 and SW1736 cells were treated with evodiamine and PXD101 at 5 μM for 24 h, and ROS production was measured. All experiments were performed in triplicate. Data are expressed as mean ± S.E. *$p<0.05$ vs. each matched control. †$p<0.05$ vs. cells treated with PXD101 alone

Repression of Akt synergistically potentiates a cytotoxic activity of evodiamine in combination with PXD101 in thyroid carcinoma cells

The aberrant activation of PI3K/Akt signaling promotes tumor formation in thyroid follicular cells and plays crucial roles in survival of thyroid carcinoma cells [18, 22, 23, 29–38]. With respect to the role of PI3K/Akt signaling in survival of thyroid carcinoma cells exposed to evodiamine and PXD101, it was

reported that evodiamine and PXD101 resulted in cell death through inhibition of PI3K/Akt signaling [18, 21–23]. Furthermore, HDAC inhibitors synergized with hsp90 inhibitors via suppression of PI3K/Akt signaling [22, 23]. In this study, the role of PI3K/Akt signaling in combination of evodiamine with PXD101 was assessed.

When cells were treated with evodiamine or PXD101 at 1, 3, and 5 μM for 24 h, phospho-Akt protein levels were diminished, while total Akt protein levels were not changed (Fig. 4a). In cells treated with both evodiamine and PXD101 at 5 μM for 24 h, compared with PXD101 alone, total and phospho-Akt protein levels were not altered (Fig. 4b).

Next, cells were administered with the PI3K inhibitor wortmannin before cotreatment of evodiamine and PXD101 at 5 μM for 24 h, and then cell viability (Fig. 4c), the percentage of viable cells (Fig. 4d), cytotoxic activity (Fig. 4e), the percentage of apoptotic cells (Fig. 4f), and ROS production (Fig. 4g) were measured. In cells treated with both evodiamine and PXD101, wortmannin diminished cell viability and the percentage of viable cells and enhanced cytotoxic activity and the percentage of apoptotic cells without change in ROS production.

Evodiamine in combination with HDAC inhibitors has synergistic cytotoxicity in thyroid carcinoma cells

To analyze the impact of evodiamine in combination with the HDAC inhibitors SAHA and TSA, cells were simultaneously treated with both evodiamine and SAHA or TSA (Fig. 5a, Table 2, Supplemental Fig. 1). After cotreatment, all of the CI values were <1.0, and the combination data points were all placed below the isobologram line at ED_{50}, suggesting the synergism between evodiamine and HDAC inhibitors in inducing death of thyroid carcinoma cells.

When cells were treated with SAHA or TSA at 5 μM for 24 h, cell viability and the percentage of viable cells were reduced, and cytotoxic activity, the percentage of apoptotic cells, and ROS production were elevated (Fig. 5b–f). In addition, the protein levels of acetyl. histone H3, and cleaved PARP were elevated and Bcl2 protein levels were reduced, whereas Bax protein levels were not altered, causing reduction of Bcl2/Bax ratio (Fig. 5g, h). In cells treated with both evodiamine and SAHA or TSA at 5 μM for 24 h, compared with SAHA or TSA alone, cell viability and the percentage of viable cells were reduced, and cytotoxic activity, the percentage of apoptotic cells, and ROS production were elevated. Moreover, cleaved PARP protein levels were elevated, and Bcl2 protein levels were reduced without change in the protein levels of Bax and acetyl. histone H3: Bcl2/Bax ratio was reduced.

Discussion

This study displays for the first time that evodiamine in combination with the HDAC inhibitors PXD101, SAHA, and TSA results in synergistic cell death via modulation of Bcl2 family proteins, DNA damage response proteins, and ROS production and inactivation of Akt further increases cell death caused by the combined treatment in thyroid carcinoma cells.

Evodiamine exerts antitumor activities in a variety of cancer cells including hormone-sensitive breast and prostate cancer cells [5]. Meanwhile, Bcl2 family proteins fulfill pivotal functions for homeostasis such as survival on the cellular level [39]. In this respect, relative expression of the prosurvival protein Bcl2 and the antisurvival protein Bax, called Bcl2/Bax switch, is associated with survival of cancer cells [40]. With regard to influence of evodiamine on survival-related proteins including Bcl2 family proteins, evodiamine leads to cell death by repressing Bcl2, Bcl-xL, survivin, inhibitor of apoptosis protein, and cyclooxygenase 2 in cancer cells [6]. Moreover, evodiamine results in cell death through mediation of Bcl2 family proteins and manipulation of Bcl2/Bax ratio in melanoma and hepatoma cells [7, 8]. Previously, it was reported that evodiamine suppressed survival and proliferation of ARO cells, thought to be ATC cells, but the cells have been identified as colon cancer cells [9, 41]. Recently, evodiamine was shown to inhibit cell proliferation with overexpression of Bcl-2, Bcl-xL, cleaved caspase-3, cleaved caspase-9, and cleaved PARP as well as underexpression of Bax, procaspase-3, procaspase-9, procaspase-PARP, PI3K, and phospho-Akt in K1 PTC cells [42]. In our recent study, evodiamine alone or in combination with chemotherapeutic agents posed cytostatic and cytotoxic properties via regulation of survival-related proteins including Bcl2 family proteins in PTC and ATC cells [18].

HDAC inhibitors such as PXD101, SAHA, and TSA exhibit pharmacological actions by repressing deacetylation of nuclear histone proteins and stimulating DNA damage response [19, 20]. In respect to single or combined effect of HDAC inhibitors on thyroid carcinoma cells, PXD101 alone or in combination with doxorubicin and paclitaxel suppresses survival and growth of ATC cells [21]. Furthermore, single or combined treatment of SAHA with doxorubicin, paclitaxel, and paraplatin abrogates survival and growth of PTC and ATC cells, and SAHA accelerates antiproliferative activities of vitamin D analog in ATC cells [24, 25]. Intriguingly, TSA enhances mRNA expression of sodium/iodide symporter and pendrin in PTC, FTC, and Hürthle cell carcinoma cells, promising effective RAI therapy [26]. In our previous studies, PXD101 in combination with the hsp90 inhibitor NVP-AUY922 synergistically induced cytotoxicity, and PXD101, SAHA, and TSA

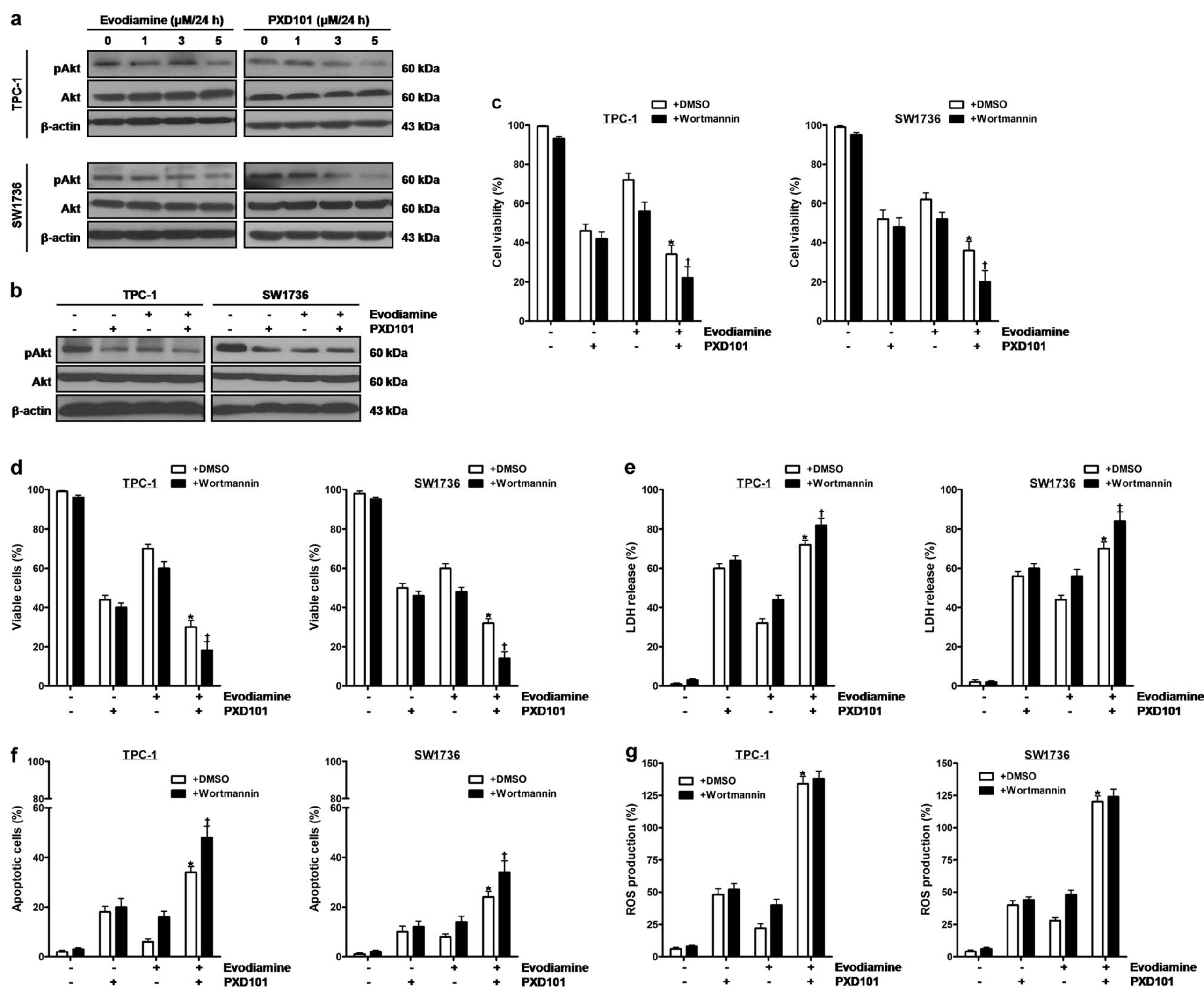

Fig. 4 The role of phosphoinositide-3 kinase (PI3K)/Akt signaling in combination of evodiamine with PXD101 in thyroid carcinoma cells. **a** TPC-1 and SW1736 cells were treated with evodiamine or PXD101 at 1, 3, and 5 μM for 24 h, and then total and phospho-Akt protein levels were measured. **b** TPC-1 and SW1736 cells were treated with evodiamine and PXD101 at 5 μM for 24 h, after which total and phospho-Akt protein levels were measured. **c**–**g** TPC-1 and SW1736 cells were administered with the PI3K inhibitor wortmannin at 1.5 μM prior to cotreatment of evodiamine and PXD101 at 5 μM for 24 h, and cell viability, the percentage of viable cells, cytotoxic activity, the percentage of apoptotic cells, and reactive oxygen species production were measured. All experiments were performed in triplicate. The blots are representative of independent experiments. Data are expressed as mean ± S.E. $^{*}p < 0.05$ vs. cells treated with PXD101 alone. $^{\dagger}p < 0.05$ vs. cells treated with both evodiamine and PXD101

had the synergism with the hsp90 inhibitor SNX5422 in induction of cytotoxic properties in ATC cells [22, 23]. In terms of impact of evodiamine combined with HDAC inhibitors, a hybrid molecule, composed of evodiamine derivative and SAHA, possesses apoptotic and anti-proliferative actions in a range of cancer cells [27]. Although it was reported that evodiamine potentiated SAHA-induced cell death through inactivation of HIF-1α in hepatoma cells under hypoxia [28], the influence of evodiamine in combination with HDAC inhibitors on survival of thyroid carcinoma cells has not been identified.

In this study, we used TPC-1 and SW1736 cells authenticated as PTC and ATC cells, respectively [41]. In cells as a result of treatment of evodiamine, PXD101, SAHA, and TSA, cell viability and the percentage of viable cells were reduced and cytotoxic activity and the percentage of apoptotic cells were elevated. In cells treated with both evodiamine and PXD101, SAHA, or TSA, compared with PXD101, SAHA, or TSA alone, reduction of cell viability and the percentage of viable cells as well as elevation of cytotoxic activity and the percentage of apoptotic cells were significantly evident. Under treatment of both evodiamine and PXD101, SAHA, or TSA, all of the CI values were <1.0 in view of death rate in combination analysis, demonstrating synergistic cell death. Correspondingly, evodiamine, PXD101, SAHA and TSA reduced Bcl2

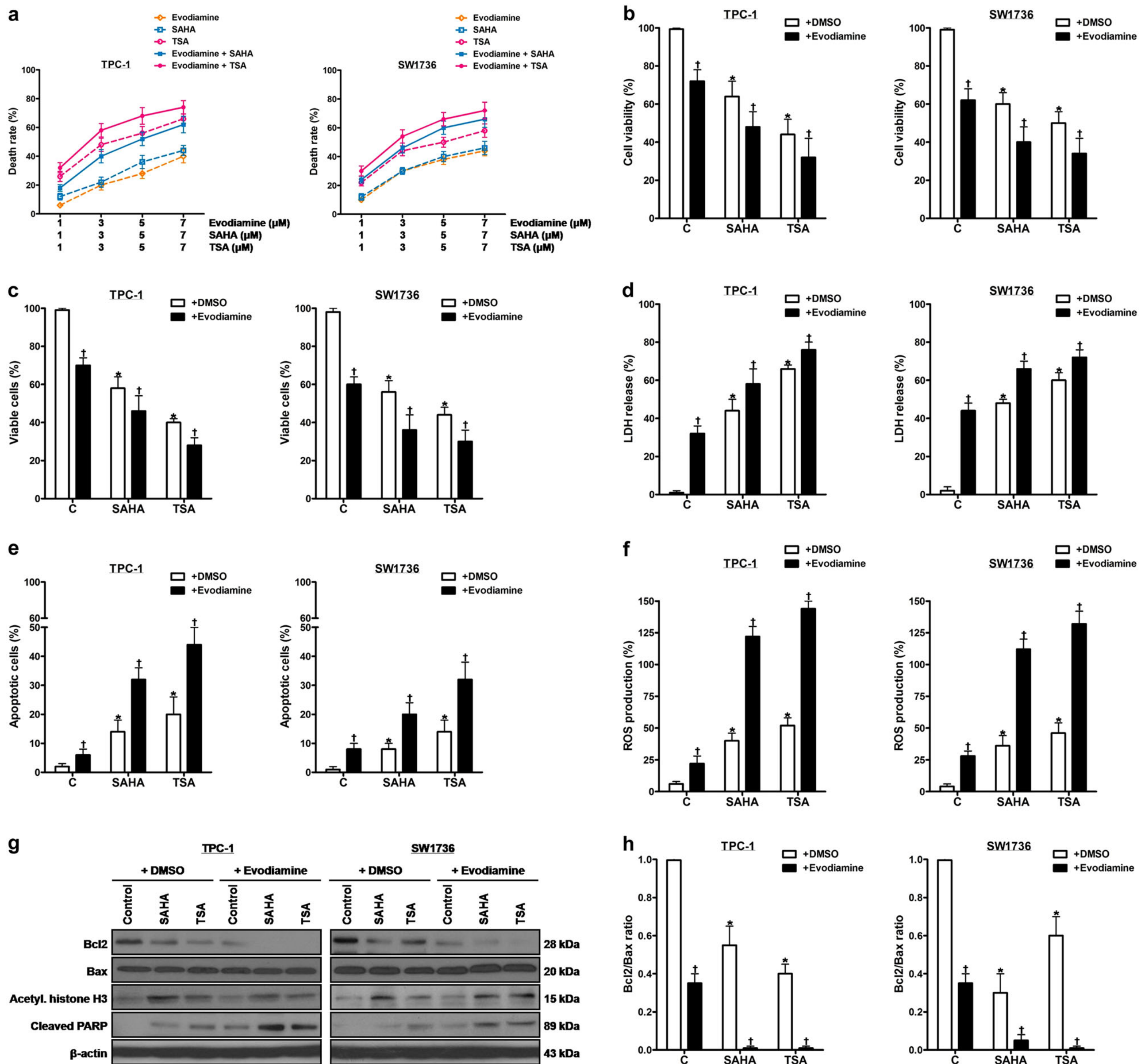

Fig. 5 The impact of evodiamine combined with suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA) on survival of thyroid carcinoma cells. **a** TPC-1 and SW1736 cells were simultaneously treated with both evodiamine and SAHA or TSA at 1, 3, 5, and 7 μM for 24 h. Cell viability was measured using CCK-8 assay, and death rate was computed as 100–cell viability (%). Combination index (CI) and isobologram were obtained. The horizontal dash lines at CI = 1.0 are drawn. **b–f** TPC-1 and SW1736 cells were treated with both evodiamine and SAHA or TSA at 5 μM for 24 h, and then cell viability, the percentage of viable cells, cytotoxic activity, the percentage of apoptotic cells, and reactive oxygen species production were measured. **g**, **h** TPC-1 and SW1736 cells were treated with evodiamine and SAHA or TSA at 5 μM for 24 h, after which the protein levels of Bcl2, Bax, acetyl. histone H3, and cleaved poly (ADP-ribose) polymerase were measured. The protein levels of Bcl2 and Bax were quantified by densitometry, and Bcl2/Bax ratio was estimated. All experiments were performed in triplicate. The blots are representative of independent experiments. Data are expressed as mean ± S.E. $^{*}p < 0.05$ vs. each matched control. $^{\dagger}p < 0.05$ vs. cells treated with SAHA or TSA alone

protein levels, whereas these did not change Bax protein levels, causing reduction of Bcl2/Bax ratio. In addition, evodiamine combined with PXD101, SAHA, or TSA, compared with PXD101, SAHA, or TSA alone, reduced Bcl2/Bax ratio. These findings reveal that evodiamine synergistically magnifies cell death induced by PXD101, SAHA, and TSA with concomitant reduction of Bcl2/Bax ratio in thyroid carcinoma cells. Moreover, these results suggest that the synergism between evodiamine and HDAC inhibitors in inducing cytotoxicity is associated with modulation of Bcl2 family proteins in thyroid carcinoma cells. Taken together, evodiamine in combination with HDAC inhibitors may be a promising therapeutic remedy in human thyroid cancer refractory to standard treatment. With this

Table 2 CI values at combined doses determined by the median effect analysis method in thyroid carcinoma cells treated with both evodiamine and SAHA or TSA

Cells	EVO (μM)	SAHA (μM)	TSA (μM)	CI	
				EVO +SAHA	EVO +TSA
TPC-1	1	1	1	0.896	0.894
	3	3	3	0.887	0.823
	5	5	5	0.917	0.861
	7	7	7	0.860	0.881
SW1736	1	1	1	0.812	0.905
	3	3	3	0.887	0.847
	5	5	5	0.832	0.794
	7	7	7	0.896	0.807

CI values <1.0, 1.0, and >1.0 indicate synergism, additivity, and antagonism, respectively

CI combination index, *EVO* evodiamine, *SAHA* suberoylanilide hydroxamic acid, *TSA* trichostatin A

regard, further studies for feasibility of clinical implications of evodiamine combined with HDAC inhibitors in thyroid cancer patients are necessary to validate whether synergistic cytotoxicity is reproducible in in vivo models.

Evodiamine multiplies γH2AX protein levels in thyroid carcinoma cells and leads to cell death via involvement of oxidative stress in colorectal and lung cancer cells [10–12, 18]. Meanwhile, HDAC inhibitors result in cell death by intensifying DNA damage response and oxidative stress in cancer cells [19–21]. In our previous studies, PXD101 synergistically amplified hsp90 inhibitor-induced cytotoxic activities in conjunction with activation of DNA damage-related proteins in ATC cells [22, 23]. In this study, after treatment of evodiamine and PXD101, the protein levels of γH2AX, acetyl. histone H3, and cleaved PARP increased. In cells treated with both evodiamine and PXD101, compared with PXD101 alone, the protein levels of γH2AX and cleaved PARP increased, whereas acetyl. histone H3 protein levels were not altered: these variations were similar to those in cells treated with SAHA and TSA. As a result of treatment of evodiamine, PXD101, SAHA, and TSA, ROS production increased, and increment of ROS production was obvious in cells treated with both evodiamine and PXD101, SAHA, or TSA, compared with PXD101, SAHA, or TSA alone. These data manifest that evodiamine, PXD101, SAHA, and TSA cause cell death through involvement of γH2AX, acetyl. histone H3, and ROS in thyroid carcinoma cells. Furthermore, these results imply that DNA damage response and oxidative stress may be a possible mechanism for the synergism between evodiamine and HDAC inhibitors in inducing cytotoxicity in thyroid carcinoma cells.

PI3K/Akt signaling regulates intracellular processes for survival in normal cells [43]. In our previous studies, it was shown that aberrant transduction of PI3K/Akt signaling was responsible for tumorigenesis in thyroid follicular cells and was essential in survival of thyroid carcinoma cells [18, 22, 23, 29–38]. In respect to role of PI3K/Akt signaling in evodiamine-induced cytotoxic properties, evodiamine inhibits cell survival and proliferation via inactivation of PI3K/Akt signaling in hepatoma and osteosarcoma cells [8, 13]. In addition, evodiamine in combination with gemcitabine improves therapeutic efficacy by repressing PI3K/Akt signaling in pancreatic cancer models [14]. In our recent study, evodiamine impeded phosphorylation of Akt and thereby induced cytotoxicity in thyroid carcinoma cells [18]. With regard to role of PI3K/Akt signaling in HDAC inhibitor-induced cytotoxic actions, PXD101 abolishes PI3K/Akt signaling and accomplishes death of thyroid carcinoma cells [21–23]. In our previous studies, HDAC inhibitors combined with hsp90 inhibitors exerted synergistic cytotoxicity through suppression of PI3K/Akt signaling in thyroid carcinoma cells [22, 23].

In this study, evodiamine and PXD101 diminished phospho-Akt protein levels without change in total Akt protein levels. Moreover, cotreatment of evodiamine and PXD101, compared with single treatment of PXD101, total and phospho-Akt protein levels were not altered. In cells treated with both evodiamine and PXD101, the PI3K inhibitor wortmannin diminished cell viability and the percentage of viable cells and enhanced cytotoxic activity and the percentage of apoptotic cells without change in ROS production. These findings corroborate that inhibition of Akt augments cell death under combination of evodiamine with PXD101 in thyroid carcinoma cells. Furthermore, these results connote that inactivation of PI3K/Akt signaling synergistically facilitates cytotoxic activities of evodiamine combined with PXD101 in thyroid carcinoma cells. Considering our previous studies that repression of Akt reinforces cytotoxicity of various agents in thyroid carcinoma cells [36–38], these results denote that suppression of Akt stimulates the synergism between evodiamine and HDAC inhibitors in inducing cytotoxic properties in thyroid carcinoma cells.

In conclusion, our results suggest that evodiamine synergizes with HDAC inhibitors in inducing cytotoxicity via involvement of survival-related proteins and ROS in thyroid carcinoma cells. In addition, inhibition of PI3K/Akt signaling intensifies synergistic cytotoxicity of evodiamine in combination with HDAC inhibitors in thyroid carcinoma cells. This study will provide the possibility of clinical implications of evodiamine combined with HDAC inhibitors as an attractive regimen in thyroid cancer patients resistant to conventional therapeutic modalities, although

our data presented herein should be scrutinized in in vivo models.

Acknowledgements This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (No. 2018R1D1A1B07044901) to S.J.L.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. M. Molina-Vega, J. García-Alemán, A. Sebastián-Ochoa, I. Mancha-Doblas, J.M. Trigo-Pérez, F. Tinahones-Madueño, Tyrosine kinase inhibitors in iodine-refractory differentiated thyroid cancer: experience in clinical practice. Endocrine **59**, 395–401 (2018)
2. B.R. Haugen, E.K. Alexander, K.C. Bible, G.M. Doherty, S.J. Mandel, Y.E. Nikiforov, F. Pacini, G.W. Randolph, A.M. Sawka, M. Schlumberger, K.G. Schuff, S.I. Sherman, J.A. Sosa, D.L. Steward, R.M. Tuttle, L. Wartofsky, 2015 American Thyroid Association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: the American Thyroid Association guidelines task force on thyroid nodules and differentiated thyroid cancer. Thyroid **26**, 1–133 (2016)
3. R.C. Smallridge, K.B. Ain, S.L. Asa, K.C. Bible, J.D. Brierley, K. D. Burman, E. Kebebew, N.Y. Lee, Y.E. Nikiforov, M.S. Rosenthal, M.H. Shah, A.R. Shaha, R.M. Tuttle, American Thyroid Association guidelines for management of patients with anaplastic thyroid cancer. Thyroid **22**, 1104–1139 (2012)
4. H. Yu, H. Jin, W. Gong, Z. Wang, H. Liang, Pharmacological actions of multi-target-directed evodiamine. Molecules **18**, 1826–1843 (2013)
5. J. Jiang, C. Hu, Evodiamine: a novel anti-cancer alkaloid from *Evodia rutaecarpa*. Molecules **14**, 1852–1859 (2009)
6. Y. Takada, Y. Kobayashi, B.B. Aggarwal, Evodiamine abolishes constitutive and inducible NF-kappaB activation by inhibiting IkappaBalpha kinase activation, thereby suppressing NF-kappaB-regulated antiapoptotic and metastatic gene expression, up-regulating apoptosis, and inhibiting invasion. J. Biol. Chem. **280**, 17203–17212 (2005)
7. C. Wang, S. Li, M.W. Wang, Evodiamine-induced human melanoma A375-S2 cell death was mediated by PI3K/Akt/caspase and Fas-L/NF-kappaB signaling pathways and augmented by ubiquitin-proteasome inhibition. Toxicol. In Vitro **24**, 898–904 (2010)
8. F. Yang, L. Shi, T. Liang, L. Ji, G. Zhang, Y. Shen, F. Zhu, L. Xu, Anti-tumor effect of evodiamine by inducing Akt-mediated apoptosis in hepatocellular carcinoma. Biochem. Biophys. Res. Commun. **485**, 54–61 (2017)
9. M.C. Chen, C.H. Yu, S.W. Wang, H.F. Pu, S.F. Kan, L.C. Lin, C. W. Chi, L.L. Ho, C.H. Lee, P.S. Wang, Anti-proliferative effects of evodiamine on human thyroid cancer cell line ARO. J. Cell. Biochem. **110**, 1495–1503 (2010)
10. C.C. Chien, M.S. Wu, S.C. Shen, C.H. Ko, C.H. Chen, L.L. Yang, Y.C. Chen, Activation of JNK contributes to evodiamine-induced apoptosis and G2/M arrest in human colorectal carcinoma cells: a structure-activity study of evodiamine. PLoS ONE **9**, e99729 (2014)
11. C. Fang, J. Zhang, D. Qi, X. Fan, J. Luo, L. Liu, Q. Tan, Evodiamine induces G2/M arrest and apoptosis via mitochondrial and endoplasmic reticulum pathways in H446 and H1688 human small-cell lung cancer cells. PLoS ONE **9**, e115204 (2014)
12. L. Lin, L. Ren, L. Wen, Y. Wang, J. Qi, Effect of evodiamine on the proliferation and apoptosis of A549 human lung cancer cells. Mol. Med. Rep. **14**, 2832–2838 (2016)
13. Z.J. Meng, N. Wu, Y. Liu, K.J. Shu, X. Zou, R.X. Zhang, C.J. Pi, B.C. He, Z.Y. Ke, L. Chen, Z.L. Deng, L.J. Yin, Evodiamine inhibits the proliferation of human osteosarcoma cells by blocking PI3K/Akt signaling. Oncol. Rep. **34**, 1388–1396 (2015)
14. W.T. Wei, H. Chen, Z.H. Wang, Z.L. Ni, H.B. Liu, H.F. Tong, H. C. Guo, D.L. Liu, S.Z. Lin, Enhanced antitumor efficacy of gemcitabine by evodiamine on pancreatic cancer via regulating PI3K/Akt pathway. Int. J. Biol. Sci. **8**, 1–14 (2012)
15. S. Wang, L. Wang, Z. Shi, Z. Zhong, M. Chen, Y. Wang, Evodiamine synergizes with doxorubicin in the treatment of chemoresistant human breast cancer without inhibiting P-glycoprotein. PLoS ONE **9**, e97512 (2014)
16. C.H. Liao, S.L. Pan, J.H. Guh, Y.L. Chang, H.C. Pai, C.H. Lin, C. M. Teng, Antitumor mechanism of evodiamine, a constituent from Chinese herb *Evodiae fructus*, in human multiple-drug resistant breast cancer NCI/ADR-RES cells in vitro and in vivo. Carcinogenesis **26**, 968–975 (2005)
17. Z.F. Zhong, W. Tan, S.P. Wang, W.A. Qiang, Y.T. Wang, Anti-proliferative activity and cell cycle arrest induced by evodiamine on paclitaxel-sensitive and -resistant human ovarian cancer cells. Sci. Rep. **5**, 16415 (2015)
18. S.H. Kim, J.G. Kang, C.S. Kim, S.-H. Ihm, M.G. Choi, S.J. Lee, Evodiamine suppresses survival, proliferation, migration and epithelial-mesenchymal transition of thyroid carcinoma cells. Anticancer Res. **38**, 6339–6352 (2018)
19. P.A. Marks, M. Dokmanovic, Histone deacetylase inhibitors: discovery and development as anticancer agents. Expert Opin. Investig. Drugs **14**, 1497–1511 (2005)
20. C. Robert, F.V. Rassool, HDAC inhibitors: roles of DNA damage and repair. Adv. Cancer Res. **116**, 87–129 (2012)
21. S.F. Lin, J.D. Lin, T.C. Chou, Y.Y. Huang, R.J. Wong, Utility of a histone deacetylase inhibitor (PXD101) for thyroid cancer treatment. PLoS ONE **8**, e77684 (2013)
22. S.H. Kim, J.G. Kang, C.S. Kim, S.-H. Ihm, M.G. Choi, H.J. Yoo, S.J. Lee, Novel heat shock protein 90 inhibitor NVP-AUY922 synergizes with the histone deacetylase inhibitor PXD101 in induction of death of anaplastic thyroid carcinoma cells. J. Clin. Endocrinol. Metab. **100**, E253–E261 (2015)
23. S.H. Kim, J.G. Kang, C.S. Kim, S.-H. Ihm, M.G. Choi, H.J. Yoo, S.J. Lee, The heat shock protein 90 inhibitor SNX5422 has a synergistic activity with histone deacetylase inhibitors in induction of death of anaplastic thyroid carcinoma cells. Endocrine **51**, 274–282 (2016)
24. Q.T. Luong, J. O'Kelly, G.D. Braunstein, J.M. Hershman, H.P. Koeffler, Antitumor activity of suberoylanilide hydroxamic acid against thyroid cancer cell lines in vitro and in vivo. Clin. Cancer Res. **12**, 5570–5577 (2006)
25. I. Clinckspoor, L. Verlinden, L. Overbergh, C. Korch, R. Bouillon, C. Mathieu, A. Verstuyf, B. Decallonne, 1,25-dihydroxyvitamin D3 and a superagonistic analog in combination with paclitaxel or suberoylanilide hydroxamic acid have potent anti-proliferative effects on anaplastic thyroid cancer. J. Steroid Biochem. Mol. Biol. **124**, 1–9 (2011)
26. R. Zarnegar, L. Brunaud, H. Kanauchi, M. Wong, M. Fung, D. Ginzinger, Q.Y. Duh, O.H. Clark, Increasing the effectiveness of radioactive iodine therapy in the treatment of thyroid cancer using trichostatin A, a histone deacetylase inhibitor. Surgery **132**, 984–990 (2002)

27. S. He, G. Dong, Z. Wang, W. Chen, Y. Huang, Z. Li, Y. Jiang, N. Liu, J. Yao, Z. Miao, W. Zhang, C. Sheng, Discovery of novel multiacting topoisomerase I/II and histone deacetylase inhibitors. ACS Med. Chem. Lett. **6**, 239–243 (2015)
28. Y.L. Li, N.Y. Zhang, X. Hu, J.L. Chen, M.J. Rao, L.W. Wu, Q.Y. Li, B. Zhang, W. Yan, C. Zhang, Evodiamine induces apoptosis and promotes hepatocellular carcinoma cell death induced by vorinostat via downregulating HIF-1α under hypoxia. Biochem. Biophys. Res. Commun. **498**, 481–486 (2018)
29. M. Xing, Genetic alterations in the phosphatidylinositol-3 kinase/Akt pathway in thyroid cancer. Thyroid **20**, 697–706 (2010)
30. S.H. Kim, J.G. Kang, C.S. Kim, S.-H. Ihm, M.G. Choi, H.J. Yoo, S.J. Lee, Hsp70 inhibition potentiates radicicol-induced cell death in anaplastic thyroid carcinoma cells. Anticancer Res. **34**, 4829–4837 (2014)
31. S.H. Kim, J.G. Kang, C.S. Kim, S.-H. Ihm, M.G. Choi, H.J. Yoo, S.J. Lee, The effect of 17-allylamino-17-demethoxygeldanamycin alone or in combination with paclitaxel on anaplastic thyroid carcinoma cells. Endocrine **48**, 886–893 (2015)
32. S.H. Kim, J.G. Kang, C.S. Kim, S.-H. Ihm, M.G. Choi, H.J. Yoo, S.J. Lee, Synergistic cytotoxicity of BIIB021 with triptolide through suppression of PI3K/Akt/mTOR and NF-κB signal pathways in thyroid carcinoma cells. Biomed. Pharmacother. **83**, 22–32 (2016)
33. S.H. Kim, J.G. Kang, C.S. Kim, S.-H. Ihm, M.G. Choi, H.J. Yoo, S.J. Lee, Synergistic cytotoxicity of the dipeptidyl peptidase-IV inhibitor gemigliptin with metformin in thyroid carcinoma cells. Endocrine **59**, 383–394 (2018)
34. S.H. Kim, J.G. Kang, C.S. Kim, S.-H. Ihm, M.G. Choi, H.J. Yoo, S.J. Lee, Gemigliptin, a novel dipeptidyl peptidase-IV inhibitor, exerts a synergistic cytotoxicity with the histone deacetylase inhibitor PXD101 in thyroid carcinoma cells. J. Endocrinol. Invest. **41**, 677–689 (2018)
35. Y.J. Kim, H.-J. Hwang, J.G. Kang, C.S. Kim, S.-H. Ihm, M.G. Choi, S.J. Lee, Enigma plays roles in survival of thyroid carcinoma cells through PI3K/AKT signaling and survivin. Anticancer Res. **38**, 3515–3525 (2018)
36. S.H. Kim, J.G. Kang, C.S. Kim, S.-H. Ihm, M.G. Choi, H.J. Yoo, S.J. Lee, Akt inhibition enhances the cytotoxic effect of apigenin in combination with PLX4032 in anaplastic thyroid carcinoma cells harboring $BRAF^{V600E}$. J. Endocrinol. Invest. **36**, 1099–1104 (2013)
37. S.H. Kim, J.G. Kang, C.S. Kim, S.-H. Ihm, M.G. Choi, H.J. Yoo, S.J. Lee, Inhibition of p21 and Akt potentiates SU6656-induced caspase-independent cell death in FRO anaplastic thyroid carcinoma cells. Horm. Metab. Res. **45**, 408–414 (2013)
38. S.H. Kim, J.G. Kang, C.S. Kim, S.-H. Ihm, M.G. Choi, H.J. Yoo, S.J. Lee, Suppression of AKT potentiates synergistic cytotoxicity of apigenin with TRAIL in anaplastic thyroid carcinoma cells. Anticancer Res. **35**, 6529–6537 (2015)
39. S. Cory, J.M. Adams, The Bcl2 family: regulators of the cellular life-or-death switch. Nat. Rev. Cancer **2**, 647–656 (2002)
40. S. Cory, J.M. Adams, Killing cancer cells by flipping the Bcl-2/Bax switch. Cancer Cell **8**, 5–6 (2005)
41. R.E. Schweppe, J.P. Klopper, C. Korch, U. Pugazhenthi, M. Benezra, J.A. Knauf, J.A. Fagin, L.A. Marlow, J.A. Copland, R.C. Smallridge, B.R. Haugen, Deoxyribonucleic acid profiling analysis of 40 human thyroid cancer cell lines reveals cross-contamination resulting in cell line redundancy and misidentification. J. Clin. Endocrinol. Metab. **93**, 4331–4341 (2008)
42. Z. Lv, D. Zhao, R. Liu, J. Guo, Y. Lin, M. Zhang, Evodiamine inhibits proliferation of human papillary thyroid cancer cell line K1 by regulating of PI3K/Akt signaling pathway. Int. J. Clin. Exp. Med. **9**, 15216–15225 (2016)
43. J.R. Testa, P.N. Tsichlis, AKT signaling in normal and malignant cells. Oncogene **24**, 7391–7393 (2005)