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A trienone analog of curcumin, 1,7-bis (3-hydroxyphenyl)-1,4,6-heptatrien-3-one, possesses ROS- and caspase-mediated apoptosis in human oral squamous cell carcinoma cells in vitro

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Abstract

The leading causes of oral cancer treatment failure are cancer metastasis and chemotherapeutic resistance. Thus, developing novel anticancer agents that are effective against those aggressive cancer cells would be important for complementary or alternative treatments. The objective of this study was to investigate cytotoxicity and anticancer mechanisms of a synthetic trienone analog of curcumin, 1,7-bis(3-hydroxyphenyl)-1,4,6-heptatrien-3-one (trienone **11**), against human oral squamous cell carcinoma (OSCC) cells exhibiting multidrug resistance (CLS-354/DX). The study of cytotoxicity showed that trienone **11** exerted threefold stronger cytotoxicity to CLS-354/DX cells than curcumin. Trienone **11** (15–30 μM) markedly induced intracellular reactive oxygen species (ROS) resulting in apoptotic cell death within 24 h, through activation of caspase-3/7 and caspase-9. A ROS inhibitor, *N*-acetylcysteine (NAC) prevented apoptotic cell death via decreasing caspase activation. Thus, the cytotoxicity of trienone **11** against CLS-354/DX cells was ROS-mediated intrinsic apoptosis. Overall, trienone **11** could be an interesting lead for developing anti-cancer agents against multidrug resistant OSCC cells.

Keywords: Trienone, Curcumin analog, Cytotoxicity, Human mouth squamous cell carcinoma cells, Reactive oxygen species, Apoptosis

Introduction

Oral squamous cell carcinoma (OSCC) is particularly serious because this cancer type is often diagnosed when cancer metastasizes in its late stages [1, 2]. Late-stage cancer cells are rendered more resistant to chemotherapy by altering biological factors such as drug transportation, metabolic reprogramming, redox status, and DNA repair that may contribute to treatment failure [3, 4]. During the last few decades, extensive studies have examined new strategies to overcome multidrug-resistant cancer cells by restraining their escape via resistance mechanisms through the use of novel anticancer drugs [5]. Currently, new anticancer agents have been developed by structurally modifying a natural compound to create analogs or derivatives with increased anticancer efficacy and relatively weak side effects [6].

Curcumin, a major chemical constituent isolated from the rhizome of turmeric (*Curcuma longa* L.), has demonstrated broad-spectrum anti-inflammatory [7], antioxidant [8], and anticancer activities [9]. Curcumin exerts anticancer effects against various types of cancer

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by interacting with multiple intracellular proteins including transcription factors that potentiate the expression of multiple genes [10]. Thus, curcumin is a popular lead compound for new drug development [9]. However, the use of curcumin is restricted by poor bioavailability, stability, solubility, and absorption [11, 12]. Synthetic curcuminoid derivatives have, therefore, been developed by modifying the parent structure to improve anticancer potency. These analogs include DK1 [13], B63 [14], EF24 [15], WZ35 [16], and those with 1,4,6-trien-one function or trienones [17].

Trienone is a rare naturally occurring curcuminoid analog; its biological activities have not been comprehensively investigated. The 1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one (trienone **4**) is a minor naturally occurring analog of curcumin that was initially isolated from *C. domestica* Val. [18]. Trienone **4** has been reported to exert anti-peroxidation [19] and anti-inflammatory activity [20], together with cytotoxicity against melanoma cells [21]. Recently, we demonstrated the successful synthesis of trienone **4** and its analogs in the laboratory to replace natural trienones for cytotoxicity evaluation in the KB cell line. Our results determined that a new trienone analog, 1,7-bis(3-hydroxyphenyl)-1,4,6-heptatrien-3-one (trienone **11**), exerted interesting cytotoxicity against the KB cell line with a very high selectivity index, suggesting potential for anticancer activity [17]. However, the exact cytotoxic mechanism of trienone **11** remains unclear and further investigation of other cancer cell types is required.

Here, the underlying anticancer activity mechanism against the multidrug-resistant human OSCC cell line (CLS-354/DX) of trienone **11** was investigated. Potency against CLS-354/DX cells of trienone **11** was compared with curcumin and trienone **4**, while mechanisms of cytotoxicity including apoptosis induction, reactive oxygen species (ROS) production, and caspase activation were also assessed. We proposed that trienone **11** is a promising candidate for developing agents against multidrug-resistant OSCCs.

Materials and methods

Curcumin, trienone, and trienone analog of curcuminoids

Curcumin was isolated from the rhizomes of *C. longa* as described previously [22]. The trienones **4** and **11** were synthesized and characterized by the literature method [17] (Fig. 1). The NMR spectra of these compounds are presented in Additional file 1. All these compounds were dissolved in dimethyl sulfoxide (DMSO) before use.

Cell lines and cell culture reagents

CLS-354/WT and CLS-354/DX cell lines were established from human OSCC cell line CLS-354 (CLS Cell line service, GmbH, Eppellheim, Germany) as has been described earlier [23, 24]. A monkey kidney epithelial cell line (Vero) was purchased from CLS Cell line service (GmbH, Eppellheim, Germany). All cell culture media and supplementary reagents were purchased from Gibco, Life Technology, USA. All cell culture materials were purchased from Nest Biotechnology, China.

Cell culture

CLS-354/DX, CLS-354/WT, and Vero cells were cultured in sterile conditions in RPMI-1640 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mM L-glutamine. The cells were maintained in a CO_2 incubator with 5% CO_2 and 95% humidity at 37 °C. Cell culture media were changed every 2 days in a sterile manner. The cells were sub-cultured when confluency was reached to 80% using 1:4 split ratio.

Cytotoxicity test

Cells were seeded in 96-well plates at a density of 1.0×10^4 cells/0.32 cm^2. The cells were grown for 48 h before treatment to reach confluency at 70%. Curcumin, trienone **4**, and trienone **11** solutions were diluted in

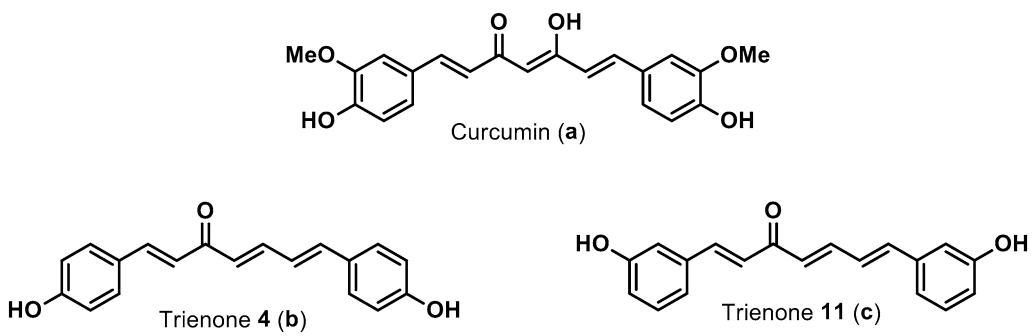

Fig. 1 Structures of curcumin (**a**), trienone **4** (**b**), and trienone **11** (**c**) determined by NMR (^{1}H and ^{13}C NMR, and 2D COSY, HMQC and HMBC) and mass spectroscopy

complete RPMI-1640 to obtain a final concentration at 80 μM, and then serially diluted to 5 μM. The cells were treated with the compounds for 24 h. In some experiments, the cells were pre-incubated with 5 mM *N*-acetyl cysteine (NAC, Sigma-Aldrich Corp., St. Louis, MO, USA) for 1 h, followed by treatment with trienone **11** at various concentrations for 24 h in order to evaluate ROS-mediated cytotoxicity. After the 24 h-treatment, the spent media were removed, 200 μl of 0.5 mg/ml 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Invitrogen, Life Technologies, USA) solution was added to each well and incubated for 4 h. The formazan crystals were dissolved in DMSO. The absorbance (Abs) was detected at 560 nm and background at 670 nm was subtracted, using a microplate reader [23]. Cell viability was calculated as follows:

$$\%Cell\ \ viability = \left(\frac{Abs.treated}{Abs.untreated}\right) \times 100$$

Determination of half maximal inhibitory concentration (IC_{50}) and selectivity index (SI)

IC_{50} was determined from a dose response curve (inhibitory effect) using Graphpad Prism 6.0 (Graphpad Software Inc., USA). Selectivity index (SI) was calculated by comparing IC_{50} values for the CLS-354/DX or CLS-354/WT cell lines against the IC_{50} of the same compound in a cell line of non-cancerous cells [25].

Measurement of intracellular ROS

Cells were seeded at $4.2 \times 10^4/cm^2$ in a 6-well plate cultured for 48 h. The cells were treated with trienone **11** at various concentrations for 12 h. In some experiments, the cells were pre-treated with NAC for 1 h, followed by trienone **11** for 12 h. Intracellular ROS generation was monitored by a flow cytometer using the peroxide-sensitive fluorescent probe 2′,7′-dichlorofluorescin diacetate (H_2DCFDA) as previously described [26]. In brief, after the incubation, cells were harvested, centrifuged at 1500 rpm for 5 min, and washed with phosphate buffer saline (PBS) twice. The suspension of cells was transferred to FACS tube, mixed with 10 μM of H_2DCFDA (Invitrogen, Life Technologies, USA), further incubated at RT for 30 min and protected from light. The cell suspension was then subjected to a flow cytometer for ROS analysis. Intracellular ROS was quantitated using CellQuest™ Pro software (BD Biosciences, San Jose, CA, USA).

Caspase activity assay

Cells were treated with the compounds for 6 h. After treatment, the cells were harvested and washed twice in cold PBS. To extract protein, the cell pellet was lysed in lysis buffer and centrifuged at 13,000 rpm for 5 min. The supernatant was collected and measured for protein content using DC-Bradford assay kit (Bio-rad Inc., USA).

Activities of caspase-3/7, caspase-8, and caspase-9 were determined using Caspase Colorimetric Activity Assay Kit (Millipore, USA) [27]. Briefly, 100 μg protein was pipetted to a 96-well plate (70 μl), followed by 50 μl reaction buffer, and 10 μl substrate (IETD-pNA for caspase-8, LEHD-pNA for caspase-9, and DEVD-pNA for caspase-3). The mixture was incubated at 37 °C for 3 and 24 h. Formation of *p*-nitroaniline was measured with an ELISA-microplate reader (Biochrom EZ Read 400, UK).

AnnexinV-FITC/Propidium iodide double staining assay

After that the cells were incubated with the compounds at various concentrations for 24 h, they were trypsinized, collected, washed with PBS, and stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). After staining, the stained cells were subjected to a flow cytometer (BD Biosciences 2 Laser FacsCalibur, USA) using FL-1 and FL-2. Percentages of Annexin V-FITC- and Annexin V-FITC/PI-positive cells were analyzed as apoptotic cells by CellQuest™ Pro (BD Biosciences) [23].

Statistical analysis

Data are expressed as mean ± standard deviation (SD) from at least three independent experiments. Statistical comparisons were performed using Student's t-test for differences between two groups and by one-way ANOVA with Bonferroni Post Hoc test for differences among multiple groups. Significance was assigned for p values < 0.05 using GraphPad Prism 6.0 (GraphPad Software, USA).

Results

Effect of trienone 11 on cell viability and cytotoxicity in CLS-354 cells

Human oral squamous carcinoma cell lines CLS-354/DX and CLS-354/WT were established as in vitro models for drug responsiveness studies. CLS-354/DX cells were found to be more aggressive and more multidrug-resistant than CLS-354/WT [23, 24]. To examine responsiveness to curcumin, trienone **4**, and trienone **11** (Fig. 1), we performed MTT assay after 24 h treatment of the three compounds at concentrations ranging from 0.01 to 80 μM. Cell proliferation of CLS-354/DX cells (Fig. 2a) and CLS-354/WT (Fig. 2b) was inhibited by curcumin, trienone **4**, and trienone **11** in a dose-dependent manner. In both cell lines, cell viability drastically decreased by 70–75% upon treatment with 20 μM of trienone **11** (Fig. 2a, b, lower panel) but not so with curcumin

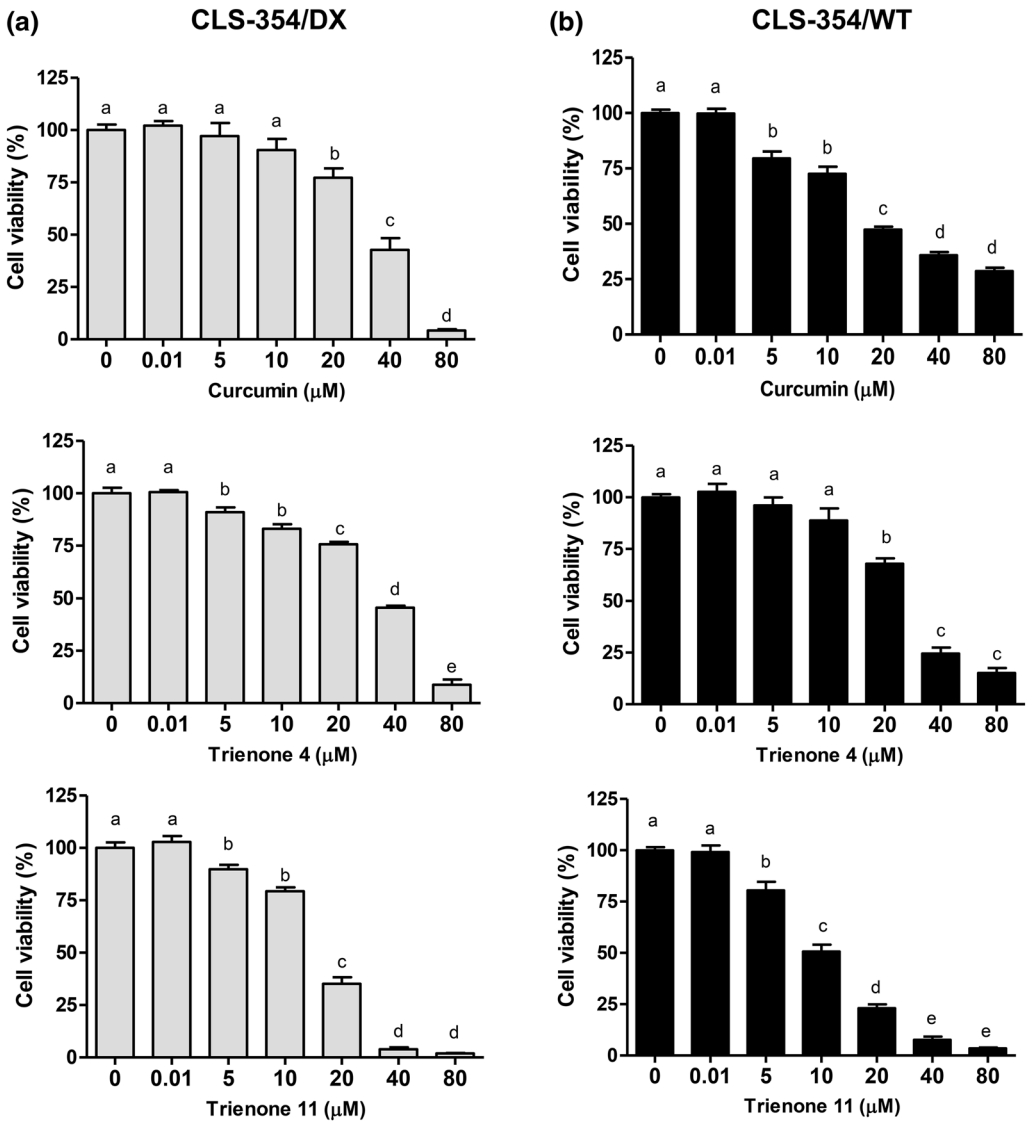

Fig. 2 The effects of curcumin, trienone **4**, and trienone **11** on cell viability of human squamous cell carcinoma CLS-354/DX and CLS-354/WT cells. CLS-354/DX cells (**a**) and CLS-354/WT cells (**b**) were treated with 0.01- 80 μM of curcumin, trienone **4**, or trienone **11** for 24 h. Cell viability was measured by MTT assay after treatment. Values are expressed as mean ± standard deviation (SD) of duplicate samples in three independent experiments. Different letters above the bar indicate the significant difference ($p < 0.05$)

(Fig. 2a, b, upper panel) and trienone **4** (Fig. 2a, b, middle panel), indicating comparatively low responsiveness to curcumin and trienone **4**. Half-maximal inhibitory concentration (IC_{50}) and selectivity index (SI) were examined to compare the potency and safety margin of the compounds. As shown in Table 1, the IC_{50} concentrations of curcumin, trienone **4**, and trienone **11** observed in CLS-354/DX cells were 47.54 ± 1.42, 36.58 ± 1.02, and 16.50 ± 1.03 μM, respectively, while IC_{50} values observed in CLS-354/WT were 13.31 ± 1.24, 23.81 ± 1.03, and 10.09 ± 1.06 μM, respectively (Table 1). DMSO (< 0.5%) was used as the treatment vehicle and did not affect cell viability. Results suggested that trienone **11** treatment provided a significantly greater IC_{50} value than curcumin (threefold) or trienone **4** (twofold) in CLS-354/DX cells ($p < 0.05$). This finding indicated that curcumin had low potential to inhibit multidrug-resistant cancer but was effective against CLS-354/WT which is non-aggressive. To evaluate the cytotoxicity of the three compounds against normal cells, African green monkey kidney cells (Vero cells) were tested as representative normal epithelial cells. We found that trienone **11** exerted cytotoxicity

Table 1 The IC_{50} and selectivity index (SI) of curcumin, trienone 4, and trienone 11 in CLS-354/DX and CLS-354/WT

Compound	IC_{50} (μM)			Selectivity index (SI)	
	CLS-354/DX	CLS-354/WT	Vero	CLS-354/DX	CLS-354/WT
Curcumin	47.54 ± 1.42^{aA}	13.31 ± 1.24^{aB}	35.41 ± 1.23^{aC}	0.7	2.6
Trienone **4**	36.58 ± 1.02^{bA}	23.81 ± 1.03^{bB}	$> 50^{bC}$	1.4	2.1
Trienone **11**	16.50 ± 1.03^{cA}	10.09 ± 1.06^{cB}	33.89 ± 6.83^{aC}	2.0	3.4

Values are expressed as mean ± standard deviation (SD) of duplicate sample in three independent experiments. Different lower-case letters above values indicate statistically significant difference ($p < 0.05$) in the same column, and different upper-case letters above values indicate statistically significant difference ($p < 0.05$) in the same row

to Vero cells at the IC_{50} concentration of 33.89 ± 6.83 μM, which was significantly weaker than the IC_{50} observed for both cancer cell lines. Moreover, trienone **11** provided the best selectivity index (SI) in both cell lines at 2.0 in CLS-354/DX and 3.4 in CLS-354/WT (Table 1). These data suggested trienone **11** as the most potent compound against CLS-354/DX cells.

Effect of trienone 11 on apoptosis induction in CLS-354/DX cells

CLS-354/DX cells were exposed to trienone **11** for 24 h and changes in cell morphology were observed including cell floating, cell shrinkage, and blebbing (Additional file 1: Fig. S2) as associated characteristics of apoptotic cells. Apoptosis induction was then investigated using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining method. Treatment with 15-30 μM of trienone **11** in CLS-354/DX cells for 24 h increased the proportions of Annexin V-FITC-positive cells (early apoptotic cells) from 2.57 to 11.60%, and Annexin V-FITC-/PI positive cells (late apoptotic cells) from 10.01 to 27.64% (Fig. 3a), while total apoptosis in CLS-354/DX cells significantly increased to 40% of the total cell population (Fig. 3b). Early apoptotic and late apoptotic CLS-354/WT cells also increased from 7.24 to 17.52% and from 5.05 to 14.56%, respectively (Fig. 3c), while total induced apoptosis rate was 30% (Fig. 3d). This data indicated that trienone **11** induced apoptosis, leading to CLS-354/DX cell death.

Effect of trienone 11 on activation of caspases

Caspases are a family of protease enzymes that play a significant role in programmed cell death by initiating two distinct apoptotic pathways as the death receptor-mediated (extrinsic) pathway or the mitochondrial-mediated (intrinsic) pathway [28]. Caspase-9 and caspase-8 are key players in the intrinsic and extrinsic pathways, respectively. These two caspases subsequently activate caspase-3 or caspase-7 (executioner), contributing to degradation of cellular components for apoptosis [29]. To investigate whether caspase was involved in apoptosis, we measured the catalytic activities of the caspases. The cells were exposed to trienone **11** for 6 h. After that, catalytic activities of caspase-3/7, caspase-8, and caspase-9 were monitored in the cell lysate at 3 and 24 h. Results showed that catalytic activity of caspase-3/7 was rapidly elevated within 3 h at 3.3-fold compared to basal level. After 24 h of incubation, activities of caspase-3/7, caspase-8, and caspase-9 significantly increased by 5.2-, 2.0- and 1.9-fold, respectively (Fig. 4). Thus, trienone **11** induced apoptosis in CLS-354/DX cells through both intrinsic and extrinsic pathways.

Effect of trienone 11 on intracellular ROS production

ROS are well-known signaling molecules that play a pivotal role in controlling cell death as well as cell survival. Here, intracellular ROS levels were examined after trienone treatment. Results indicated that trienone **11** at 15 and 30 μM increased ROS levels in CLS-354/DX cells dose-dependently, as shown by right-shifted signals (Fig. 5a). Elevated intracellular ROS levels significantly increased 1.5- and 3.1-fold after treatment with 15 and 30 μM of trienone **11**, respectively (Fig. 5b). Furthermore, *N*-acetyl cysteine (NAC), an antioxidant, was used as a ROS inhibitor to confirm trienone **11**-induced ROS formation in CLS-354/DX cells. We found that NAC treatment reversed ROS levels to the basal level (Fig. 5a, b), suggesting that trienone **11** had ROS-inducing potency. We further compared the ROS-inducing potency between trienone **11** and trienone **4**. Results determined that trienone **4** was not able to induce ROS (Additional file 1: Fig. S3), while trienone **11** was successful.

Involvement of ROS in trienone 11-induced apoptosis

To further demonstrate the involvement of ROS in caspase activation and its anti-proliferative effects, we determined whether NAC prevented caspase catalytic activity and cell death in CLS-354/DX cells. All caspase catalytic activities significantly increased after trienone **11** treatment at 2–5-fold from the basal level (Fig. 6). When the cells were exposed to trienone **11** (30 μM) in the presence of NAC (5 mM) for 6 h, catalytic activities of caspase-3/7 and caspase-9 significantly reduced, similar to

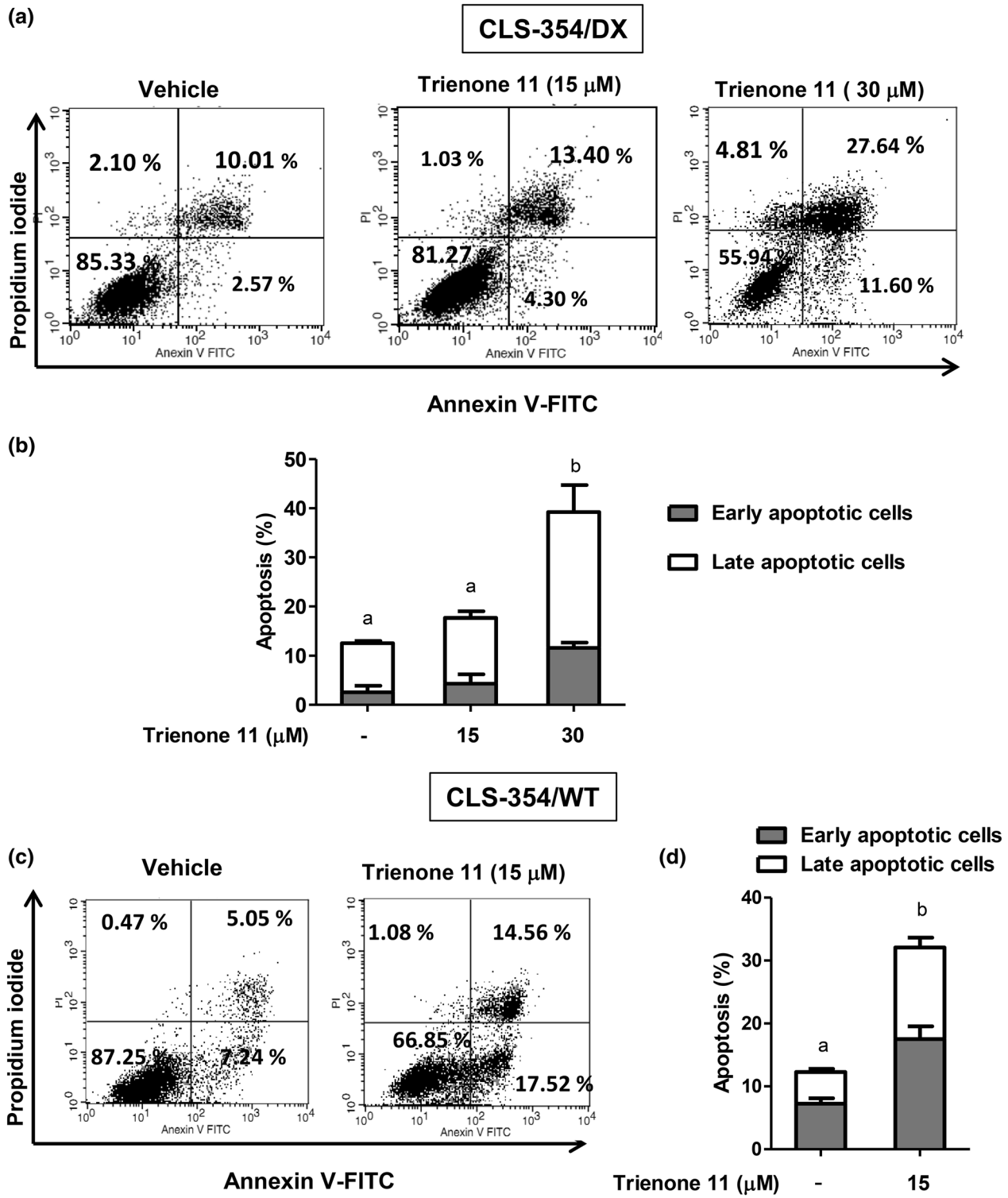

Fig. 3 The effects of trienone **11** on the induction of apoptosis in CLS-354/DX and CLS-354/WT cells. CLS-354/DX cells (**a**, **b**) and CLS-354/WT (**c**, **d**) were treated with trienone **11** at 15–30 μM and 15 μM, respectively, for 24 h. Apoptosis was determined by flow cytometric analysis using double staining with Annexin V-FITC and propidium iodide, and the results are represented as scatter plots (**a, c**) and as percentages of apoptotic cells (**b**, **d**). Values are expressed as mean ± standard deviation (SD) of duplicate samples in three independent experiments. Different letters above the bar indicate the significant difference ($p < 0.05$)

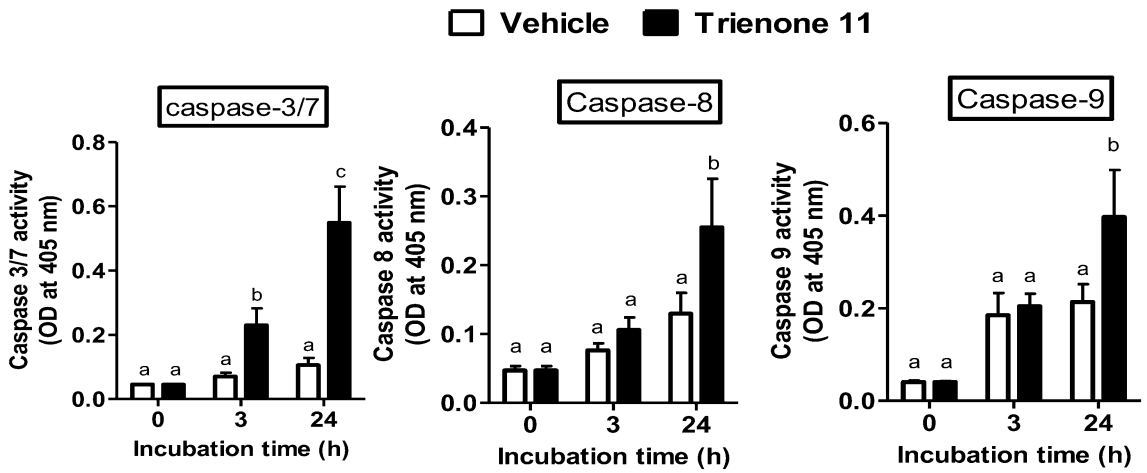

Fig. 4 The effects of trienone **11** on the activation of caspase-3/7, caspase-8, and caspase-9 in CLS-354/DX cells. The cells were treated with trienone **11** (30 μM) for 6 h. After treatment, protein lysates (100 μg protein) were incubated with the substrates of caspase-3/7, caspase-8, and caspase-9 for indicated times at 37 °C for 3 and 24 h. Activities of caspase-3/7 (**a**), caspase-8 (**b**), and caspase-9 (**c**) were measured by a microplate reader. Values are expressed as mean ± standard deviation (SD) from four independent treatments. Different letters above the bar indicate the significant difference ($p < 0.05$)

observation levels in untreated cells (Fig. 6a). Furthermore, the presence of NAC (2.5–10 mM) completely prevented trienone **11**-induced cell death with significantly increased cell viability from 50 to 90% (Fig. 6b). These findings indicated that ROS induced by trienone **11** was decisively involved in the mitochondrial-mediated (intrinsic) pathway of apoptosis.

Discussion

Extensive studies have demonstrated that curcumin exhibits various biological activities including anticancer activity [7–10]; however, curcumin had moderate-to-low cytotoxic efficacy against human cancer cells due to its poor bioavailability [11, 12]. Recently, structural modification of curcumin has offered a potential approach for optimizing new leads for anticancer drug development [30]. Structural modification studies of curcumin have suggested β-diketone moiety as the position leading to chemical instability, while modification to mono-ketone moiety improved stability as well as cytotoxicity [31]. Accordingly, curcuminoid analogs exerting mono-ketone moiety such as trienones could be used as curcumin-based leads for anticancer drug development. Trienones are found in very small amounts in nature [18] but some have been synthesized in the laboratory by various methods including key fragment substituted 4-phenylbut-3-en-2-ones and 3-phenylacrylaldehydes [17], dehydrogenation of the enone with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone and THF [22], and Wittig reaction and aldol condensation [32], thereby overcoming the limitation of trienone preparation.

Several trienones and modified analogs have demonstrated more potent cytotoxicity than curcumin toward cancer cells including melanoma cells (B16F10) [21], prostate cancer cells (PC-3, DU-45, and LNCaP) [32], cervical cancer cells (HeLa) [32] and the KB cell line [17]. Recently, we found that trienone **11** as modified trienone **4** exerted the highest cytotoxic potency in KB cells [17]. Here, trienone **11** was investigated for its anticancer effect against the multidrug-resistant OSCC cell line CLS-354/DX and the parental cell line CLS-354/WT in comparison to curcumin and trienone **4**. We found that curcumin poorly inhibited multidrug-resistant cancer cells with the high IC_{50} at 47.54 μM, whereas curcumin was still active in parental cells with IC_{50} at 13.31 μM. This occurred because CLS-354/DX cells are more aggressive than CLS-354/WT cells by expressing multidrug-resistant protein (MRP), increased drug pump activity, and reduced redox status [22, 23]. As expected, trienone **11** was more potent than both trienone **4** and curcumin in inhibiting cell growth in CLS-354/DX cells, similar to results for KB cells in our previous report [17]. Interestingly, the IC_{50} of trienone **11** inhibiting CLS-354/DX cells (16.5 μM) indicated higher potency than cisplatin (a platinum-based anticancer drug) (33.2 μM) [22]. This finding suggested the notion that trienone **11** may be useful for eliminating multidrug-resistant cancer cells.

Several studies reported that curcuminoids induced apoptotic cell death by increasing the intracellular ROS level over the threshold in OSCC cell lines [33–35]. Similar to our observation, trienone **11** treatment increased intracellular ROS levels and cell death which could be

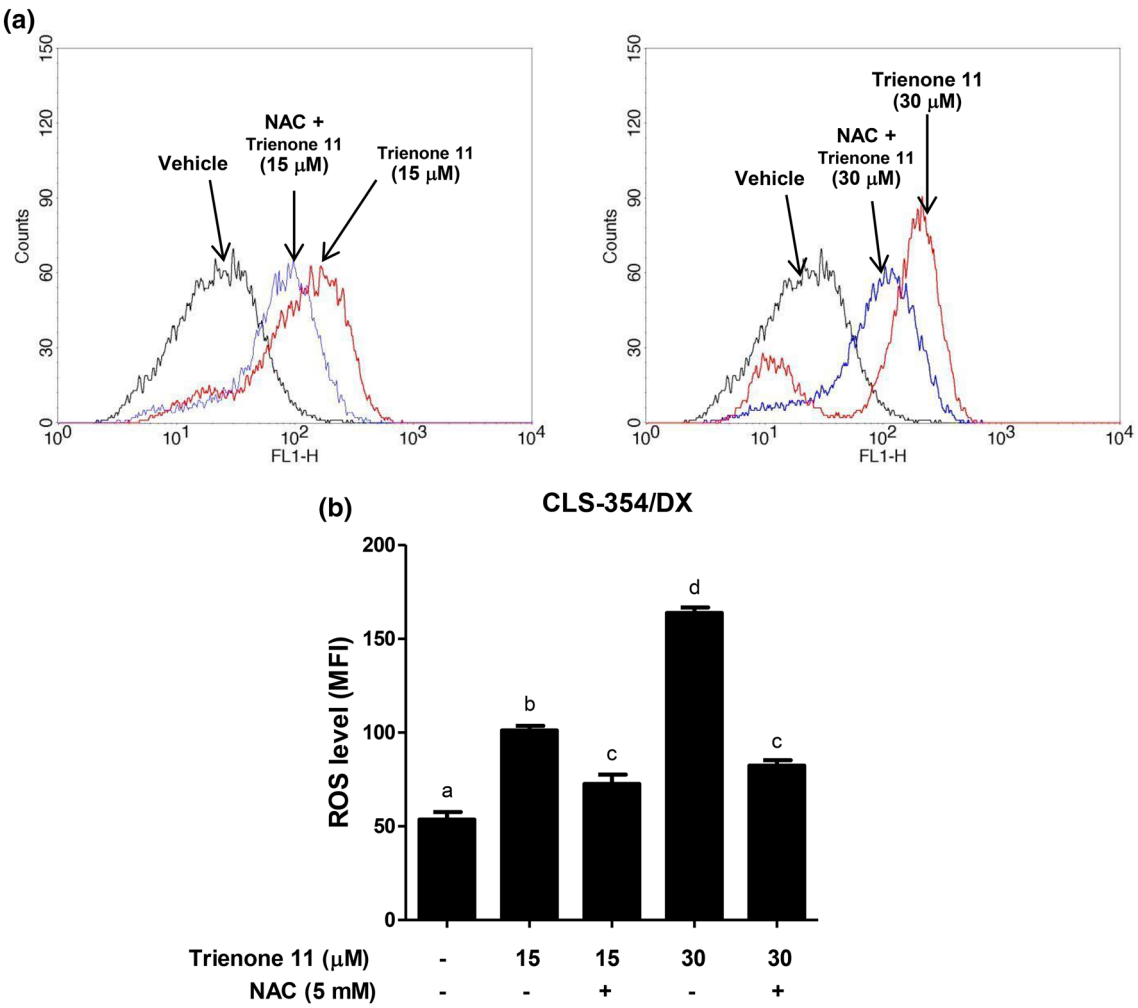

Fig. 5 The effects of trienone **11** on production of reactive oxygen species (ROS) in CLS-354/DX cells. CLS-354/DX cells were treated with 15–30 μM trienone **11** for 12 h with/without 5 mM *N*-acetyl cysteine (NAC) pre-treatment. Intracellular ROS was detected using H_2DCFDA probe followed by flow cytometric analysis. ROS levels are represented in a histogram (**a**) and mean ± standard deviation (SD) of fluorescent intensities (MFI) (**b**). Values are expressed as mean ± standard deviation (SD) of duplicate samples in three independent experiments. Different letters above the bar indicate the significant difference ($p < 0.05$)

reversed by supplementation with NAC, indicating ROS-mediated cell death. ROS-metabolic enzymes have been proposed as the main biological targets of curcuminoids. Curcuminoids and analogs can bind to several ROS-metabolic enzymes including carbonyl reductase 1, glutathione-S-transferase, glyoxalase I, NAD(P)H dehydrogenase, quinone 1, and thioredoxin reductase 1 (TrxR1), thereby inactivating the enzymes and leading to ROS accumulation [16, 36]. Additionally, a differential ROS-inducing ability was related, in part, to the compound's structure. Trienone **11** possesses the free phenolic hydroxyl group at the *meta*-position which can activate stronger oxidation than analogs having a methoxy group [17]. This may induce mitochondrial membrane damage and subsequently mitochondrial ROS leakage.

When the ROS level climbs above cellular tolerability thresholds, excessive ROS causes oxidative damage to deoxyribonucleic acid (DNA), lipids, and proteins, leading to activation of cell death processes mainly as the mitochondrial pathway of apoptosis [37]. Initially, damaged DNA leads to activation of p53 protein and the p53 signaling pathway to upregulate pro-apoptotic proteins and downregulate anti-apoptotic proteins in the mitochondria. Overexpression of pro-apoptotic Bax induces cytochrome c release from the mitochondria to form complexes with Apaf-1 and caspase-9 that consequently activate caspase-3 and apoptosis [37]. ROS-derived curcuminoid treatment causes DNA damage, change in

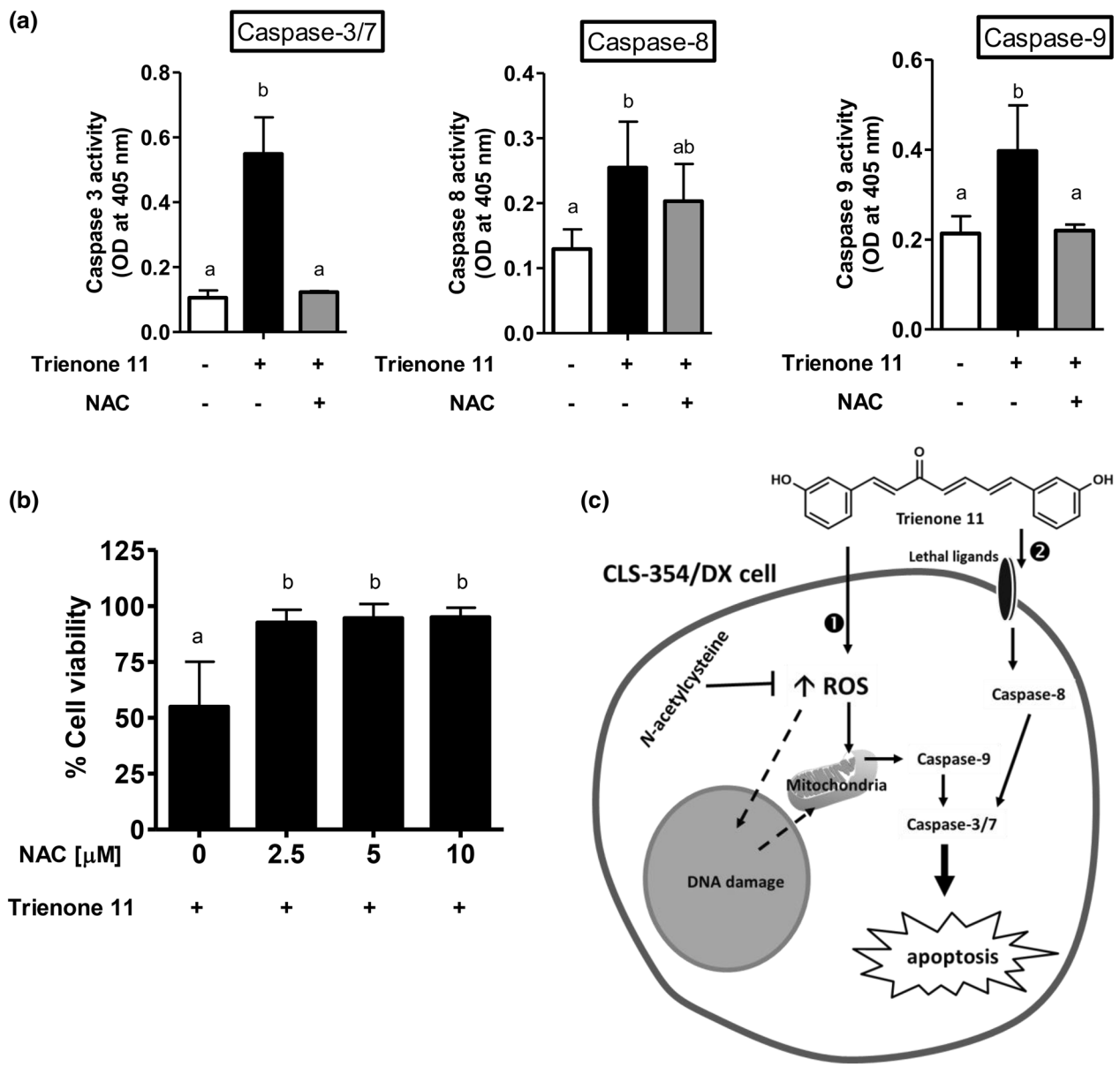

Fig. 6 Trienone **11**-induced cell death is ROS mediated and caspase-3/7, -9 dependent in CLS-354/DX. The activities of caspase-3/7, -8, and -9 of CLS-354/DX cells upon pre-treatment with 5 mM *N*-acetyl cysteine (NAC), followed by 30 μM trienone **11** for 12 h (**a**). Values are expressed as mean ± standard deviation (SD) from four independent treatments. Cell viability (**b**) of CLS-354/DX cells upon pre-treatment with NAC at the indicated concentrations and trienone **11** (30 μM) treatment for 24 h was measured using MTT assay. Values are expressed as mean ± standard deviation (SD) of triplicate samples in three independent experiments. Different letters above the bar indicate the significant difference ($p < 0.05$). The proposed cytotoxic mechanism (**c**) of trienone **11** in CLS-354/DX cells; ❶ trienone **11** markedly induced intracellular ROS production, which may directly damage mitochondria or DNA resulting in caspase-9 and -3/7 activation through intrinsic pathway of apoptosis; ❷ trienone **11** can trigger extrinsic pathway of apoptosis via ROS-independent caspase-8 activation

mitochondrial membrane potential (MMP), increased expression of pro-apoptotic proteins and decreased expression of anti-apoptotic proteins, leading to caspase-9 and -3 activation in oral cancer SAS cells [33]. Thus, caspase-9 and -3 activation could be an indicator of the mitochondrial pathway of apoptosis. Our results showed that ROS-derived trienone **11** revealed activation of caspase-9 and caspase-3, suggesting that trienone **11** might induce apoptosis through a mitochondrial pathway [36]. Furthermore, curcumin has also been reported to induce apoptosis via a Fas receptor/caspase-8 or extrinsic pathway [38]. Consistent with our findings, trienone

11 also increased caspase-8 catalytic activity; however, it is ROS-independent. Trienone **11** may interact with the Fas receptor and form a complex on the cell surface that directly triggers a signaling cascade to activate caspase-8. The proposed cytotoxic mechanism of trienone **11** in CLS-354/DX cells is illustrated in Fig. 6c.

Our studies are the first to indicate that the trienone **11** analog of curcumin shows promise as a potent cytotoxic agent, more powerful than curcumin and the natural trienone **4** against the multidrug-resistant OSCC cell line CLS-354/DX. The compound induced apoptotic cell death via ROS and caspase-3/7, -8, and -9 activations. We also demonstrated the mechanism by which trienone **11** activates ROS to mediate caspase activation and eventually apoptosis via the intrinsic pathway. To further develop trienone **11** as an effective anticancer agent for OSCCs, the therapeutic potential of an in vivo model, as well as the main molecular targets, require a more detailed and comprehensive analysis.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13765-020-0491-8.

Additional file 1. ^{1}H and ^{13}C NMR spectra of compounds (Fig. S1), cell morphology (Fig. S2), and intracellular ROS levels in CLS-354/DX cells upon treatment with trienone 4 (Fig. S3).

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Authors' contributions
TU, TC, WC, performed experiments and wrote the paper. PB and AS helped the preparation of experiments. TU, WC, AS, PB revised the manuscript. AS, WC supervised the work. All authors read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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