Suberoylanilide hydroxamic acid sensitizes neuroblastoma to paclitaxel by inhibiting thioredoxin-related protein 14-mediated autophagy

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Neuroblastoma (NB) is an aggressive malignant tumor commonly seen in childhood, and the long-term survival rate of high-risk patients is still poor.1,2 NB is a chemotherapy-sensitive tumor, and chemotherapy plays an important role in the treatment of patients with NB.3–5 The effective chemotherapy regimens for NB primarily include cyclophosphamide, ifosfamide, adriamycin, vincristine, etoposide, and platinum. However, few effective drugs are available for patients who are resistant to front-line chemotherapy.6 Paclitaxel (PTX), which is a cytotoxic agent that can bind to the spindle microtubules of tumor cells, causes mitotic arrest and cell death,7 and has been widely used to treat many adult tumors.6–9 In a phase II investigational window study, an objective response was documented in 25% of children with NB who received PTX alone.10 This finding suggests that PTX is not as effective as most front-line chemotherapeutics in the treatment of NB. The sensitization of NB to paclitaxel would provide more choices in clinical practice.

Autophagy is a physiological process that occurs during metabolic stress, such as in times of energy deficiency and hunger.11 It is also closely associated with tumor progression.12 Recent studies have found that autophagy is one of the modalities of cell death that occurs after chemotherapy and that it is one of the mechanisms underlying chemoresistance in tumor cells.13–16 The Beclin 1 gene, which was the first gene reported to be associated with autophagy in mammalian cells, is homologous to the autophagy-related Atg6 gene in yeast.17 Currently, studies on the Beclin 1 protein are primarily focused on autophagy regulation.18 Unfortunately, the association between autophagy and NB sensitivity to chemotherapy has yet to be investigated.

Thioredoxin-related protein 14 (TRP14) is a disulfide reductase with a molecular mass of 14 kDa that was recently found to be expressed in the human HeLa cell line; however, this protein is also expressed in multiple types of human tissues.19 A crystal structure study showed that the sequence similarity between TRP14 and human thioredoxin (Trx) 1 is only 37%, but these two proteins have similar spatial topological structures and active centers. Thus, TRP14 belongs to the Trx family.20 The function of TRP14 has not yet been fully elucidated, but TRP14 may participate in the regulation of the tumor necrosis factor alpha (TNF-α) signaling pathway,21 which is associated with the process of autophagy. We hypothesize that TRP14 plays a role in Beclin1-mediated autophagy in NB.

Suberoylanilide hydroxamic acid (SAHA) is a broad-spectrum hydroxamic histone deacetylase (HDAC) inhibitor that may inhibit Trx activity by increasing the expression level of the endogenous Trx suppressor thioredoxin-binding protein-2.22 SAHA can also slow cell growth and induce cell differentiation and apoptosis.23 Furthermore, SAHA may be used in combination with various chemotherapeutics to promote...
chemotherapy sensitivity and produce a synergistic effect in tumors. Whether SAHA can inhibit TRP14-induced autophagy has not been investigated. The present study explores the regulatory mechanism that underlies PTX-induced autophagy and whether SAHA can sensitize neuroblastoma to paclitaxel through the inhibition of TRP14-mediated autophagy.

Methods

Ethics approval. Ethics approval was obtained from the Institutional Review Board of Sun Yat-Sen University in China, and the study was conducted according to the principles of the Declaration of Helsinki.

Materials. SK-N-SH and QDDQ-NM cells, in which MYCN was amplified and expressed at normal levels, respectively, were supplied by the Cell Bank of the Chinese Academy of Sciences (Beijing, China). The methyl thiazolyl tetrazolium (MTT), monodansylcadaverine (MDC) and Alexa Fluor 430-A were purchased from Sigma (San Francisco, CA, USA). The microplate reader and MetaMorph offline 7.7.8.0 software package were purchased from Bio-Rad (Hercules, CA, USA). The transmission electron microscope was purchased from NIKON (TYK, Japan). The Beclin 1 siRNA and TRP14 siRNA were both purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the suberoylanilide hydroxamic acid was purchased from Selleck Chemicals (Houston, TX, USA).

Cell culture. Cells were cultured in DMEM-F12 medium supplemented with 10% fetal calf serum. The cells were maintained in a 5% CO2 incubator under saturated humidity at 37°C and were passaged as previously described. Cells in the logarithmic growth phase were used for the experiment.

MTT assay. After digestion and centrifugation, SK-N-SH and QDDQ-NM cells were seeded in a 96-well plate where each well contained 100 μL culture solution and 1 × 10⁴ cells. The plates were maintained in an incubator overnight in an atmosphere of CO2 to achieve full cell attachment. PTX was added at a final concentration of 0, 2, 5, 10, 50, or 100 nM in triplicate. After 24 h of cell culture, 20 μL methyl thiazolyl tetrazolium (MTT) was added to each well and the cells were allowed to incubate for 4-6 h, after which the culture solution was discarded. A total of 150 μL dimethyl sulfoxide was then added to each well. The plate was subjected to vibration on an oscillator for 10 min to fully dissolve the formazan. The absorbance at 570 nm was then measured in a microplate reader.

Western blot. The cells in each group were collected, rinsed with PBS, and centrifuged before the addition of lysis buffer to extract the protein. The protein purity was evaluated by the Bradford method. After albuminous degeneration, an equal amount of protein (20 μg) from each group was separated by SDS-PAGE. The separated proteins were transferred onto PDVF membranes and blocked with skim milk powder for 4 h. The primary antibody was added at an appropriate diluted concentration; the blots were incubated overnight, rewarmed for 1 h, and then rinsed with TBS-T three times for 15 min each time. Then, the second antibody was added at an appropriate diluted concentration; the blots were incubated for 2 h, rinsed with TBS-T, stained, and exposed.

Scanning transmission electron microscopy. SK-N-SH and QDDQ-NM cells in the logarithmic growth phase were seeded in a 6-well plate at a density of 1 × 10⁵ cells/well for 20 h. After digestion and centrifugation, the cells were seeded in a 6-well plate at a density of 1 × 10⁵ cells/well for 20 h. After digestion and centrifugation, the cells were seeded in a 6-well plate at a density of 1 × 10⁵ cells/well for 20 h. After digestion and centrifugation, the cells were seeded in a 6-well plate at a density of 1 × 10⁵ cells/well for 20 h. After digestion and centrifugation, the cells were seeded in a 6-well plate at a density of 1 × 10⁵ cells/well for 20 h. After digestion and centrifugation, the cells were seeded in a 6-well plate at a density of 1 × 10⁵ cells/well for 20 h.
overnight culture. After they were cultured with PTX (10 nM) for 24 h, the cells were collected and fixed in 2.5% glutaraldehyde and 1% osmic acid before elution with an ethanol gradient. After infiltration, embedding, and aggregation, ultrathin sections (50 nm) were obtained and double-stained with uranyl acetate and lead citrate. Finally, the cell ultrastructure was observed under a transmission electron microscope and was video-recorded.

Flow cytometry and confocal microscopy. Flow cytometric analysis was performed to identify apoptotic cells as described. To quantify the cells with autophagic vacuoles, the fluorochrome MDC was used to stain the vacuoles. Staining was detected by flow cytometry, which measured the absorbance at 488 nm as previously described. To further determine the autophagic vacuoles, LysoTracker staining method was used and detected by confocal microscopy as previously described.

RNA transference. Tumor cells were seeded in a 6-well plate at a density of $4 \times 10^5$ cells/well. After 24 h of culture, tumor cells were transfected with two small interfering RNA (siRNA)
SH and QDDQ-NM was evaluated by MTT assay. The combination. After 24 h of culture, the viability of the SK-N-SH, 50, or 100 nM and 0.25 μmol/L SAHA, either alone or in combination. The cells were exposed to PTX at doses of 0, 2, 5, 10, 50, or 100 nM PTX and 0.25 μmol/L SAHA. After 24 h of culture, the expression of LC3-I, LC3-II, Atg 5, Beclin 1, TRP14 and LC3-II-to-LC3-I ratio in NB cells were increased after PTX exposure in a dose-dependent manner (Fig. 1c–e). PTX induced vacuole formation and increased autophagic flux in NB cells. To further analyze the autophagy induced by PTX in NB cells, the formation of autophagic vesicles was evaluated by scanning transmission electron microscopy. Large vacuoles with a double-membrane were observed in the cytoplasm of SK-N-SH and QDDQ-NM cells after PTX exposure. 3-MA, 3-methyladenine. The results are presented as the mean ± SEM of three independent experiments. *P < 0.01, two-tailed t-test.

to produce NB cells with siBeclin 1a and siBeclin 1b, as previously described.(32) Similarly, tumor cells were transfected with siRNA to produce NB cells with siTRP14a and siTRP14b. After transfection for 72 h, the cells in each group were harvested, and the expression of Beclin 1 and TRP14 was detected by western blot.

SAHA exposure. Tumor cells in the logarithmic growth phase were seeded in a 96-well plate at a density of 1.0 × 10^4 cells/well, after which the plates were maintained in an incubator overnight. The experiment was conducted after cell attachment occurred. The cells were exposed to PTX at doses of 0, 2, 5, 10, 50, or 100 nM and 0.25 μmol/L SAHA, either alone or in combination. After 24 h of culture, the viability of the SK-N-SH and QDDQ-NM was evaluated by MTT assay. The expression of LC3-I, LC3-II, Beclin 1, and TRP14 in the cells in each group was investigated by western blot. To further determine the inhibitory effect of SAHA on TRP14, the viability of TRP14low cells was reevaluated by MTT assay after exposure to PTX and SAHA, either alone or in combination.

Transduction. SK-N-SH and QDDQ-NM cells were seeded in a 24-well plate at a density of 1 × 10^4 cells/well in 500 μL complete culture solution. When cell confluence reached 60–80%, the cells were transduced according to the lentivirus transduction manual; the purpose of the transduction was to upregulate the Beclin 1 and TRP14 genes and to establish Beclin 1high and TRP14high tumor cells, as previously described.(33) The empty vector LacZ was used as a control construct. Beclin 1high and TRP14high NB cells were treated with 10 nM PTX and 0.25 μmol/L SAHA. After 24 h of culture, the expression of LC3-I, LC3-II, Beclin 1, and TRP14 was detected by western blot. To determine the effect of SAHA on the chemotherapy sensitivity of Beclin 1high and TRP14high cells to PTX, these cells were treated with PTX at doses of 0, 2, 5, 10, 50, or 100 nM SAHA and PTX, either alone or in combination. Cell viability was then evaluated by MTT assay.

Statistical analysis. Data are given as the means ± SEM. For statistical comparisons, a one-way ANOVA followed by Tukey’s test was performed using SPSS software (SPSS GmbH Software, Munich, Germany). P < 0.05 was considered significant.

Results

PTX induced the expression of autophagy-associated proteins in NB cells. First, the viability of tumor cells treated with PTX was analyzed by MTT assay. As the PTX dose increased, the viability of both SK-N-SH and QDDQ-NM cells decreased, which demonstrates dose dependence (Fig. 1a). Flow cytometry also showed that apoptosis was enhanced as the PTX dose increased (Fig. 1b). However, PTX at a concentration as high as 100 nM could not completely inhibit the growth of NB cells, and the SK-N-SH and QDDQ-NM cells still had viability rates of 43.7% and 45.6%, respectively. Similarly, the apoptosis was not further enhanced concurrent with the increased PTX dose at high levels. A western blot showed that the levels of LC3-II were increased as the PTX dose increased (Fig. 1a). LysoTracker and MDC staining found that the percentage of cells with vacuoles was higher in NB cells exposed to PTX compared with cells that were not exposed to PTX (Fig. 2a). To determine the increased cellular autophagic flux, the expression of LC3-II and p62 proteins was detected by western blot. We found that the expression of LC3-II was consistent with that of p62 in NB cells exposed to PTX, with or without the addition of the lysosomal inhibitor BafA1 (Fig. 2e, f). However, the increased expression levels of LC3-II and p62 that resulted from BafA1 treatment were higher in NB cells exposed to PTX compared with cells that were not exposed to PTX (Fig. 2e, f). Thus, the autophagic vacuoles induced by PTX exposure in NB cells were increased due to the increased cellular autophagic flux and were not
increased due to the inhibition of the degradation of the autophagic vacuoles.

Inhibition of autophagy increased the sensitivity of tumor cells to PTX. To elucidate the underlying mechanisms of the autophagy induced by PTX, the expression of Beclin 1 and TRP14 was interrupted by specific siRNAs. Western blot showed that the expression of LC3-I, LC3-II, Atg 5, Beclin 1, TRP14 and LC3-II-to-LC3-I ratio was not increased in SK-N-SH cells exposed to SAHA alone or SAHA combined with PTX; this is the opposite result to that obtained in cells exposed to PTX alone. GAPDH was used as the loading control. LC3-II and p62 expression that resulted from BafA1 treatment were similar in cells exposed to SAHA (with or without PTX) compared with cells that were not exposed to SAHA (with or without PTX). The apoptosis was enhanced in QDDQ-NM cells exposed to PTX plus SAHA compared to PTX alone. MTT assay showed that the viability of SK-N-SH cells treated with PTX combined with SAHA was lower than that of cells treated with PTX or SAHA alone at the doses of 2, 5, 10, 50 and 100 nM. MTT assay showed that the viability of siTRP14-treated QDDQ-NM cells that were exposed to PTX alone at each PTX dose level was similar to that of cells exposed to PTX plus SAHA. BC, blank control, neuroblastoma (NB) cells without PTX and SAHA exposure. The results are presented as the mean ± SEM of three independent experiments. *P < 0.01, **P < 0.01, two-tailed t test.
However, the viability of SK-N-SH and QDDQ-NM cells exposed to PTX at the doses of 2, 5, 10, 50 or 100 nM combined with SAHA was significantly lower than the viability of cells treated with PTX or SAHA alone (Fig. 4f). Concurrently, the apoptosis was enhanced in NB cells exposed to PTX plus SAHA compared to PTX alone (Fig. 4e). SAHA alone or combined with PTX exerted no effect on cellular autophagic flux (Fig. 4c,d).

To functionally determine whether the inhibition of TRP14 was important for the sensitization of NB cells to PTX after SAHA treatment, the anti-tumor effects of PTX alone or those of PTX combined with SAHA were reevaluated in NB cells treated with siRNA against TRP14. The viability of TRP14low cells exposed to PTX alone was similar to that of cells exposed to PTX plus SAHA, which indicates that SAHA sensitized NB cells to PTX via the inhibition of TRP14 (Fig. 5g).

Upregulation of Beclin 1 or TRP14 reversed the inhibition of autophagy by SAHA. To elucidate whether SAHA inhibited autophagy via the TRP14/Beclin 1/LC3-II pathway, the Beclin 1 and TRP14 genes were upregulated via plasmid transfection. Western blot showed that SK-N-SH and QDDQ-NM cells, in which the Beclin 1 and TRP14 genes were upregulated, highly expressed the Beclin 1 and TRP14 proteins and increased the levels of LC3-I, LC3-II, Atg 5 and LC3-II-to-LC3-I ratio.

![Western Blot Images](image)

**Fig. 5.** Upregulation of Beclin 1 or TRP14 Reversed the Inhibition of Autophagy by SAHA. (a,b) Western blot showed that the levels of LC3-I, LC3-II, Atg 5 and LC3-II-to-LC3-I ratio were increased in Beclin 1high SK-N-SH cells. (c) LC3-II and p62 in Beclin 1high QDDQ-NM cells measured by western blot. (d) MTT assay showed that the viability of Beclin 1high SK-N-SH cells and TRP14high QDDQ-NM cells exposed to PTX alone was similar to that of cells exposed to PTX plus SAHA. (e) MTT assay showed that SAHA could not sensitize ordinary SK-N-SH cells to PTX when the cells were treated with a pro-autophagic drug Bredeldin A followed by PTX plus SAHA. (f,g) Western blot showed that the levels of LC3-I, LC3-II, Atg 5 and LC3-II-to-LC3-I ratio were increased in TRP14high QDDQ-NM cells. (h) LC3-II and p62 in TRP14high SK-N-SH cells measured by western blot. The increased levels of LC3-II and p62 expression that resulted from BafA1 treatment were higher in Beclin 1high cells compared with LacZ control. LacZ, an empty vector used as a control construct. BC, blank control, Beclin 1high or siTRP14high cells that were not exposed to PTX and SAHA. Bredeldin A, a pro-autophagic drug. The results are presented as the mean ± SEM of three independent experiments. *P < 0.01, two-tailed t-test.
(Fig. 5a, b, f, g). Beclin 1\textsuperscript{high} and TRP14\textsuperscript{high} increased cellular autophagic flux (Fig. 5c). The viability of Beclin 1\textsuperscript{high} or TRP14\textsuperscript{high} cells exposed to PTX alone was similar to that of cells exposed to PTX plus SAHA (Fig. 5d). Thus, upregulation of Beclin 1 or TRP14 reversed the SAHA-induced inhibition of autophagy. To further support the definitive role of autophagy in the response of NB cells to PTX, ordinary NB cells were pretreated with a pro-autophagic drug Bredeldin A followed by PTX plus SAHA. SAHA could not sensitize NB cells to PTX in this setting (Fig. 5e).

**Discussion**

Autophagy is one of the underlying mechanisms of tumor resistance to chemotherapy.\(^{13-16, 34}\) In the present study, NB showed moderate sensitivity to PTX, as demonstrated in vivo in a previous study.\(^{10}\) Notably, the tumor cells still had a viability rate of 43.7–45.6\% at a high PTX dose of 100 nM and the apoptosis was not further enhanced concurrent with the increased PTX dose at high levels. We further demonstrated that PTX resistance resulted from autophagy in NB cells. The levels of the autophagy-associated proteins LC3-I, LC3-II, Atg 5, Beclin 1 and LC3-II to LC3-I ratio were significantly increased in tumor cells exposed to PTX. Increased numbers of autophagosomes and increased autophagic flux were also observed in the PTX-exposed cells. Similar findings have been noted in lung cancer\(^{35, 36}\) and breast cancer cells.\(^{37}\) However, the opposite result was obtained here in that PTX induced mitotic arrest and significantly inhibited the formation of autophagosomes in tumor cells.\(^{38}\) These findings indicate that the induction of autophagy by PTX varies among different cell types.

To demonstrate the key role of Beclin 1 in the regulation of PTX-induced autophagy, Beclin 1 expression was interrupted in NB cells using specific siRNA. We found that LC3-1, LC3-II and Atg 5 in PTX-exposed NB cells were downregulated and that the sensitivity of NB cells to PTX was increased. The Beclin 1 gene has been shown to be located on chromosome 17q21, and the Beclin 1 protein forms a complex by combining with the class III PI3K catalytic subunit to mediate other autophagy-associated proteins positioned on the autophagosome.\(^{39}\) Moreover, Beclin 1 contains a BH3 protein region, which could combine with either Bcl-2 or Bcl-XL, to inhibit apoptosis.\(^{40}\) Thus, this protein exerts its functions at an important intersection of the autophagy and apoptosis pathways. A previous study also demonstrated that the inhibition of Beclin 1 can increase the chemotherapeutic sensitivity of liver cancer.\(^{41}\)

Surprisingly, the newly defined protein TRP14 was concurrently upregulated along with Beclin 1. When TRP14 expression was inhibited by siRNA, LC3-I, LC3-II, Atg 5 and Beclin 1 were not upregulated in PTX-exposed cells, and the sensitivity of NB cells to PTX increased at each dosage level. This finding confirmed that Beclin 1-induced autophagy is regulated by TRP14. TRP14 contains Trx-like active center proteins, functions as a disulfide reductase and peroxidase, and maintains redox homeostasis in cells.\(^{19}\) In addition, TRP14 is involved in the regulation of the TNF-α signal transduction pathway. TNF-α may activate c-Jun N-terminal kinase and p38 mitogen-activated protein kinases, and may enhance the activation of TNF-α-induced caspases and the subsequent apoptotic process.\(^{42}\) Thus, TRP14 may regulate the expression of the Beclin 1 gene and autophagy via the TNF-α pathway.

SAHA is a novel HDACI that increases the acetylation of histones by binding to HDACs. This leads to abnormal gene transcription, which further induces apoptosis in cancer cells. SAHA has been shown to exert synergistic antitumor effects in some tumors when given with cis-platinum\(^{43}\) or tamoxifen,\(^{25}\) but the anticancer mechanism through which SAHA exerts its effects is still unknown. We found that PTX and SAHA have additive antitumor effects in NB. Interestingly, SAHA could inhibit autophagy and reverse resistance to PTX in common control SK-N-SH and QDDQ-NM cells, but not in Beclin 1\textsuperscript{high}, TRP14\textsuperscript{high} cells or common NB cells pretreated with a autophagy inducer, which further indicates that TRP14 regulates PTX-induced autophagy via the Beclin 1/LC3-II pathway.

In conclusion, our findings showed that PTX-induced autophagy in NB cells is regulated by TRP14 and that the TRP14 inhibitor SAHA can sensitize NB cells to PTX. TRP14 is therefore a novel treatment target in NB and is worthy of further investigation.

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**Disclosure Statement**

The authors declare no conflict of interest.

**Abbreviations**

- BafA1: Bafilomycin A1
- HDAC: hydroxamic histone deacetylase
- MDC: monodansylcadaverine
- MTT: methyl thiazolyl tetrazolium
- NB: neuroblastoma
- PTX: paclitaxel
- SAHA: suberoylanilide hydroxamic acid
- TGF-α: tumor necrosis factor alpha
- TRP14: thioredoxin-related protein 14
- Trx: thioredoxin

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