Cancer Letters 451 (2019) 58–67

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Combination of 5-aminosalicylic acid and hyperthermia synergistically enhances apoptotic cell death in HSC-3 cells due to intracellular nitric oxide/peroxynitrite generation

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ARTICLE INFO

Keywords:
Cancer cell death
Reactive oxygen and nitrogen species
Drug repurposing
OSCC

ABSTRACT

The repurposing of existing FDA-approved non-cancer drugs is a potential source of new treatment options for cancer patients. An anti-inflammatory drug, 5-aminosalicylic acid (5-ASA), has been clinically used to treat inflammatory bowel disease. Hyperthermia (HT) is widely applicable addendum therapy with the existing cancer treatment modalities. Here, we addressed how 5-ASA combined with HT induces lethal effects in human oral squamous cell carcinoma (OSCC) HSC-3 cells. We found that 5-ASA/HT combination significantly inhibited the viability of HSC-3 cells, while cytotoxic effects in primary human dermal fibroblast cells were minor. Apoptotic endpoints were significantly increased by the 5-ASA/HT combined treatment, as evidenced by presence of Annexin V-FITC/PI positive cells, loss of MMP, Bcl-2/Bax ratio alteration, and increased Fas, cleaved Bid, and caspase expression. Interestingly, the enhancement of apoptosis was reversed in the presence of ON/$ONOO^-$ scavengers. These findings indicate that the combination treatment enhances apoptosis via ON/$ONOO^-$ mediated ER stress-Ca^{2+}-mitochondria signaling and caspase-dependent apoptotic pathways. Our findings provide novel evidence that the combination of 5-ASA and HT is a promising approach for the enhancement of apoptosis; it may serve as an effective strategy for treating human OSCC.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most frequently diagnosed human malignant neoplasm in the oral cavity; it constitutes more than 90% of all oral cancers [1,2]. Despite the advancement of mainstay treatment strategies, the prognosis of OSCC, including survival rates, remains poor [3,4]. Thus, there is an urgent need to explore a more effective strategy that aids the prognostic prediction of patients with OSCC.

The anti-inflammatory drug 5-ASA is known to reduce the incidence of colitis-associated colorectal cancer (CRC) in patients with ulcerative colitis [5]. Previous studies have revealed that 5-ASA is degraded to quinone and ammonia (NH_3) during its electrochemical oxidation in aqueous solutions [6]. It is known that quinone can induce apoptosis by generating intracellular reactive oxygen species (ROS) [7]. On the other hand, ammonia (NH_3) is oxidized by heating with oxygen and catalyzed to form nitric oxide (NO) and water [8]. These biochemical properties of 5-ASA can be exploited for therapeutic benefits with regards to cancer treatment. Although 5-ASA induces apoptosis in a wide variety of CRC cell lines in *vitro*, the chemopreventive potential of 5-ASA alone has been shown to vary in different cancer cell types [9,10].

Hyperthermia (HT), i.e., raising the temperature of a tumor to 40–45 °C via the application of an exogenous heat source, has long been used for cancer treatment, either alone or in combination with existing tumor treatment modalities [11,12]. Research has shown that these high temperatures can damage and kill several types of cancer cells (either by directly damaging proteins and structure within cells or improving the thermal-sensitivity of tumor cells to anticancer agents via the increase of microvascular permeability and blood flow), usually with minimal injury to normal tissues. This fact makes HT an interesting topic in oncology [13,14]. Previous reports have demonstrated that HT exerted its cytotoxic effects via the generation of reactive

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https://doi.org/10.1016/j.canlet.2019.03.004
Received 12 December 2018; Received in revised form 19 February 2019; Accepted 1 March 2019

Abbreviations

5-ASA	5-aminosalicylic acid
HT	Hyperthermia
DMSO	Dimethyl sulfoxide
OSCC	Human oral squamous cell carcinoma
HDF	Primary human dermal fibroblast
CRC	colorectal cancer
ROS	Reactive oxygen species
ROS/RNS	Reactive oxygen and nitrogen species
GSH	Glutathione
HE	Hydroethidine
DAF-2 DA	Diaminofluorescein-2 diacetate
APF	Aminophenyl-fluorescein
HPF	Hydroxyphenyl fluorescein
$^{\bullet}O_2^-$	Superoxide anion
NO	Nitric Oxide
ONOO	Peroxynitrite
OH	Hydroxyl radical
OCl	Hypochlorite
FITC	Fluorescein isothiocyanate
c-PTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide
PBS	Phosphate-buffered saline
CCK-8	Cell counting kit-8
$[Ca^{2+}]i$	Intracellular Ca^{2+} concentration
Mit	Mitochondria
ER	Endoplasmic reticulum
MMP	Mitochondrial membrane potential
TMRM	Tetramethylrhodamine methyl ester
TCA	Trichloroacetic acid
h	Hours
min	Minutes
sec	Seconds
Fig	Figure
NS	Not significant

oxygen and nitrogen species (ROS/RNS) including superoxide anions ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and nitric oxide (NO), resulting in the initiation of oxidative stress and subsequent apoptosis in various cell types [15,16]. Although HT has been proven to be effective for the treatment of many tumor types alone or in combination with radio and/or chemotherapy, this approach has been limited because the efficiency of HT treatment is directly related to the temperature achieved during the heating, the exposure time, tumor cell characteristics, and tumor volume [12]. As a result, in many circumstances, the cytotoxic effect of HT is often insufficient for quantitative cancer cell death due to the biological and technical problems. In order to overcome these challenges, chemical thermosensitizers that are non-toxic at normal temperatures but could be cytotoxic at hyperthermic temperatures may be beneficial. Therefore, it is conceivable that non-toxic doses of 5-ASA may possess the ability to synergize with HT, thereby inducing cancer cell death. Nonetheless, no study investigating the anti-tumor effects of the 5-ASA/HT combination therapy have been conducted thus far. Here, we, report that 5-ASA became a thermosensitizer when combined with HT, and the 5-ASA/HT combination synergistically enhances apoptotic cell death by increasing ROS/RNS generation, ER stress, mitochondrial dysfunction, and the expression of the death receptor Fas, and the subsequent activation of caspase-dependent apoptotic pathways. The results of the present study may provide guidance for the future clinical application of 5-ASA as an adjunctive therapy to HT for treating cancers such as OSCC.

2. Material and methods

2.1. Reagents

5-Aminosalicylic acid (5-ASA), dimethyl sulfoxide (DMSO), and Uric acid were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (c-PTIO) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). The experiments were carried under protection from light.

2.2. Cell culture and treatment

The human oral squamous cell carcinoma cell line HSC-3, T lymphoblast cell line Molt-4, and the colon carcinoma cell line HCT-116 were obtained from the Human Sciences Research Resource Bank (Japan Human Sciences Foundation, Tokyo, Japan). The primary human dermal fibroblasts (HDF) were a kind gift from Dr. T. Shimizu, Department of Dermatology, University of Toyama. The HSC-3, Molt-4, HCT-116, and HDF were grown in Eagle's minimal essential medium (EMEM), RPMI 1640 culture medium, McCoy's 5a medium, and Dulbecco's modified Eagle's medium (DMEM), respectively. All media (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were supplemented with 10% heat-inactivated fetal bovine serum (FBS). The cell cultures were maintained at 37 °C in humidified air with 5% CO_2. 5-ASA was dissolved in DMSO and further added to the culture medium to prepare the desired concentration for experimental use. Cells were plated at a density of 1.25×10^5 cells/well overnight and treated with 5-ASA for 1 h; then, they were exposed to HT. For HT treatment, plastic vessels containing the attached cells were sealed with paraffin film and then immersed in a water bath at 44 °C for 60 min, followed by incubation for 0–24 h at 37 °C. For Molt-4 cells, at 1 h after 5-ASA treatment, the cells were transferred to plastic tubes and then exposed to HT at 44 °C for 10 min by immersing the tubes containing the cell suspensions into a precision-controlled water bath; the cells were then incubated for followed by 12 h.

2.3. Apoptosis assay by flow cytometry

Apoptotic cells were analyzed using the Annexin V-FITC/PI apoptosis detection kit (Immunotech, Marseille, France). In brief, after the combination treatment, the cells were incubated at 37 °C for 12 h and 24 h, and then collected, washed with PBS, and centrifuged at 1200 rpm for 3 min. The resulting pellet was mixed with the binding buffer of the Annexin V-FITC kit. Annexin V-FITC (5 μl) and PI (5 μl) were added to 490 μl of each cell suspensions, followed by gentle mixing. After incubation at 4 °C for 30 min in the dark, the cells were analyzed by flow cytometry (Epics XL, Beckman-Coulter, Miami, FL, USA).

2.4. DNA fragmentation assay

For the detection of apoptosis, a quantitative DNA fragmentation assay was performed according to the method described by Sellins and Cohen [17]. In brief, at 24 h after the combination treatment, HSC-3 cells were collected and lysed using a lysis buffer (10 mM Tris, 1 mM EDTA and 0.2% Triton X-100, pH 7.5) and then centrifuged at 13,000 × *g* for 10 min. Subsequently, each DNA sample in the supernatant and the resulting pellet was precipitated in 25% trichloroacetic acid (TCA) at 4 °C and quantified using the diphenylamine reagent after hydrolysis in 5% TCA at 90 °C for 20 min. The percentage of DNA fragmentation refers to the ratio of DNA in the supernatant ("fragmented") to the total DNA recovered in both the supernatant and pellet ("fragmented plus intact").

2.5. Morphological detection of apoptosis

The morphological changes in the HSC-3 cells were examined using a microscope. In brief, the cells were incubated with or without 5-ASA for 1 h, and then exposed to HT, followed by incubation for 24 h at 37 °C. After the 24 h incubation, the cell morphology was photographed under a light microscope.

2.6. Cell viability assay

Cell survival was determined using the colorimetric cell counting kit-8 assay (CCK-8; Dojindo Laboratories Co., Ltd., Kumamoto, Japan). Cells were seeded into 96-well plates in 100 µl of medium for overnight. Next, the HSC-3 cells were incubated in the absence or presence of 5-ASA for 1 h, and then exposed to HT. At 24 h after the combination treatment, the medium was replaced with fresh medium containing 10% CCK-8. After 2 h of incubation at 37 °C, the absorbance of the samples was measured at 450 nm using a Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA). The trypan blue exclusion test was performed by mixing 50 µl of each cell suspension with an equal amount of 0.3% trypan blue solution (Sigma, St. Louis, MO, USA) in PBS. After 3 min of incubation at room temperature, the stained and unstained cells were counted using a Burker Turk hemocytometer to estimate the number of intact non-viable cells and viable cells, respectively, at 12, 24, 48, and 72 h post-treatment.

2.7. Cell counting assay

The cells were counted to determine the increase in cell number after the combination treatment. In brief, the cells were incubated with or without 5-ASA for 1 h, and then exposed to HT. Samples subjected to each treatment were collected, and the number of cells in each was counted using a Burker Turk hemocytometer.

2.8. Measurement of intracellular ROS/RNS production

Fluorescent probes, which are differentially sensitive to different ROS/RNS, were employed to detect the extent of change in intracellular oxidative stress in HSC-3 cells following their exposure to the 5-ASA/HT combination treatment. The cells were preloaded with a probe in phosphate-buffered saline (PBS) for 15 min at 37 °C. The probes included were: HE (5 µM) for superoxide anion ($^{\cdot}O_2^-$); DAF-2 DA (10 µM) for nitric oxide (NO); APF (2.5 µM) for hydroxyl radical ($^{\cdot}OH$), peroxynitrite ($ONOO^-$), and hypochlorite (OCl^-); and HPF (2.5 µM) for hydroxyl radical ($^{\cdot}OH$) and peroxynitrite ($ONOO^-$). The fraction of fluorescence positive cells was measured by flow cytometry as the proportion of cells with intracellular ROS/RNS.

2.9. Assessment of intracellular glutathione (GSH)

Intracellular GSH was measured using an intracellular GSH kit (Abcam; ab112132) according to the manufacturer's protocol. In brief, HSC-3 cells were incubated in the absence or presence of 5-ASA for 1 h, and then exposed to HT. After that, the cells were collected and washed with PBS, then loaded with green dye (50 nmol/l) for 30 min. The fluorescence intensity of the green dye was detected with a flow cytometer using the FL1 channel.

2.10. Determination of mitochondrial membrane potential (MMP)

The HSC-3 cells were incubated in the absence or presence of 5-ASA for 1 h, and then exposed to HT. After incubation for 24 h, the cells were collected and treated with 10 nM tetramethylrhodamine methyl ester perchlorate (TMRM) (Molecular Probes, Eugene, OR, USA) in 1 ml of 1% fetal bovine serum in PBS, for 15 min at 37 °C. As TMRM is a cationic fluorophore, which is widely used for staining cellular mitochondria and the mitochondrial matrix, the percentage of cells with mitochondrial membrane potential (MMP) loss was analyzed by flow cytometry gated on red TMRM fluorescence (excitation at 488 nm; emission at 575 nm).

2.11. Measurement of intracellular free calcium ions [Ca2+]i

The intracellular free Ca^{2+} concentration was measured using the calcium probe Fluo-3/AM (Dojindo Laboratories Co., Ltd., Kumamoto, Japan). In brief, HSC-3 cells were incubated with or without 5-ASA for

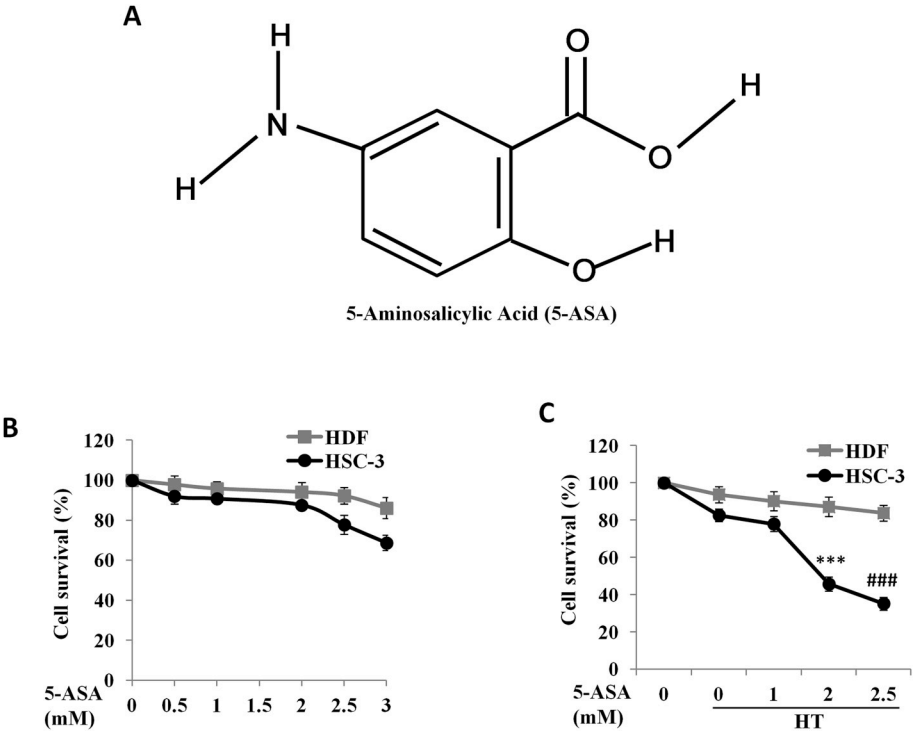

Fig. 1. The effects of the 5-ASA/HT combination treatment on the induction of cell death in HSC-3 and HDF cells. (A) The chemical structure of 5-amino-2-hydroxybenzoic acid (5-Aminosalicylic acid, 5-ASA). (B) Cytotoxic effect of 5-ASA in HSC-3 and HDF cells as determined by the CCK-8 assay at 24 h. (C) HSC-3 and HDF cells were incubated with or without 5-ASA for 1 h, and then exposed to HT. After incubation for 24 h, cell survival was examined by the CCK-8 assay. The data are represented the mean ± SD of three independent replicates, as determined by one-way ANOVA with Bonferroni multiple comparison test. ***P < 0.0001 vs HT with combination treatment (2 mM + HT). ###P < 0.0001 vs HT with combination treatment (2.5 mM + HT).

1 h, and then exposed to HT. The cells were harvested after incubation for 24 h, followed by treatment with 5 μM Fluo-3/AM for 30 min at 37 °C. Excess Fluo-3/AM was removed by washing the cells thrice with PBS. The fluorescence intensity, which represents the free Ca^{2+} levels, was measured by flow cytometry.

2.12. Western blot analysis

After harvest and washing with PBS, cells were lysed in a RIPA buffer [50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40 (v/v), 1% sodium deoxycholate, 0.05% SDS, 1 μg of each aprotinin, pepstatin, and leupeptin, and 1 mM phenylmethylsulfonyl fluoride] for 20 min. Following sonication for a brief period, the lysates were centrifuged at 12,000 x *g* for 10 min at 4 °C, and the protein content in the supernatant was measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). The protein lysates were mixed with 2 μl SDS-loading buffers and denatured at 96 °C for 5 min. They, were then loaded onto an SDS-polyacrylamide gel for electrophoresis, and the protein bands were transferred onto a nitrocellulose membrane. Western blot analysis was performed to detect the expression of cleaved caspase-3 and -8, truncated-Bid (t-Bid), Bax, Bcl-2, Bcl-xl, Chop, Bip, Fas, HSP70, JNK, phosphor-JNK (p-JNK), Erk1/2, phosphor-Erk1/2 (p-Erk1/2), and β-actin using specific antibodies. All antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). The blots were then probed with either secondary horseradish peroxide (HRP) -conjugated anti-rabbit or antimouse IgG antibodies. Band signals were visualized on a luminescent image analyzer (LAS 4000, Fujifilm Co., Tokyo, Japan) by using chemiluminescence ECL detection reagents (Amersham Biosciences, Buckinghamshire, UK).

2.13. Statistical analysis

The results are expressed as the mean ± standard deviation (SD). The statistical significance was assessed either by using one way ANOVA followed by the Bonferroni method for multiple comparison or unpaired Student's *t*-test (two-tailed). P values < 0.05 were considered statistically significant. All experiments were performed at least in triplicate.

3. Results

3.1. The 5-ASA/HT combination treatment significantly suppressed the survival of cancer cells

To investigate the potential of 5-ASA to inhibit the growth of human OSCC cells, HSC-3 and HDF were incubated with different concentrations of 5-ASA for 24 h, and the cell viability was then detected by the CCK-8 assay. We found that above concentrations of 2.5 mM, 5-ASA notably inhibited the viability of the HSC-3 and HDF cells (Fig. 1B). Based on these data, 2.5 mM of 5-ASA was selected as the maximum 5-ASA concentration to be used in this study. To investigate the effects of HT, HSC-3 cells were incubated at different temperatures (39, 42, 43, and 44 °C) for 60 min, and then allowed to recover at 37 °C for 24 h. Flow cytometry analysis indicated a slight enhancement of apoptosis at 44 °C (Supplementary Fig. 1). Next, HSC-3 and HDF cells were treated with different concentrations of 5-ASA for 1 h and then exposed to HT (44 °C for 60 min), followed by incubation for 24 h. The results showed that in combination with HT, 5-ASA (at concentrations of 2 mM and 2.5 mM) significantly suppressed the survival of HSC-3 cells, compared to either treatment alone; in contrast, only a small percentage of cell death was observed following the combination treatment in the HDF cells (Fig. 1C). To confirm this effect, we employed two other cancer cell lines Molt-4 and HCT-116. The trypan blue assay was used to detect cell viability; the 5-ASA/HT combination treatment was found to suppress the viability of Molt-4 and HCT-116 cells (Supplementary Fig. 2A and B). Importantly, the viability of HSC-3 cells showed a dramatic time-dependent decrease following the 5-ASA/HT combination treatment; this decrease was not observed when the cells received either the 5-ASA or the HT treatment alone (Supplementary Fig. 3A). We also observed that the 5-ASA/HT combination treatment completely inhibited the cell growth, while no significant inhibition of the cell growth was seen when the HT treatment was administered alone (Supplementary Fig. 3B). Therefore, we selected the 2 mM and 2.5 mM concentrations of 5-ASA, and HT (44 °C, 60 min) for use in the following experiments.

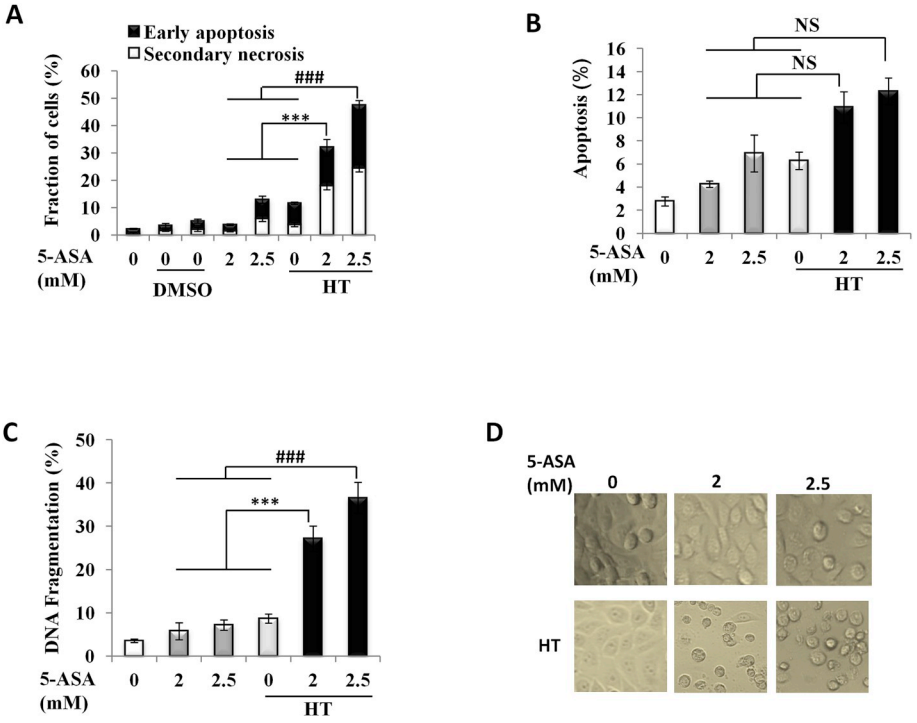

Fig. 2. The 5-ASA/HT combination treatment synergistically enhanced apoptosis in HSC-3 cells. Apoptotic features of HSC-3 cells were monitored at 24 h after combination treatment by using various indexes. The percentage of early apoptosis and secondary necrosis were measured by flow cytometry using Annexin V-FITC/PI double staining (A) HSC-3 cells and (B) HDF cells. (C) Enhancement of apoptosis after the combination treatment was confirmed by the DNA fragmentation assay. (D) The cell morphology of untreated cells and cells exposed to the 5-ASA/HT combination treatment was imaged under a light microscope (20x magnification) after incubation of the cells for 24 h, Scale bar: 100 μm. One representative photomicrograph from three independent experiments is shown here. The results are presented as the mean ± SD (n = 3), as determined by one-way ANOVA with Bonferroni multiple comparison test. ***P $<$ 0.0001 vs 5-ASA, HT with combination treatment (2 mM + HT). ###P $<$ 0.0001 vs 5-ASA, HT with combination treatment (2.5 mM + HT).

3.2. Synergistic enhancement of cancer cell death following the 5-ASA/HT combination treatment

To explore the effect of co-treatment of the 5-ASA/HT combination treatment *in vitro*, HSC-3 and HDF cells were incubated with or without 5-ASA for 1 h and then subjected to HT, with subsequent recovery at 37 °C for 24 h. We examined the cell death (early apoptosis and secondary necrosis) using flow cytometry via annexin V-FITC/PI double staining. The 5-ASA and HT treatments alone slightly induced apoptosis, yielding levels of 10 ± 2%; when the cells were treated with the 5-ASA/HT combination, the apoptosis level in HSC-3 cells was increased to 31.9% and 47.4%, in case of treatment with 2 mM and 2.5 mM of 5-ASA, respectively (Fig. 2A). However, no significant apoptosis was observed in normal HDF cells after the combination treatment or after each treatment was administered alone (Fig. 2B). Flow cytometry analysis also indicated a significant enhancement of apoptosis in the other cancer cell lines (Molt-4 and HCT-116) (Supplementary Fig. 4A and B). The 5-ASA/HT combination treatment also significantly increased the number of apoptotic HSC-3 cells as manifested by DNA fragmentation (Fig. 2C). Furthermore, significant cell shrinkage and decreased cellular attachment were observed after the combination treatment than either treatment alone (Fig. 2D). Our results indicated that the 5-ASA/HT combination treatment can selectively enhance cell death in cancerous cells such as HSC-3, Molt-4, and HCT-116 cells, while crucial cytotoxic effects in non-cancerous cells are minor.

3.3. The 5-ASA/HT combination treatment increases ROS/RNS accumulation and the modulation of intracellular glutathione (GSH) in HSC-3 cells

HT has been reported to induce apoptosis by increasing the generation of ROS/RNS [15,16]. Therefore, we were interested to evaluate the role of ROS/RNS in the effects of the combination treatment. Immediately after the treatment of cells with both HT and 5-ASA alone and their combination, increased superoxide ($^{\bullet}O_2^-$) formation was observed, as detected by HE staining, compared to the untreated control cells (Fig. 3A). It is interesting to note that the superoxide formation induced by the 5-ASA and HT treatments alone subsided in a fraction of cells after 1 h, but remained elevated in cells subjected to the combination treatment. In addition, the NO production also increased synergistically after the combination treatment, compared to the case when either treatment was administered (Fig. 3B). Furthermore, the APF and HPF fluorescence intensities increased slightly in response to HT treatment alone, and increased significantly in the presence of 5-ASA (Fig. 3C). Mounting evidence suggests that ROS/RNS can oxidize cellular GSH (GSH is the most abundant intracellular antioxidant and plays an important role in protection against ROS/RNS) or induce its extracellular export, leading to the loss of intracellular redox homeostasis and the activation of the apoptotic signaling cascade [18,19]. HT treatment substantially increased the low intracellular GSH levels, and this increase was significantly enhanced in the presence of 5-ASA (Fig. 3D). These results suggest that the ROS/RNS-dependent intracellular oxidative stress resulting from the combination treatment induced apoptosis in cancer cells.

3.4. The 5-ASA/HT combination treatment increases ER stress and mitochondrial dysfunction, which contributes to the induction of apoptosis via the Ca^{2+}-mitochondria-dependent pathway

An excess cellular level of ROS/RNS has been linked to ER stress and the unfolded protein response (UPR) [20]. Thus, we examined the expression of ER stress-related proteins, such as CHOP and BIP, in the 5-ASA/HT-treated HSC-3 cells. The 5-ASA/HT combination treatment caused a significant up-regulation in the expression of CHOP and Bip, compared to either treatment alone (Fig. 4A). Prolonged or irreparable ER stress disturbs the Ca^{2+} homeostasis in the ER, which may, in turn, exacerbate Ca^{2+} release from the ER, as well as enhance the intracellular Ca^{2+} levels [21]. Our results showed that the combination treatment significantly enhanced the intracellular $[Ca^{2+}]i$ levels, compared to the case when either treatment was administered alone (Fig. 4B). Increased ROS/RNS levels and Ca^{2+} concentrations cooperatively induce mitochondrial dysfunction, followed by the initiation of mitochondria-mediated apoptosis [22]. Thus, we next examined the role of combination treatment-induced apoptosis by triggering mitochondrial dysfunction. In our current study, MMP loss increased

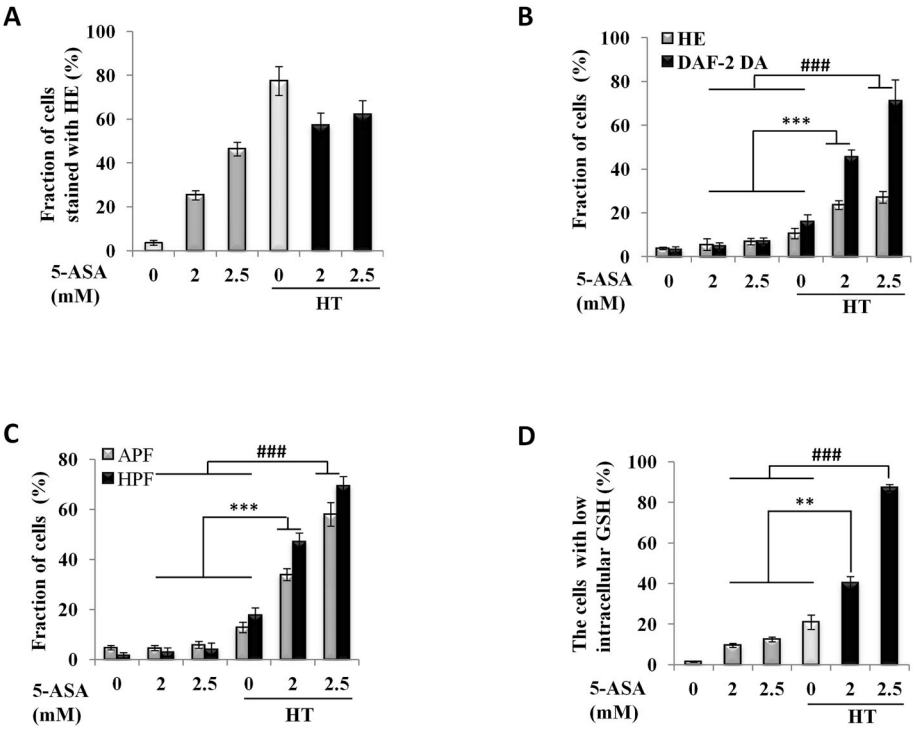

Fig. 3. The 5-ASA/HT combination treatment significantly enhanced intracellular ROS/RNS generation followed by the depletion of intracellular GSH levels. Elevated levels of ROS/RNS were analyzed flow cytometrically using a number of fluorescent probes with different affinities. (A) HE staining immediately after combination treatment. (B) HE and DAF-2 DA staining at 1 h after combination treatment. (C) APF and HPF staining at 1 h following combination treatment. Low intracellular GSH levels were measured by flow cytometry using an intracellular GSH kit. (D) GSH synthesis was significantly reduced following the combination treatment, compared with either treatment alone. The results are presented as mean ± SD (n = 3), as determined by one-way ANOVA with Bonferroni multiple comparison test. ***P < 0.0001 vs 5-ASA, HT with combination treatment (2 mM + HT). ###P < 0.0001 vs 5-ASA, HT with combination treatment (2.5 mM + HT).

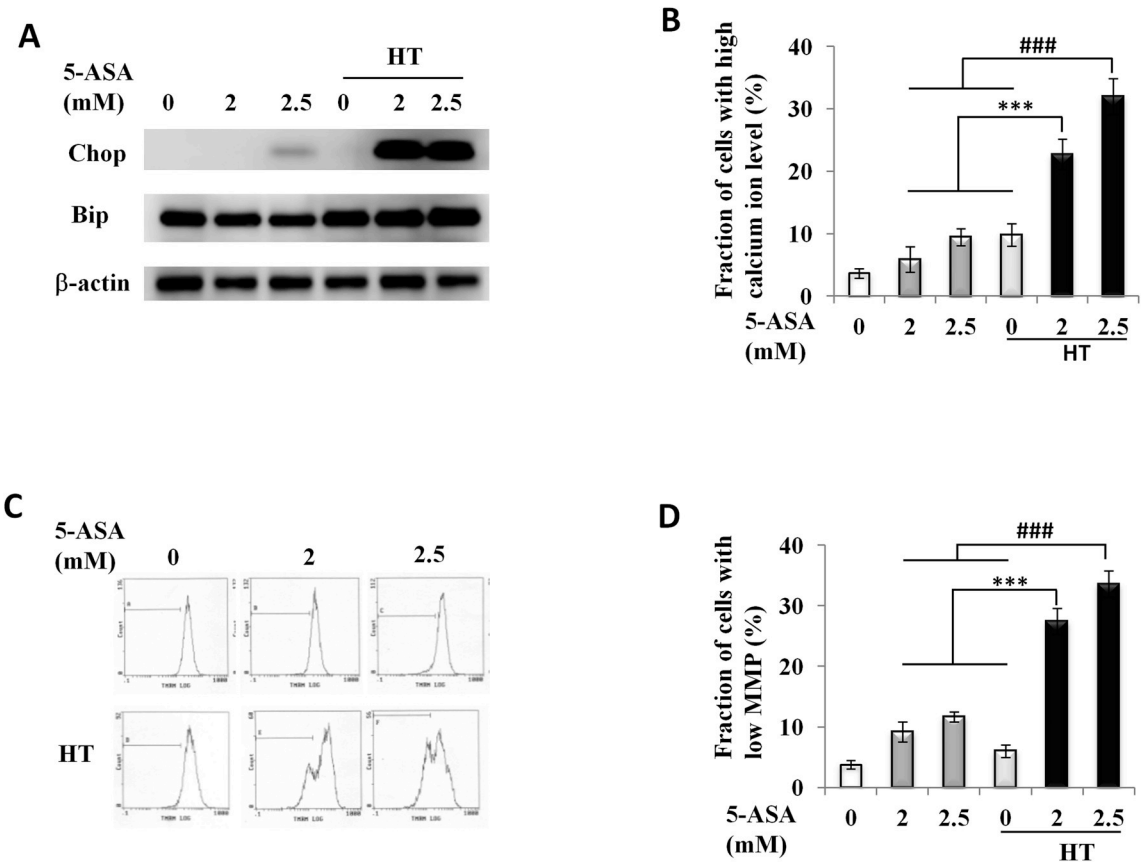

Fig. 4. In the presence of 5-ASA, HT initiated ER–stress, followed by an increase in the intracellular Ca^{2+} levels and mitochondrial dysfunction in HSC-3 cells. (A) The expression of ER–stress-associated proteins Chop and Bip was evaluated by western blot analysis. β-actin was used to normalize the expression levels of proteins in each sample. (B) After routine treatment, cells were loaded with 5 μM calcium probe Fluo-3/AM for 30 min and the increase in intracellular $[Ca^{2+}]i$ concentrations was assessed by flow cytometry. (C) Increased fluorescence intensity with regards to MMP loss was measured using flow cytometry with TMRM staining. (D) Representative flow cytometry histogram of MMP loss. The results are presented as mean ± SD (n = 3), as determined by one-way ANOVA with Bonferroni multiple comparison test. ***P < 0.0001 vs 5-ASA, HT with combination treatment (2 mM + HT). $^{###}$P < 0.0001 vs 5-ASA, HT with combination treatment (2.5 mM + HT).

significantly when the cells were exposed to the combination treatment (Fig. 4C and D). Collectively, these results indicated that ROS/RNS production may be linked to the above process, and the 5-ASA/HT combination treatment could enhance apoptosis through the induction of ROS/RNS-mediated ER stress and Ca^{2+}-dependent mitochondrial pathway.

3.5. The 5-ASA/HT combination treatment causes the induction of caspase-dependent cell death

To assess the molecular insights involved in the 5-ASA/HT combination treatment-induced apoptotic cell death in HSC-3 cells, we employed western blotting to detect the expression levels of apoptosis-related proteins including Bcl-2 family proteins, Caspase-3, and HSP70. The 5-ASA/HT combination treatment dramatically suppressed the expression levels of the antiapoptotic protein Bcl-2, whereas the proapoptotic protein Bax was highly expressed (Fig. 5A). In the presence of any cell death-related stimuli, Bid transforms into its activated or truncated form, t-Bid. As shown in Fig. 5B, the combination treatment notably increased the expression of truncated Bid (tBid) in HSC-3 cells. Caspases are the key executioners involved in the activation of the caspase-dependent apoptosis pathway. However, the activation of cleaved caspase-3 (an active form of caspase-3) increased significantly in response to the 5-ASA/HT combination treatment than in 5-ASA and HT treatment alone (Fig. 5C). Previous studies revealed that heat shock is involved in the upregulation of heat shock proteins (HSPs) such as HSP70; HSP70 knockdown led to significant decreases in the cell survival and increased the thermo-sensitivity of cells in response to stresses [23]. We found that the expression of HSP70 decreased slightly after the combination treatment (Supplementary Fig. 5). These findings revealed that the synergistic enhancement of apoptosis in HSC-3 cells following 5-ASA/HT combination treatment was associated with the activation of mitochondria-mediated caspase-dependent (intrinsic) pathway of apoptotic cell death.

3.6. The 5-ASA/HT combination treatment induces death receptor-mediated apoptotic signaling pathway

Fas (CD95/APO-1) is a death domain-containing receptor that activates the extrinsic apoptotic pathway in response to extracellular stress stimuli [24]. Therefore, we; evaluated whether Fas expression is involved in the combination treatment-induced apoptosis of HSC-3 cells. It was interesting to note that the cells treated with the 5-ASA/HT combination showed Fas expression, but those treated with either 5-ASA alone or HT alone did not (Fig. 5D). It is believed that caspase-8 is required for the initiation of the Fas-induced apoptotic cascade [25]. After the 5-ASA/HT combination treatment, cleaved caspase-8 expression was markedly elevated (Fig. 5D). Fas has been reported to activate members of the mitogen-activated protein (MAP) kinase family, including Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), which is responsible for apoptosis induction; on the other hand, the extracellular signal-regulated kinases Erk1/2 protect against apoptosis and promote cell proliferation [26]. Interestingly, the observations of the current study are consistent with these findings; JNK was

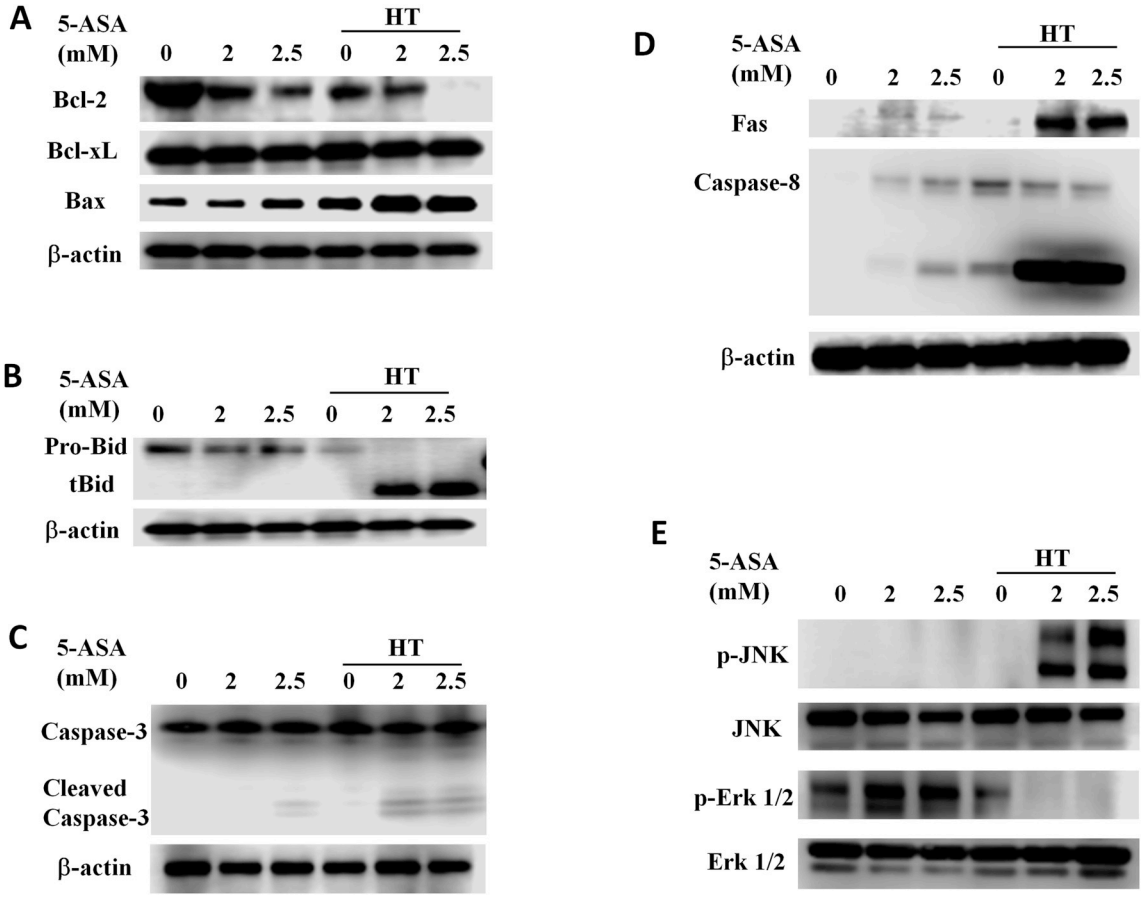

Fig. 5. Effect of the 5-ASA/HT combination treatment on the expression of intrinsic and extrinsic apoptotic pathways. Human oral squamous cell carcinoma (OSCC) HSC-3 cells were incubated in the presence or absence of 5-ASA (2 mM and 2.5 mM) for 1 h and then exposed to HT at 44 °C for 60 min, followed by incubation for 24 h. Western blot analysis was performed to observe the changes in the expression of apoptosis-regulating proteins. (A) The expression levels of anti-apoptotic (Bcl-2 and Bcl-xl) and pro-apoptotic (Bax) proteins. (B) Bid transforms into the activated or truncated form t-Bid. (C) Changes in the expression of cleaved caspase-3. (D) The expression levels of extrinsic apoptotic pathway-associated proteins Fas and cleaved caspase-8. (E) The expression of the phosphorylated form of JNK and Erk1/2. β-actin was used to normalize the expression levels of proteins in each sample.

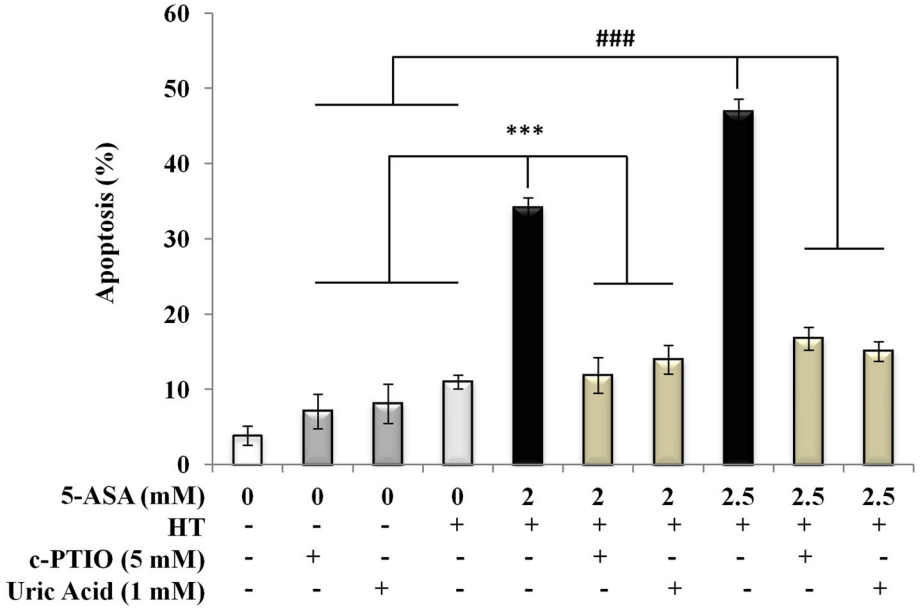

Fig. 6. Pre-treatment with NO/ONOO$^-$ scavengers significantly decreased the 5-ASA/HT combination treatment-induced. Cells were pre-cultured in the absence or presence of the NO scavenger c-PTIO (5 mM) and ONOO$^-$ scavenger uric acid (1 mM) for 1 h prior to the 5-ASA treatment and apoptosis was evaluated using flow cytometry with Annexin-V FITC and PI dual staining 24 h after HT treatment (Fig. 6). Synergistic enhancement of apoptosis induced by the combination treatment was reversed in the presence of c-PTIO and uric acid. The results are presented as mean ± SD (n = 3), as determined by one-way ANOVA with Bonferroni multiple comparison test. ***P < 0.0001 vs 5-ASA, HT, c-PTIO, uric acid, (2 mM + c-PTIO), (2 mM + uric acid) and with combination treatment (2 mM + HT). ###P < 0.0001 vs 5-ASA, HT, c-PTIO, uric acid, (2.5 mM + c-PTIO), (2.5 mM + uric acid) and with combination treatment (2.5 mM + HT).

significantly phosphorylated, and Erk1/2 was dephosphorylated following combination therapy (Fig. 5E). These results indicate the involvement of the extrinsic pathway in the synergistic enhancement of apoptosis induced by the 5-ASA/HT combination treatment in OSCC cells.

3.7. Effects of NO/ONOO$^-$ scavengers in the enhancement of combination treatment-induced apoptosis in HSC-3 cells

The rapid reactions of NO with superoxide ($^{\bullet}O_2^-$) form peroxynitrite (ONOO$^-$). The significance of OONO$^-$ is that it has a high diffusibility across cell membranes and reacts instantaneously with cellular macromolecules like DNA, proteins, and lipids, as well as damages nucleic acids, leading to the induction of lethal DNA damage and subsequent apoptosis in a variety of cell types [27]. To verify the role of NO/ONOO$^-$ generation in 5-ASA/HT combination treatment-induced apoptosis, a specific NO scavenger c-PTIO, and ONOO$^-$ scavenger uric acid, were utilized. The flow cytometry results showed that c-PTIO and uric acid significantly suppressed the enhancement of apoptosis induced by the combination treatment (Fig. 6). Collectively, the results indicated that the generation of NO/ONOO$^-$ is an essential step for the 5-ASA/HT-mediated synergistic enhancement of apoptosis in OSCC cells.

4. Discussion

Elimination of cancer by selectively targeting and destroying cancer cells without affecting normal tissues is the main therapeutic strategy of most cancer therapies. Clinically, it is very hard to treat cancer with a single modality because of micro-environmental barriers; moreover, the combination of two or more therapeutic treatments has shown a promising outlook to specifically achieve cancer cell death [28]. Thus, recent research has focused on exploring multimodal treatments for oncological therapy. HT is clinically used as a thermal sensitizer during the existing cancer treatment modalities (e.g. chemotherapy and/or radiotherapy) for various solid tumors [12]. During the heating period, HT can enhance cell membrane permeability; this modification facilitates drug delivery and an influx of ROS radicals, resulting in an increase of drug cytotoxicity [29,30]. Thermal enhancement of drug cytotoxicity in the tumor region is accompanied by cellular death such as apoptosis, which is an effective way for the treatment of cancer [30,31]. Hence, chemical thermosensitizers may play a crucial role in enhancing the HT-induced lethal effects on cancer cells. Particularly, it was of interest to employ 5-ASA as an adjuvant therapy to HT; we aimed to delineate how the combination treatment synergistically enhanced apoptosis in HSC-3 cells. To the best of our knowledge, this is the first study to have provided evidence that 5-ASA is an effective thermosensitizer and when combined with HT treatment, it could represent a promising anticancer strategy for the treatment of OSCC. We conducted several approaches to establish the mechanistic basis for this thermosensitization.

Compared with normal cells, cancer cells exhibit various differences in their cellular biological activities, including metabolic changes, increased levels of ROS/RNS, and increased inducetion of oxidative stress due to an imbalanced redox status [19,32]. These biochemical properties of cancer cells can be exploited for therapeutic benefits because elevated ROS/RNS levels render cancer cells more susceptible to agents that further increase the ROS/RNS and oxidative stress levels; as a result, different types of cell death processes, including apoptosis, can be initiated in cancer cells [33]. Electron paramagnetic resonance (EPR) results have indicated that HT increased the ROS/RNS generation, and the resultant oxidative stress may mediate, in part, heat-induced cellular damage [16]. In this study, treatment with 5-ASA and HT alone caused an early transient elevation of superoxide ($^{\bullet}O_2^-$) generation, but apoptosis induction was not observed because the $^{\bullet}O_2^-$ generation lasted only for a short time period and was below the threshold to activate the apoptotic machinery. This result is consistent with those of previous reports indicating that the steady-state level of $^{\bullet}O_2^-$ is estimated to be low, and its activity is spatially limited because the sustained elevation of $^{\bullet}O_2^-$ levels is believed to be due to the activation of superoxide-producing enzymes such as NADPH oxidase and xanthine oxidase at elevated temperatures [34,35]. Importantly, sustained increase in intracellular nitric oxide (NO) and peroxynitrite (ONOO$^-$) generation was observed in the cells of a prolonged period after the combination treatment, compared to the case when either the 5-ASA or HT treatments were administered. Consistent with our findings, recent studies have shown that the overproduction of $^{\bullet}O_2^-$ can cause it to react with NO, to generate the highly reactive oxidant ONOO$^-$, which mediates the cytotoxic effects of NO [36,37]. The origin of NO has not yet been identified and needs further investigation. However, ROS/RNS are highly reactive molecules due to the presence of unpaired valence shell electrons or non-static bonds and their rapid passage across membranes via certain aquaporins or other specific proteins [38]. This may result in a significant increase in the intracellular ROS/RNS concentrations, causing oxidative damage to cellular biomolecules including lipids, proteins, and DNA, and finally resulting in the induction of cancer cell death both *in vitro* and *in vivo* [39]. In the present study, the assumption that the 5-ASA/HT combination synergistically enhanced apoptosis corresponds well with the observed intracellular ROS/RNS generation levels. The possible mechanism underlying in this synergistic enhancement involves the disruption of cytoskeleton structures like microtubules and microfilaments due to HT-induced heat stress, leading to the alteration of membrane permeability towards several compounds, as has been observed in case of many therapeutic agents, including anti-cancer drugs [14]. Thus, it was speculated that HT, in combination with 5-ASA, increases the generation of ROS/RNS, mediating, in part, the cytotoxicity by facilitating the penetration of 5-ASA into cells, causing the therapeutically appropriate response, i.e. the induction of cell death.

Excess generation of ROS/RNS has been closely linked to ER–stress; it also disrupts the protein folding mechanism in the ER [20]. However, prolonged or irreparable ER stress can disturb the ER response, which may, in turn, exacerbate Ca^{2+} release from the ER, and therefore, increase the intracellular Ca^{2+} concentrations [21]. Intracellular ROS/RNS and Ca^{2+} overload could eventually result in the induction of mitochondrial depolarization, which is the hallmark of the initiation of different types of cell death processes particularly the apoptotic pathway [22,40]. The Bcl-2 protein family either has pro-apoptotic (i.e. Bax, Bak, and Bid) or anti-apoptotic (i.e. Bcl-2 and Bcl-xL) proteins, which activate and regulate the mitochondrial pathway of apoptosis by governing the mitochondrial outer membrane permeabilization (MOMP). The impairment of Bcl-2 family proteins results in the increase of the Bax/Bcl-2 ratio, which elicits the activation of the caspase cascade [41,42]. The caspases are the central components of the apoptotic response; they are activated either through the 'intrinsic' pathway, triggered by stimuli from the mitochondria, or the 'extrinsic' apoptosis pathway, initiated by stimuli from the cell surface 'death receptors' such as Fas [43–45]. Fas has been regarded as the most abundant transmembrane receptor in the membrane raft domains [46]. Disruptions of membrane fluidity and lipid rafts have been linked to the course of apoptosis. Previous reports have shown that heat treatment can increase the membrane fluidity and alteration of lipid raft contents by disrupting lipid the raft integrity leading to the activation of death receptors and apoptotic cell death; however, in this study, we observed Fas expression only after the combination treatment [47–49]. Based on our data, it was assumed that HT treatment alone is not sufficient for the cells to attain the Fas-inducing threshold, whereas, the 5-ASA/HT combination treatment can force the induction of Fas expression due the increased intracellular NO/ONOO$^-$ generation [50,51]. It is reported that in the Fas-induced apoptosis pathway, caspase-8 activation is required, which may lead to either the direct activation of executioner caspases (caspase-3), or their activation through the induction of

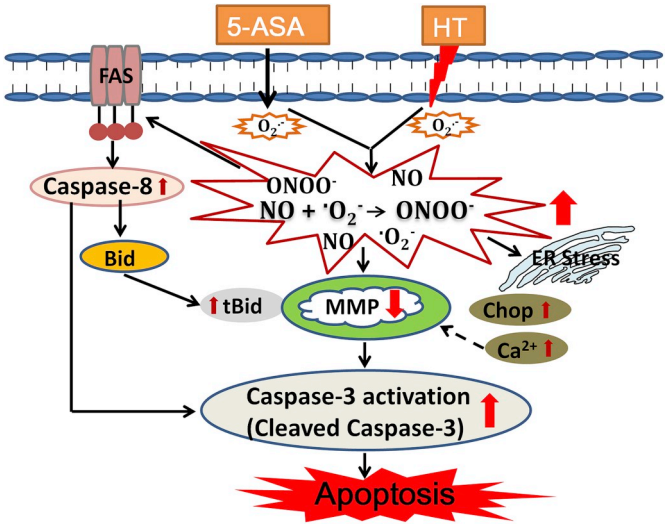

Fig. 7. Schematic diagram of the multiple signal pathways involved in the enhancement of 5-ASA/HT combination treatment-induced apoptosis. HT and 5-ASA alone induce the excessive generation of intracellular $^{\cdot}O_2^{-}$, but are unable to induce a sufficient amount of apoptosis. In contrast, 5-ASA/HT combination treatment generates excessive $^{\cdot}O_2^{-}$ and NO; further, these radicals may they react one other to form $ONOO^{-}$. However, NO/$ONOO^{-}$ induces mitochondrial dysfunction directly or via the externalization of Fas, subsequently activating caspase-8, tBid, and caspase-3, thereby sensitizing the cells to intrinsic pathway- and extrinsic pathway-mediated apoptosis. In addition, the combination treatment-induces ER stress leads to elevated intracellular Ca^{2+} levels, also contributing to the enhancement of apoptosis.

the intrinsic apoptotic pathway via the cleavage of the BH3-interacting domain death agonist (Bid) into truncated-Bid (t-Bid) [52,53]. Therefore, Bid is considered as a bridging element between the two distinct apoptotic pathways and both these pathways converge at the executioner caspases such as caspase-3 [54,55]. Importantly, scavengers of ON and $ONOO^{-}$ significantly inhibited the enhancement of apoptosis induced by the combination treatment, and indicating the involvement of high ON/$ONOO^{-}$ generation. Therefore, we speculate the possibility that HT enhances the delivery and efficacy of 5-ASA by increasing vascular perfusion and permeability and causing intracellular NO/$ONOO^{-}$ accumulation, resulting in increased combination treatment-induced cytotoxicity; this could mediate, in part, the synergistic enhancement of apoptotic cell death in response to the 5-ASA/HT combination treatment. In the present study, we found that the 5-ASA/HT combination treatment caused caspase activation and a significant loss of MMP, with an increase in intracellular NO/$ONOO^{-}$ generation, ER stress marker levels, intracellular $[Ca^{2+}]i$ concentrations, Bax/Bcl-2 ratio, and expression of Fas, and truncated-Bid (t-Bid), and subsequent synergistic enhancement of apoptotic cell death (Fig. 7). This study provides the initial piece of evidence that the anti-inflammatory agent 5-ASA is a potent thermo-sensitizer and the 5-ASA/HT combination may be a potential therapeutic strategy against OSCC.

In conclusion, the current study is the first to show that combining 5-ASA and HT synergistically enhances cell death via activation of the intrinsic, extrinsic, and ER stress-Ca^{2+}-mediated apoptotic signaling pathways, resulting in massive apoptotic cell death in cells exposed to the combination treatment. Our approach of combining 5-ASA and HT as a therapeutic strategy for treating OSCC achieved satisfying results *in vitro*. Our further research will be focused on the clinical applications of such a combination therapy for treating cancer.

Conflicts of interest

The authors declare that they have no conflict of interests.

Acknowledgements

This work was supported by the JSPS KAKENHI Grant Number JP15H04904 and a Grant-in-Aid for Scientific Research on Innovative Area, (15H00892) from the Ministry of Education, Culture, Sports, Science and Technology.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.004.

References

[1] J. Bagan, G. Sarrion, Y. Jimenez, Oral cancer: clinical features, Oral Oncol. 46 (2010) 414–417 https://doi.org/10.1016/j.oraloncology.2010.03.009.
[2] A. Almangush, M. Pirinen, I. Heikkinen, A.A. Mäkitie, T. Salo, I. Leivo, Tumour budding in oral squamous cell carcinoma: a meta-analysis, Br. J. Canc. 118 (2018) 577–586 https://doi.org/10. 1038/bjc.2017.425.
[3] S.A. Gharat, M. Momin, C. Bhavsar, Oral squamous cell carcinoma: current treatment strategies and nanotechnology-based approaches for prevention and therapy, Crit. Rev. Ther. Drug Carrier Syst. 4 (2016) 363–400 https://doi.org/10. 1615/CritRevTherDrugCarrierSyst.2016016272.
[4] S.A. Liu, C.C. Wang, R.S. Jiang, F.Y. Lee, W.J. Lin, J.C. Lin, Pathological features and their prognostic impacts on oral cavity cancer patients among different subsites - a singe institute's experience in Taiwan, Sci. Rep. 7 (2017) 7451 https://doi.org/10. 1038/s41598-017-08022-w.
[5] L.N. Zhao, J.Y. Li, T. Yu, G.C. Chen, Y.H. Yuan, Q.K. Chen, 5-Aminosalicylates reduce the risk of colorectal neoplasia in patients with ulcerative colitis: an updated meta-analysis, PLoS One 9 (2014) e94208https://doi.org/10.1371/journal.pone.0094208.
[6] R.K. Palsmeier, D.M. Radzik, C.E. Lunte, Investigation of the degradation mechanism of 5-aminosalicylic acid in aqueous solution, Pharm. Res. (N. Y.) 9 (1992) 933–938 https://doi.org/10.1023/A:1015813302412.
[7] S. Sagar, I.R. Green, Pro-apoptotic activities of novel synthetic quinones in human cancer cell lines, Cancer Lett. 285 (2009) 23–27 https://doi.org/10.1016/j.canlet.2009.04.036.
[8] G.B. Taylor, Oxidation of ammonia, Ind. Eng. Chem. 11 (1927) 1250–1252 https://doi.org/10.1021/ie50215a017.
[9] A. Reinacher-Schick, A. Schoeneck, U. Graeven, I. Schwarte-Waldhoff, W. Schmiegel, Mesalazine causes a mitotic arrest and induces caspase-dependent apoptosis in colon carcinoma cells, Carcinogenesis 24 (2003) 443–451 www.ncbi.nlm.nih.gov/pubmed/12663503.
[10] P.J. Bus, I.D. Nagtegaal, H.W. Verspaget, C.B. Lamers, H. Geldof, J.H. Van Krieken, At el., Mesalazine-induced apoptosis of colorectal cancer: on the verge of a new chemopreventive era? Aliment. Pharmacol. Ther. 11 (1999) 1397–1402 https://doi.org/10.1046/j.1365-2036.1999.00652.x.
[11] N.R. Datta, S.G. Ordóñez, U.S. Gaipl, M.M. Paulides, H. Crezee, J. Gellermann, At el., Local hyperthermia combined with radiotherapy and-/or chemotherapy: recent advances and promises for the future, Cancer Treat Rev. 41 (2015) 742–753 https://doi.org/10.1016/j.ctrv.2015.05.009.
[12] T. Mantso, S. Vasileiadis, I. Anestopoulos, G.P. Voulgaridou, E. Lampri, S. Botaitis, et al., Hyperthermia induces therapeutic effectiveness and potentiates adjuvant therapy with non-targeted and targeted drugs in an in vitro model of human malignant melanoma, Sci. Rep. 8 (2018) 10724 https://doi.org/10.1038/s41598-018-29018-0.
[13] F.K. Storm, W.H. Harrison, R.S. Elliott, D.L. Morton, Normal tissue and solid tumor effects of hyperthermia in animal models and clinical trials, Cancer Res. 39 (1979) 2245–2251.
[14] S. Jha, P.K. Sharma, R. Malviya, Hyperthermia: role and risk factor for cancer treatment, Achiev, Life Sci. 10 (2016) 161–167 https://doi.org/10.1016/j.als.2016.11.004.
[15] D.M. Katschinski, K. Boos, S.G. Schindler, J. Fandrey, Pivotal role of reactive oxygen species as intracellular mediators of hyperthermia-induced apoptosis, J. Biol. Chem. 275 (2000) 21094–21098 https://doi.org/10.1074/jbc.M001629200.
[16] Y. Tabuchi, K. Ahmed, T. Kondo, Induction of oxidative stress by hyperthermia and enhancement of hyperthermia-induced apoptosis by oxidative stress modification, in: S. Kokura, T. Yoshikawa, T. Ohnishi (Eds.), Hyperthermic Oncology from Bench to Bedside, 2 Springer, 2016, pp. 7–18.
[17] K.S. Sellins, J.J. Cohen, Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes, J. Immunol. 139 (1987) 3199–3206 http://www.jimmunol.org/content/139/10/3199.
[18] M.L. Circu, T.Y. Aw, Reactive oxygen species, cellular redox systems, and apoptosis, Free Radic. Biol. Med. 48 (2010) 749–762 https://doi.org/10.1016/j.freeradbiomed.2009.12.022.
[19] M.L. Circu, T.Y. Aw, Glutathione and modulation of cell apoptosis, Biochim. Biophys. Acta 1823 (2012) 1767–1777 https://doi.org/10.1016/j.bbamcr.2012.06.019.
[20] R. Ozgur, B. Uzilday, Y. Iwata, N. Koizumi, I. Turkan, Interplay between unfolded protein response and reactive oxygen species: a dynamic duo, J. Exp. Bot. 69 (2018) 3333–3345 https://doi.org/10.1093/jxb/ery040.

[21] A. Deniaud, O. Sharaf el dein, E. Maillier, D. Poncet, G. Kroemer, C. Lemaire, et al., Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis, Oncogene 27 (2008) 285–289 https://doi.org/10.1038/sj.onc.1210638.
[22] N. Tajeddine, How do reactive oxygen species and calcium trigger mitochondrial membrane permeabilisation? Biochim. Biophys. Acta 1860 (2016) 1079–1088 https://doi.org/10.1016/j.bbagen.2016.02.013.
[23] C. Li, K. Sunderic, S.B. Nicoll, S. Wang, Downregulation of heat shock protein 70 impairs osteogenic and chondrogenic differentiation in human mesenchymal stem cells, Sci. Rep. 8 (2018) 553 https://doi.org/10.1038/s41598-017-18541-1.
[24] L. Galluzzi, I. Vitale, S.A. Aaronson, J.M. Abrams, D. Adam, P. Agostinis, et al., Molecular mechanisms of cell death: recommendations of the nomenclature committee on cell death 2018, Cell Death Differ. 25 (2018) 486–541 https://doi.org/10.1038/s41418-017-0012-4.
[25] S.M. Aouad, L.Y. Cohen, E. Sharif-Askari, E.K. Haddad, A. Alam, R.P. Sekaly, Caspase-3 is a component of Fas death-inducing signaling complex in lipid rafts and its activity is required for complete caspase-8 activation during Fas-mediated cell death, J. Immunol. 172 (2004) 2316–2323 https://doi.org/10.4049/jimmunol.172.4.2316.
[26] S.E. Tran, T.H. Holmstrom, M. Ahonen, V.M. Kahari, J.E. Eriksson, MAPK/ERK overrides the apoptotic signaling from Fas, TNF, and TRAIL receptors, J. Biol. Chem. 276 (2001) 16484–16490 https://doi.org/10.1074/jbc.M010384200.
[27] R.P. Patel, J. McAndrew, H. Sellak, C.R. White, H. Jo, B.A. Freeman, et al., Biological aspects of reactive nitrogen species, Biochim. Biophys. Acta 1411 (1999) 385–400 https://doi.org/10.1016/S0005-2728(99)00028-6.
[28] R. Bayat Mokhtari, T.S. Homayouni, N. Baluch, E. Morgatskaya, S. Kumar, B. Das, et al., Combination therapy in combating cancer, Oncotarget 8 (2017) 38022–38043 https://doi.org/10.18632/oncotarget.16723.
[29] R. Engelhardt, Rational for clinical application of hyperthermia and drugs, Rev. Med.-Chir. Soc. Med. Nat. Iasi 91 (1987) 347–352.
[30] J.P. May, S.D. Li, Hyperthermia-induced drug targeting, Expert Opin. Drug Deliv. 10 (2013) 511–527 https://doi.org/10.1517/17425247.2013.758631.
[31] A. Dart, Cell death: a better way to die, Nat. Rev. Canc. 17 (2017) 575 https://doi.org/10.1038/nrc.2017.90.
[32] V. Nogueira, N. Hay, Molecular pathways: reactive oxygen species homeostasis in cancer cells and implications for cancer therapy, Clin. Cancer Res. 19 (2013) 4309–4314 https://doi.org/10.1158/1078-0432.CCR-12-1424.
[33] D. Trachootham, J. Alexandre, P. Huang, Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat. Rev. Drug Discov. 8 (2009) 579–591 https://doi.org/10.1038/nrd2803.
[34] S.I. Dikalov, D.G. Harrison, Methods for detection of mitochondrial and cellular reactive oxygen species, Antioxidants Redox Signal. 20 (2014) 372–382 https://doi.org/10.1089/ars.2012.4886.
[35] M.G. Battelli, L. Polito, M. Bortolotti, A. Bolognesi, Xanthine oxidoreductase-derived reactive species: physiological and pathological effects, Oxid. Med. Cell. Longev. 2016 (2016) 3527579 https://doi.org/10.1155/2016/3527579.
[36] D.D. Thomas, Breathing new life into nitric oxide signaling: a brief overview of the interplay between oxygen and nitric oxide, Redox Biol 5 (2015) 225–233 https://doi.org/10.1016/j.redox.2015.05.002.
[37] C. Szabó, H. Ischiropoulos, R. Radi, Peroxynitrite: biochemistry, pathophysiology and development of therapeutics, Nat. Rev. Drug Discov. 6 (2007) 662–680 https://doi.org/10.1038/nrd2222.
[38] A. Ozcan, M. Ogun, Biochemistry of reactive oxygen and nitrogen species, Basic Principles and Clinical Significance of Oxidative Stress 3 (2015) 37–58 https://doi.org/10.5772/61193.
[39] M. Redza-Dutordoir, D.A. Averill-Bates, Activation of apoptosis signalling pathways by reactive oxygen species, Biochim. Biophys. Acta 1863 (2016) 2977–2992 https://doi.org/10.1016/j.bbamcr.2016.09.012.
[40] C. Wang, R.J. Youle, The Role of mitochondria in apoptosis, Annu. Rev. Genet. 43 (2009) 95–118 https://doi.org/10.10.1146/annurev-genet-102108-134850.
[41] J. Kale, E.J. Osterlund, D.W. Andrews, BCL-2 family proteins: changing partners in the dance towards death, Cell Death Differ. 25 (2018) 65–80 https://doi.org/10.1038/cdd.2017.186.
[42] P.E. Czabotar, G. Lessene, A. Strasser, J.M. Adams, Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy, Nat. Rev. Mol. Cell Biol. 15 (2014) 49–63 https://doi.org/10.1038/nmr3722.
[43] K. McArthur, B.T. Kile, Apoptotic caspases: multiple or mistaken identities? Trends Cell Biol. 28 (2018) 475–493 https://doi.org/10.1016/j.tcb.2018.02.003.
[44] M.E. Peter, A. Hadji, A.E. Murmann, S. Brockway, W. Putzbach, A. Pattanayak, et al., The role of CD95 and CD95 ligand in cancer, Cell Death Differ. 22 (2015) 885–886 https://doi.org/10.1038/cdd.2015.25.
[45] R.M. Siegel, J.K. Frederiksen, D.A. Zacharias, F.K. Chan, M. Johnson, D. Lynch, et al., Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations, Science 288 (2000) 2354–2357 https://doi.org/10.1126/science.288.5475.2354.
[46] A.O. Hueber, A.M. Bernard, Z. Herincs, A. Couzinet, H.T. He, An essential role for membrane rafts in the initiation of Fas/CD95-triggered cell death in mouse thymocytes, EMBO Rep. 3 (2002) 190–196 https://doi.org/10.1093/embo-reports/kvf022.
[47] D. Scheel-Toellner, K. Wang, R. Singh, S. Majeed, K. Raza, S.J. Curnow, et al., The death-inducing signalling complex is recruited to lipid rafts in Fas-induced apoptosis, Biochem. Biophys. Res. Commun. 297 (2002) 876–879 https://doi.org/10.1016/S0006-291X(02)02311-2.
[48] S. Baritaki, P. Kanellou, S. Apostolakis, D.A. Spandidos, B. Bonavida, Role of plasma membrane fluidity alterations in tumor cell sensitization to apoptosis-inducing stimuli: therapeutic implications, Proc. Am. Assoc. Canc. Res. 47 (2006).
[49] B. Csoboz, G.E. Balogh, E. Kusz, I. Gombos, M. Peter, T. Crul, et al., Membrane fluidity matters: hyperthermia from the aspects of lipids and membranes, Int. J. Hyperth. 29 (2013) 491–499 https://doi.org/10.3109/02656736.2013.808765.
[50] T. Esaki, T. Hayashi, E. Muto, H. Kano, T.N. Kumar, Y. Asai, et al., Expression of inducible nitric oxide synthase and Fas/Fas ligand correlateswith the incidence of apoptotic cell death in atheromatous plaques of humancoronary arteries, Nitric Oxide 4 (2000) 561–571 https://doi.org/10.1006/niox.2000.0311.
[51] J. Liversidge, A. Dick, S. Gordon, Nitric oxide mediates apoptosis throughformation of peroxynitrite and Fas/Fas-ligand interactions in experimental autoimmune uveitis, Am. J. Pathol. 160 (2002) 905–916 https://doi.org/10.1016/S0002-9440(10)64913-9.
[52] T. Kaufmann, A. Strasser, P.J. Jost, Fas death receptor signalling: roles of Bid and XIAP, Cell Death Differ. 19 (2012) 42–50 https://doi.org/10.1038/cdd.2011.121.
[53] S.W. Tait, D.R. Green, Mitochondria and cell death: outer membrane permeabilization and beyond, Nat. Rev. Mol. Cell Biol. 11 (2010) 621–632 https://doi.org/10.1038/nrm2952.
[54] X.M. Yin, Signal transduction mediated by Bid, a pro-death Bcl-2 family proteins, connects the death receptor and mitochondria apoptosis pathways, Cell Res. 10 (2000) 161–167 https://doi.org/10.1038/sj.cr.7290045.
[55] A.G. Porter, R.U. Jänicke, Emerging roles of caspase-3 in apoptosis, Cell Death Differ. 6 (1999) 99–104 https://doi.org/10.1038/sj.cdd.4400476.