



# Nanoencapsulation of Docetaxel Induces Concurrent Apoptosis and Necroptosis in Human Oral Cancer Cells (SCC-9) via TNF- $\alpha$ /RIP1/RIP3 Pathway

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**Abstract** Human oral squamous cell carcinoma is the sixth most frequent malignant cancer, with an unacceptably high death rate that affects people's health. Albeit, there are several clinical approaches for diagnosing and treating oral cancer they are still far from ideal. We previously synthesised and characterised the docetaxel nanoformulation (PLGA-Dtx) and discovered that docetaxel nanoencapsulation may suppress oral cancer cells. The goal of this study was to figure out the mechanism involved in the suppression of oral cancer cell proliferation. We discovered that PLGA-Dtx inhibited SCC-9 cell growth considerably as compared to free docetaxel (Dtx), and that the viability of SCC-9 cells treated with PLGA-Dtx was decreased dose-dependently. MTT assay showed that PLGA-Dtx selectively inhibited the growth of PBMCs from oral cancer patients while sparing PBMCs from normal healthy controls. Further, flow cytometry analysis showed that PLGA-Dtx induced apoptosis and necroptosis in SCC-9 cells. G2/M cell cycle arrest

has been confirmed on exposure of PLGA-Dtx for 24 h in SCC-9 cells. Interestingly, western blot investigation found that PLGA-Dtx increased the amounts of necroptotic proteins and apoptosis-related proteins more efficiently than Dtx. Furthermore, PLGA-Dtx was more effective in terms of ROS generation, and mitochondrial membrane potential depletion. Pretreatment with necroptosis inhibitor Nec-1 efficiently reversed the ROS production and further recover MMP caused by PLGA-Dtx. Overall, this study revealed a mechanistic model of therapeutic response for PLGA-Dtx in SCC-9 cells and proposed its potency by inducing cell death via activation of concurrent apoptosis and necroptosis in SCC-9 cells via TNF- $\alpha$ /RIP1/RIP3 and caspase-dependent pathway.

**Keywords** Oral cancer · Apoptosis · Reactive oxygen species · Mitochondrial membrane potential

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## Introduction

One of the most prevalent forms of cancer in the head and neck region is oral squamous cell carcinoma (OSCC), which develops in the oral cavity and lips. According to International Agency for Research on Cancer (IARC), 377,713 new cases of OSCC were reported globally with Asia (248,360) having the largest number [1]. Asia has the highest rate of mortality from OSCC which was found to be 131,610 (74%). Despite recent breakthroughs in cancer treatment, the expectancy for many cancer patients remains dismal, and current treatment options including surgery, radiotherapy, and chemotherapy continue to have severe side effects [2]. Chemotherapeutic medicines offer a lot of promise as agents, however, due to their low water solubility, fast degradation, and metabolism, they can't deliver the required results. In

addition, drug resistance limits the efficiency of chemotherapeutic therapy that primarily acts by inducing apoptosis or necrosis [3, 4]. Acquired resistance to drugs resulted from different mechanisms, including malfunctioning apoptotic machinery and the activation of the prosurvival cascade [5]. Thus, it's critical to develop complementary medicines that have low systemic toxicity while also improving therapeutic responses in cancer sparing normal cells.

Nanomedicine is a potential option for improving cancer selectivity, antitumorigenic impact, antimetastatic effect, and drug resistance reversal effect, among other things [6]. Nanomedicines might greatly boost the therapeutic impact of tumors by triggering or suppressing regulated cell death (RCD), according to a burgeoning series of studies, possibly paving the way for novel cancer therapies [7]. Different types of nanoparticles including selenium nanoparticles, and zinc oxide nanoparticles are reported to induce ROS-mediated necroptosis and apoptosis in various types of cancer cells [8, 9].

Docetaxel is a taxanes-family chemotherapeutic drug that has been used to treat different types of cancers such as HNSCC, lung cancer, breast cancer, and colorectal cancer. Docetaxel has been proven to expedite apoptosis, trigger autophagy, promote cell cycle progression arrest, and inhibit tumor invasion in the majority of studies [10]. However, little study has been done on nanoformulations of docetaxel eliciting various types of cell death, including necroptosis in HNSCC.

Apoptosis is a highly regulated type of cell death that helps to eliminate undesirable and malfunctioning cells and is crucial in cancer prevention and treatment. The apoptotic process can be triggered by several factors, including DNA damage or excessive growth [11]. Apoptosis can be triggered by two different mechanisms involving extrinsic cell death and intrinsic cell death. Both pathways activate the caspase cascade, which induces cell death [12]. Dysregulation of apoptotic machinery is a hallmark of cancers and other pathological conditions. Therefore, addressing different types of cell death might be useful in cancer treatment.

Necroptosis has recently been reclassified as a kind of controlled necrosis since it can be triggered when apoptosis is disrupted. The same factors that trigger apoptosis, including ROS, hypoxia, tumor necrosis factor (TNF) family, irradiation, or DNA damaging compounds, can also cause necroptosis [13]. Necroptosis is mediated by two distinct signaling cascades: the RIP1/RIP3 regulated mechanism, which is triggered by extracellular signaling molecules like tumor necrosis factor (TNF), and the poly (ADP-ribose) polymerase-1 (PARP-1) signaling, which is triggered by prolonged DNA damage [14].

In our previous study, we discovered that docetaxel nanoencapsulation (PLGA-Dtx) inhibited oral cancer cells more effectively than free Dtx [15]. The mechanism of the

improved antitumor effect has not been elucidated there. In the present study, we evaluated the underlying mechanism of action for PLGA-Dtx in oral cancer cells, identifying the involvement of both apoptosis and necroptosis in PLGA-Dtx induced cell death.

## Materials and Methods

### Materials

Docetaxel, DMEM, DMEM/F-12, MTT (3-(4,5-Dimethylthiazol-2-yl), and dimethyl sulphoxide (DMSO) were obtained from Sigma Chemical Company Ltd. (Burlington, MA, United States). The antibiotic–antimycotic solution, Phosphate buffered saline (PBS), and fetal bovine serum (FBS) were gotten from Gibco, Invitrogen Cor. (Waltham, Massachusetts, USA). Annexin V-FITC Apoptosis Staining / Detection Kit is procured from BD Biosciences (Franklin Lakes, New Jersey, U.S). Primary antibodies, such as caspase-3, caspase-8, caspase-9, bcl-2, cytochrome-c,  $\beta$ -actin, RIPK1, RIPK3, bcl-2, and TNF- $\alpha$  were procured from Santa Cruz (Santa Cruz, California, United States). Necroptosis inhibitor necrostatin-1 (Nec-1) and apoptosis inhibitor Z-VAD-fmk were purchased from Santa Cruz (Santa Cruz, California, United States). Cell culture-grade plastic wares and plates were purchased from Nunc (Roskilde, Sjælland, Denmark). DCFDA and JC-1 dye were bought from Sigma Chemical Company Ltd. (Burlington, MA, United States).

### Cells and Cell Culture

SCC-9 human tongue squamous carcinoma cell lines were procured from American Type Culture Collection (Manassas, Virginia, United States). Five healthy control donors and five oral cancer patients provided peripheral blood samples for in vitro investigations. Samples were collected during standard diagnostic evaluations from the department of Otorhinolaryngology & Head Neck Surgery, King George Medical University (2018–2019). The study was approved by the ethical advisory board and the ethical approval number is 86th ECM II B-Thesis/P22. Before enrolling, all participants signed informed written consent forms. Density centrifugation (Ficoll-Paque) was used to separate PBMCs from the blood of healthy donors and patients. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, HEPES buffer, and 1% antimycotic solution in a humidified chamber.

### Cell Viability

MTT assay was used to determine the viability of cultured cells following manufacturer instructions.  $1 \times 10^4$  cells were

plated in 96 well cell culture plate in 200  $\mu$ l of medium and incubated with varying concentrations (0.125–1  $\mu$ g/ml) of drug docetaxel alone and PLGA-Dtx nanoconjugate containing the same amount of drug as equivalent to the free drug for 24 h. The range of the docetaxel concentration has been selected based on previous literature [16, 17]. After incubation for desired time 10  $\mu$ l of MTT solution was added to each well and waited for the next 3 h. The cell viability was assessed at 550 and 660 nm utilizing a microplate reader.

### Analysis of Apoptosis and Cell Cycle

Apoptosis in SCC-9 and PBMCs cells was analyzed using Annexin V-FITC Apoptosis Staining / Detection Kit. Cells were treated with Dtx and PLGA-Dtx nanoconjugate for twenty-four hours. After treatment, all cells were collected by trypsinization and centrifuged at 2000 rpm; after that, the cells were washed using PBS and harvested in 1 ml of annexin binding buffer that was provided in the kit and incubated with 5  $\mu$ l of annexin-V-FITC dye for 30 min. After incubation with annexin dye, we suspended cells in 1  $\mu$ g/ml PI (propidium iodide dye). Thereafter, 1 ml of annexin binding buffer was used for further dilution of the sample. The sample was acquired at BD Influx and at least 10,000 cells were used for acquisition. BD FACS Diva software was used for the analysis of the acquisition data. Cell cycle detection was done by using the propidium iodide (PI) dye as instructed by the manufacturer.

### Western Blot Analyses

Western blot analysis was done using a vertical electrophoresis unit. Proteins isolated from cells were quantified by BCA kit and further run on 10% SDS gel (sodium dodecyl sulfate poly-acrylamide gel). Afterward, the resolved proteins gel was transferred to PVDF membranes. Next, the protein blotted PVDF membranes were blocked for preventing any unspecific binding with 5 percent BSA (Bovine serum albumin) and further incubated with the desired antibodies for 4 to 6 h or overnight at dilutions instructed by the manufacturer. After completion of desired incubation with primary antibody, the blots were probed with HRP conjugated secondary antibody for 2 h. HRP conjugated secondary antibody was detected by enhancing chemiluminescence using BIO-RAD western blotting detection reagents as described in the manufacturer's protocol. To ensure equivalent protein loading, all blots were stripped and reprobed with  $\beta$ -actin.

### Intracellular ROS Measurement

SCC-9 ( $1 \times 10^5$  cells/well) were cultured in 12-well black flat-bottom cell culture plate. Cells were treated with Dtx/PLGA-Dtx nanoconjugate and Nec-1 for (12 and 24 h).

After desired incubation, cells were washed two times by suspending in PBS. Afterward, 20  $\mu$ M of 2, 7'-dichlorodihydrofluorescein diacetate ( $H_2DCFDA$ ) dye was added to each well. The plate was further incubated for 30 min and after completion of the incubation period medium was discarded. 200  $\mu$ l PBS was added and fluorescence was analyzed by a flow cytometer.

### Mitochondrial Membrane Potential (MMP) Assay

Mitochondrial membrane potential was examined using the mitochondrial staining dye, JC-1. Cells were treated with PLGA-Dtx nanoconjugate and inhibitors for 24 h in a 12-well cell culture plate. Cells were washed with PBS two times and further incubated with 0.3  $\mu$ M JC-1 dye for 30 min. After completion of the incubation period cells were washed two times with PBS and again suspended in 500  $\mu$ l of PBS. Fluorescence intensity was measured from a flow cytometer.

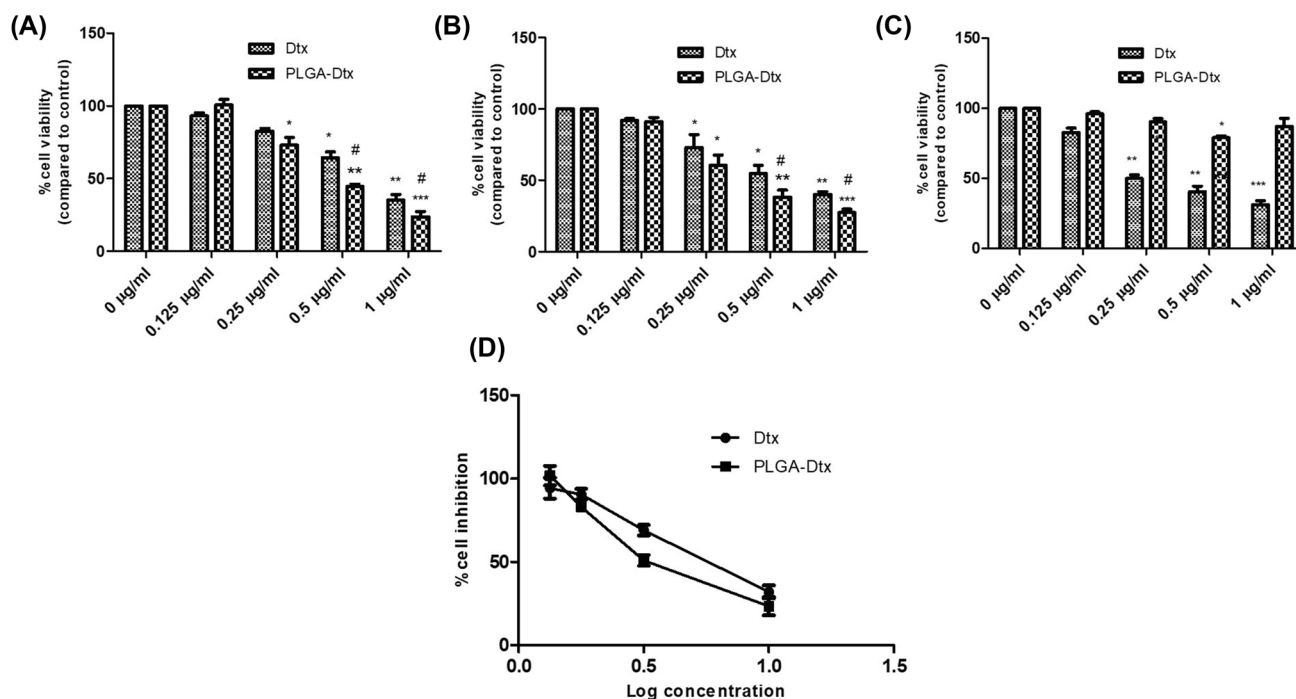
### Statistical Analysis

The data from this study were statistically evaluated to get meaningful results using GraphPad Prism6 (GraphPad Software Inc, San Diego, CA, USA). The results were presented as means with standard deviations. Statistical significance was estimated by Student's t-test, and two-way annova.  $P < 0.05$  was considered significant.

## Results

### Nanoencapsulation Augmented Docetaxel Effect on the Growth of SCC-9 Cells and PBMCs from HNSCC Patients

To examine the effect of nanoencapsulation on docetaxel-induced growth inhibition, we treated SCC-9 cells with increasing doses of docetaxel (Dtx) or nano encapsulated docetaxel (PLGA-Dtx) and the MTT test was performed to determine cell viability. As shown in Fig. 1a PLGA-Dtx significantly affected the viability of SCC-9 cells as compared to control. The effect was more pronounced in the PLGA-Dtx treated cells ( $IC_{50} = 0.6612 \pm 0.06$   $\mu$ g/ml) as compared to free drug docetaxel treated cells ( $IC_{50} = 0.903 \pm 0.1$   $\mu$ g/ml). To verify if the observed effects on SCC-9 cells were also valid for human primary cells, the effect of Dtx or PLGA-Dtx was evaluated in PBMCs isolated from oral cancer patients and healthy volunteers. PLGA-Dtx, as shown in Fig. 1b, had a comparable effect on PBMCs derived from oral cancer patients as it did on SCC-9 cells. PLGA-Dtx, on the other hand, had no effect on the viability of PBMCs isolated from healthy persons until the maximum dose was



**Fig. 1** Cytotoxicity evaluation of Dtx, PLGA-Dtx in SCC-9 Human tongue squamous cell carcinoma cell lines and PBMCs isolated from oral cancer patients and normal healthy donors. **a** Dose dependent effects of Dtx/PLGA-Dtx on SCC-9 cell viability as determined by MTT assay at 24 h, **b** Cytotoxicity of Dtx/PLGA-Dtx on PBMCs isolated from oral cancer patients as detected by MTT assay after 24 h of

treatment, **c** Cytotoxicity of Dtx/PLGA-Dtx on PBMCs isolated from normal healthy donors, **d** IC<sub>50</sub> determination following treatment with Dtx/PLGA-Dtx in SCC-9 cells for 24 h. (\*P < 0.05; \*\*P < 0.01; and \*\*\*P < 0.001 compared with the control group, (#P < 0.05, ##P < 0.01, and ###P < 0.001 compared with the Dtx)

employed, as shown in Fig. 1c, whereas free Dtx was toxic to these cells. These results establish the biosafety profile of PLGA-Dtx as it was more toxic to cancer cells sparing normal primary cells from healthy volunteers. The IC<sub>50</sub> value calculated from the dose response curve was used in further experiments.

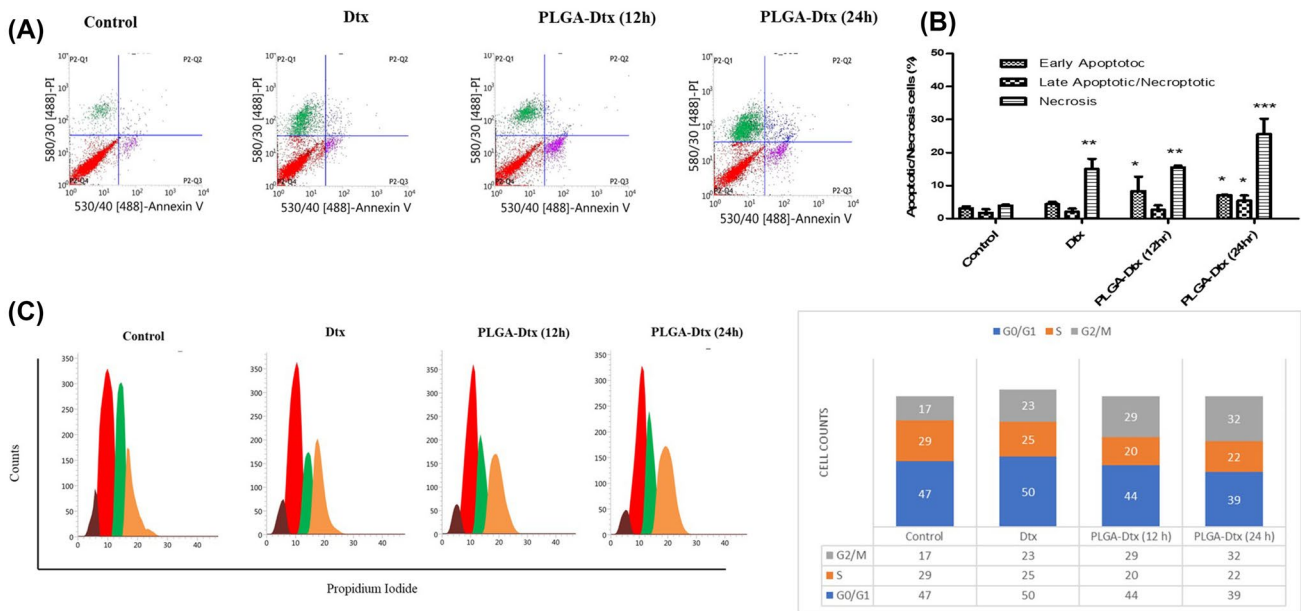
### PLGA-Dtx Triggered Apoptosis and Necroptosis in SCC-9 Cells

To identify the type of cell death produced by Dtx or PLGA-Dtx in SCC-9 cells, a flow cytometry assay with Annexin V-FITC and PI was used. PLGA-Dtx triggered the apoptosis and necrosis of SCC-9 cells in a time-dependent manner. The percentage of apoptotic, late apoptotic/necroptotic, and necrotic cells was calculated using flow cytometer histograms, as shown in Fig. 2a. The percentages of necrotic SCC-9 cells treated with Dtx for 24 h were  $15.045 \pm 3.045\%$  while the percentage of necroptotic and apoptotic cells were  $2.075 \pm 0.925\%$  and  $4.4 \pm 0.6\%$  respectively. The percentages of necrotic SCC-9 cells treated with PLGA-Dtx for 24 h were  $25.62 \pm 4.62\%$ , while the percentage of late apoptosis/necroptotic cells was  $5.495 \pm 1.50\%$  as compared to control. Interestingly percentages of apoptotic cells were also

increased in PLGA-Dtx treated cells by  $7.09 \pm 0.09\%$ . From these results, it can be demonstrated that nanoencapsulation potentiates the effect of free Dtx by inducing both apoptosis and necrosis. Since docetaxel is known to elicit cell cycle arrest in many malignancies, flow cytometry was utilized to see if the reduction of cell growth was impacted by the cell cycle. As seen in Fig. 2c, PLGA-Dtx increased G2/M-phase accumulation, as well as an increase in subG0/G1 content indicating increased cell death and effect was more prominent than free Dtx.

### PLGA-Dtx Activated Caspase-Dependent Apoptosis and TNF- $\alpha$ /RIP1/RIP3 Mediated Necroptosis

To investigate the possible molecular mechanism of PLGA-Dtx triggered apoptosis and necroptosis, expression levels of apoptosis and necroptosis related proteins were examined by Western blotting. We first examined the protein levels of apoptotic markers caspase-3, caspase-9, caspase-8, bcl-2, and cytochrome c in SCC-9 cells treated with Dtx or PLGA-Dtx using western blot analysis. As shown in Fig. 3a PLGA-Dtx treatment significantly increased the levels of caspase-3 and caspase-9. In contrast to caspase-3 and caspase-9 total protein expression level of caspase-8 decreased



**Fig. 2** Apoptotic and necroptotic response of SCC-9 cell lines treatment with Dtx/PLGA-Dtx treatments **a** represents histograms of treated SCC-9 cells **b** Graphical representation of percentage of

apoptotic cells in PLGA-Dtx treated SCC-9 cells **c** Dtx/PLGA-Dtx induced cell cycle arrest in SCC-9 cells analysed by flow cytometer. (\* $P < 0.05$ ; \*\* $P < 0.01$ ; and \*\*\* $P < 0.001$ )

in PLGA-Dtx treated cells. Interestingly, the expression level of anti-apoptotic protein bcl-2 was significantly decreased and the expression level of cyt c was increased in PLGA-Dtx treated cells. All these protein levels significantly changed more potently as compared to Dtx. We suggested that necroptosis may be involved in the death induced by PLGA-Dtx in SCC-9 cells, based on the above flow cytometry data. We then tested whether treatment with PLGA-Dtx could affect the protein levels of necroptosis-associated markers (TNF- $\alpha$ , RIP1, and RIP3) in SCC-9 cells. We discovered that PLGA-Dtx treatment enhanced the protein levels of all of these indicators when compared to control. Finally, our results might suggest that PLGA-Dtx causes both apoptosis and necroptosis in SCC-9 cells.

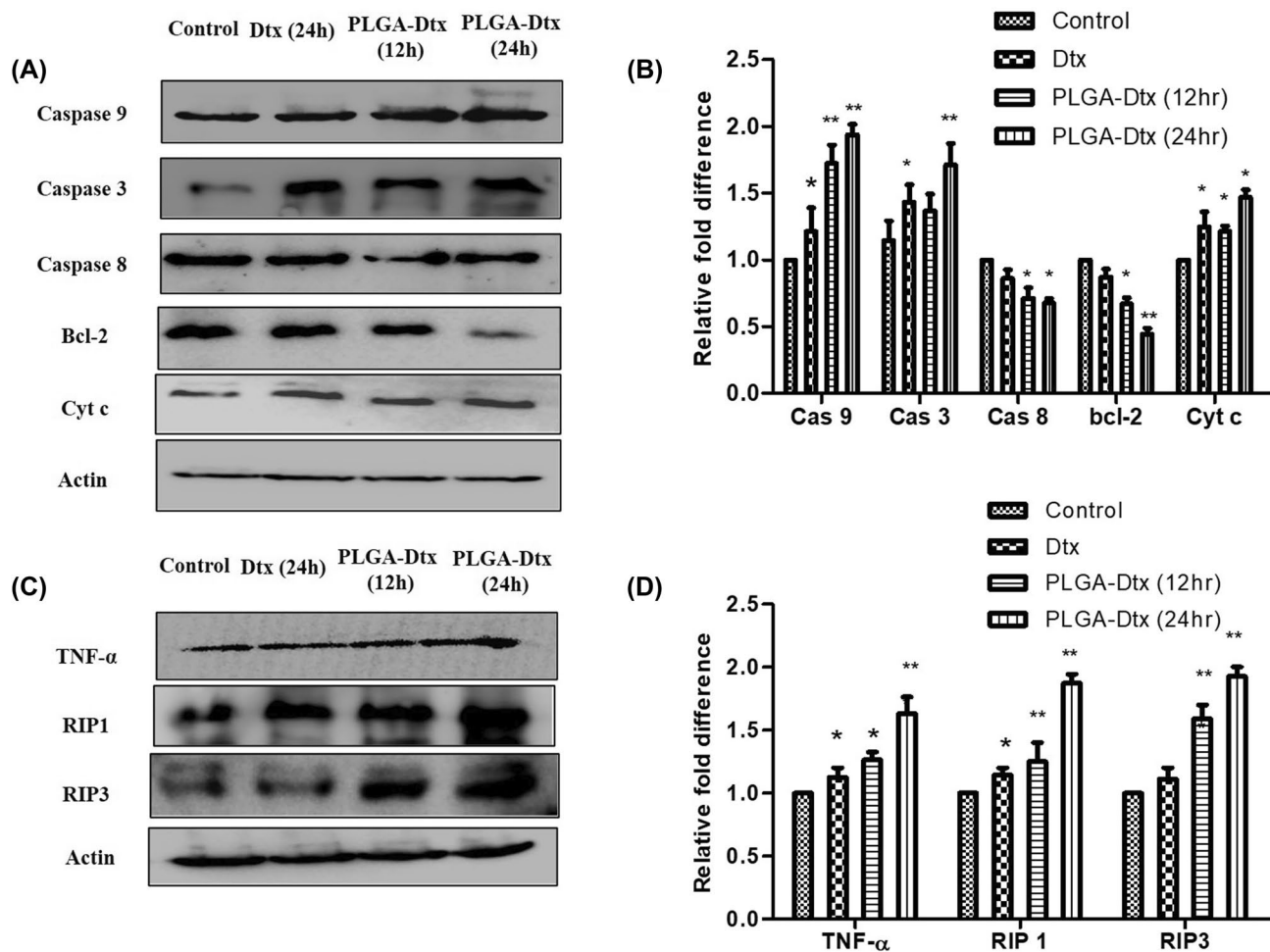
**PLGA-Dtx Enhanced Cellular ROS and Mitochondria Membrane Potential**

ROS has been implicated in several types of cell death by disrupting mitochondrial polarisation and causing ATP depletion. We used the oxidation-sensitive fluorescent dye DCFDA to monitor the redox status of SCC-9 cells to see if PLGA-Dtx induced cell death by triggering the production of ROS. As illustrated in Fig. 4a intracellular ROS levels in PLGA-Dtx-treated cells tended to rise after 12 h, but spiked dramatically after 24 h. Remarkably, pre-treatment with Nec-1 ameliorated the PLGA-Dtx induced ROS production in SCC-9 cells. This finding suggested

that necroptosis mediated by RIP1/RIP3 played a critical role in regulating ROS generation. Next, we examined the change in mitochondrial membrane potential ( $\Delta\Psi_m$ ) by quantifying the change in JC-1 fluorescence dye from red to green. Figure 4c demonstrates that PLGA-Dtx lowered mitochondrial membrane potential by decreasing the red to green ratio, whereas pretreatment with Nec-1 significantly reduced PLGA-Dtx-induced mitochondrial depolarization, boosting SCC-9 cell MMP recovery. Taken together these results showed that oxidative stress-mediated mitochondria membrane potential played a significant role in the increased toxicity of PLGA-Dtx in SCC-9 cells.

**PLGA-Dtx Induced Cell Death Reversed by Necrostatin-1**

We examined the dose–response curve of SCC-9 cells treated with PLGA-Dtx conjugate in the presence of pharmacological inhibitors of apoptosis (pan-caspase inhibitor z vad-fmk) and/or necroptosis (RIP1 kinase inhibitor necrostatin-1). Figure 5 showed the involvement of both apoptosis and necroptosis as cells rescued from PLGA-Dtx conjugate induced cell death. Surprisingly Nec-1 treatment reversed the cell death more efficiently than z vad-fmk. Based on these results it can be demonstrated that both pathways apoptosis, as well as necroptosis, play a key role in PLGA-Dtx conjugate persuaded cell death.



**Fig. 3** **a** The proteins of SCC-9 cells treated with Dtx/PLGA-Dtx for 24 h were prepared and quantified, the levels caspase-3, caspase-9, caspase-8, bcl-2, cytochrome-c were measured by western blot analysis, **b** Quantification of apoptotic protein levels were done

using ImageJ software, **c** Levels of necroptotic proteins TNF- $\alpha$ , RIP1 and RIP3 were measured by western blot analysis, **d** Quantification of necroptotic protein levels were done using ImageJ software. (\*P < 0.05; \*\*P < 0.01; and \*\*\*P < 0.001)

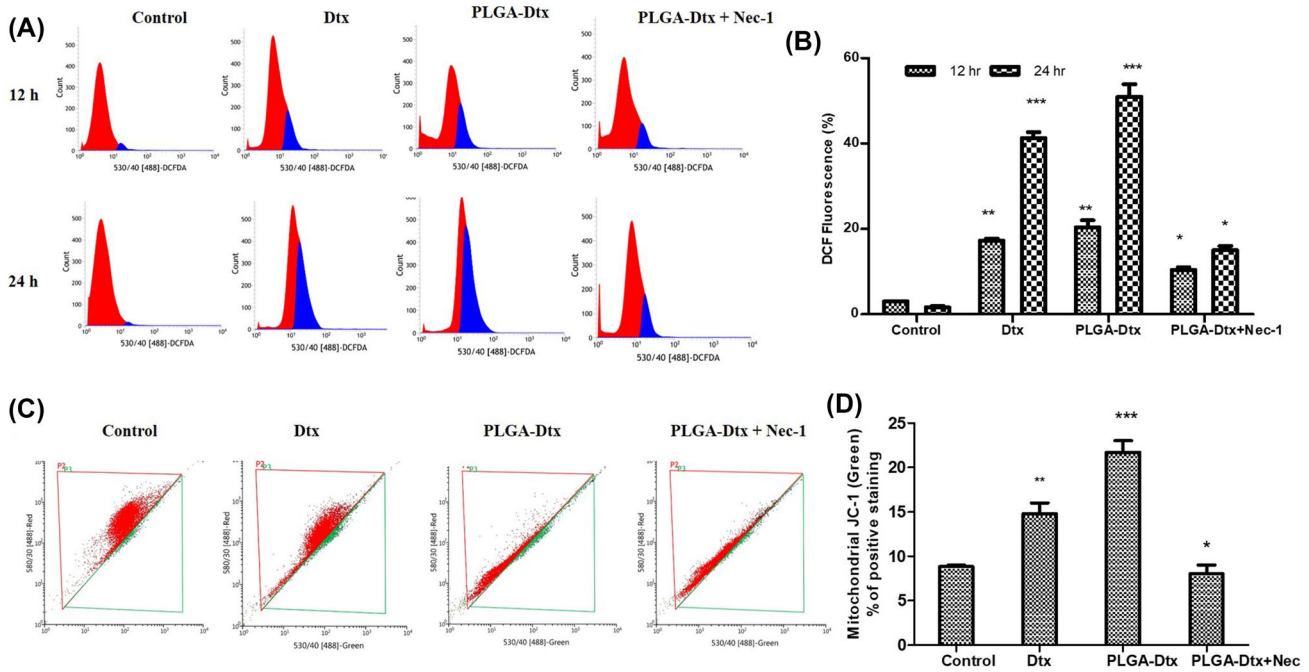
## Discussion

Necroptosis has received a lot of interest as an alternate type of programmed cell death. It provides potential new anti-cancer approaches, particularly for overcoming drug resistance [15]. Compromised apoptosis and resistance to cell death is a major hindrance in the treatment of cancer [5]. Necroptosis was primarily considered as a fallback and substitute for apoptosis; in other words, it is incompatible with apoptosis [5]. It is evident from the study that demonstrated the cell death in L929 cells was caused by necroptosis when cells were exposed to apoptosis inhibitor [18]. Necroptotic cell death involves the cell membrane rupture and subsequent release of various DAMPs that are capable to induce immune-mediated cell death. The potential of necroptosis to recruit the immune mediators for assistance in cell death presents a promising opportunity for cancer treatment [19]. Several studies are in different phases that

exploit necroptosis as the key death mechanism to increase the effectiveness of chemotherapeutic drugs [20].

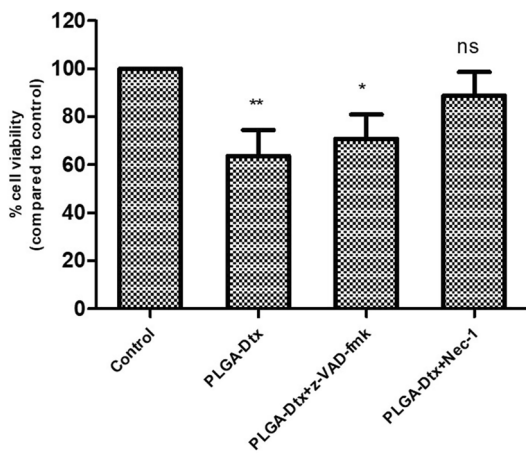
Recent shreds of evidence recommend that chemotherapeutic agents can induce different types of cell death whether apoptotic, necroptotic, or necrosis in cancer [5, 19]. Taxanes are the first line of drugs for treating various solid cancer. However, the application is limited largely attributed to its hydrophobic nature and various side effects, and less specificity. Docetaxel is one of the taxanes that is widely used nowadays for treating cancer and is an antimetabolic drug that primarily affects the microtubules [21]. There is a need to make taxanes more effective since they only provide palliative benefits. Nanoparticles are thought to have improved anticancer effects due to the activation of different cell death modes.

Here, we assessed the effects of PLGA-Dtx and Dtx on SCC-9 cells (human oral cancer cell line) and PBMCs isolated from oral cancer patients and normal healthy donors.



**Fig. 4** Dtx/PLGA-Dtx induced generation of oxygen radicals in SCC-9 cells **a** SCC-9 cells were treated with Dtx or PLGA-Dtx for 12 and 24 h in the presence or absence 20  $\mu$ M Nec-1 and then stained with DCFDA DYE and analysed by flow cytometry, **b** Graphical representation of ROS induced by Dtx or PLGA-Dtx, **c** SCC-9

cells were treated were treated with Dtx or PLGA-Dtx in the presence or absence of 20  $\mu$ M Nec-1 for 24 h followed by JC-1 staining and analysed by flow cytometry, **d** Graphical representation of loss of mitochondrial membrane potential. (\*P < 0.05; \*\*P < 0.01; and \*\*\*P < 0.001)



**Fig. 5** Effect of different death inhibitors z-VAD-fmk or Nec-1 on the viability of SCC-9 cells analysed by MTT assay. (\*P < 0.05; \*\*P < 0.01; and \*\*\*P < 0.001)

In our results, we confirmed that PLGA-Dtx has superior anticancer therapeutic potential as it inhibited cancer cell growth more significantly than the free drug docetaxel. PLGA-Dtx nanoconjugate was also found to be more effective in PBMCs from oral cancer patients whereas it imparted minimal toxicity in the PBMCs isolated from normal healthy donors. Moreover, its free counterpart Dtx

exhibited significant toxicity in cells from healthy donors. This may be because the PLGA nanoparticles efficiently target cancer cells while the release of drug from nanocarrier is sustainable. These results are in accordance with the previous studies where the nanoencapsulation of drug exhibited improved potential against cancer cells leaving normal cells less affected [21–25].

Docetaxel has been shown to exhibit its anticancer effect especially by activating apoptosis and tubulin damage [26]. Previous research data assessing the anti-tumor effects of docetaxel and its nanoformulation have engrossed on apoptosis induction, inhibition of migration of cancer cells, diminished angiogenesis, and cell cycle arrest [27, 28]. Nonetheless, no specific research on the effects and mechanisms of PLGA-Dtx-induced cell death in oral cancer cells has been published. In the present study, PLGA-Dtx was identified as both apoptosis and necroptosis inducer in human lung cancer cells. Annexin V-PI double-staining assay revealed both early and late apoptotic/necroptotic cell death. Indeed, apoptotic and necroptotic cells were increased more prominently in PLGA-Dtx treated SCC-9 cells as compared to Dtx. Further, we elucidated the cell cycle arrest by using propidium iodide dye and it was shown that cells were accumulated in the G2/M phase of the cell cycle in PLGA-Dtx treated cells.

Results from Annexin V-PI double-staining were consistent with the observation of the western blot analysis assay that proteins associated with apoptosis (caspase-3, caspase-9, and cytochrome-c) were elevated and the level of non-apoptotic protein (bcl-2) was diminished significantly in PLGA-Dtx treated cells. Surprisingly caspase-8 expression was found to be compromised in the PLGA-Dtx treated cells indicating the involvement of necroptosis also. The most well-studied form of necroptosis cell death is TNF-driven necroptosis [19]. For activation of necroptosis, receptors of TNF- $\alpha$  are entangled with corresponding ligands present on the cell membrane. Following the binding of the receptor to its ligands downstream signaling cascade which includes the formation of necrosomes and membrane-associated complex I, complex II was activated resulting in necroptosis. The activation of necroptosis was linked to a decrease in caspase-8 levels and phosphorylation levels of RIP1, RIP2, and MLKL [29]. In our findings, we noticed that the level of proteins involved in the necroptosis process TNF- $\alpha$ , RIP1, and RIP3 was elevated in oral cancer cells treated with PLGA-Dtx and the effect was more prominent than in Dtx. Therefore, we suggested that PLGA-Dtx could induce both apoptosis and necroptosis by activating the expression of caspases and TNF- $\alpha$ , RIP1, and RIP3. These findings are consistent with prior studies with nanoparticles, which showed that nanoformulations can trigger several types of cell death [30, 31].

Exploiting reactive oxygen species (ROS) as a cancer treatment is a revolutionary chemotherapeutic technique. Cancer cells frequently exhibited elevated levels of intracellular ROS that sometimes helped the cancer cells to promote cell growth and even provide therapeutic resistance [32]. Increased ROS may have negative consequences for essential macromolecules including proteins, ribonucleic acids (DNA and RNA), and lipids, ultimately leading to cell death [33]. Oxidative stress is caused by an increase in ROS production or a reduction in ROS scavenging capability, resulting in a buildup of ROS. Redox adaptation may be triggered by oxidative stress, resulting in a change in redox dynamics with increased ROS synthesis and clearance to keep ROS levels below lethal levels. As a result, cancer cells are more susceptible to increased oxidative stress, and any extracellular chemical that may boost oxidative stress in cancer cells while reducing antioxidant levels would be extremely beneficial in the fight against cancer cells [34, 35]. ROS production has been suggested to be the executioner and mediator of necroptosis [36]. In a study, the production of ROS was required for necroptotic cell death mediated by TNF and the Smac mimic BV6, and ROS has been suggested as an important regulator of necroptotic signaling [37]. TNF-induced non-apoptotic cell death in mouse embryonic fibroblasts has also been linked to RIP1-mediated cellular ROS production [38]. In our study, PLGA-Dtx was found to be effective at elevating ROS levels for 12 and 24 h, resulting

in a considerable loss of mitochondrial membrane potential ( $\Delta\Psi_m$ ). In a study by Chen et al., they discovered that MAM, a natural naphthoquinone, caused colon cancer cell death by triggering necroptosis with enhanced ROS production and defective mitochondrial potential [39]. To establish the function of necroptosis in PLGA-Dtx-induced cell death, cells were pre-treated with the necroptosis inhibitor Nec-1 and the effect on ROS and MMP was investigated. Interestingly Nec-1 treatment reverted the elevated ROS level and balanced the mitochondrial membrane potential. More research is needed to define any cross-talk between the apoptotic and necroptosis pathways in cancer cells treated with nano encapsulated medicines.

## Conclusion

In conclusion, our study has established that the PLGA-Dtx can inhibit cancer cell proliferation more efficiently than free drug and induce both apoptosis and necroptosis in oral cancer cells. This finding may provide a new therapeutic strategy for the treatment of cancer when the chemotherapeutic drug is conjugated with nanoparticles in the future. Examination of the multiple death modalities of cancer cells concurrently may present a good idea for understanding a more inclusive understanding of the effects of various chemotherapeutic drugs. However, this study represents very primarily information regarding the involvement of both cell death pathways, further investigation can be done to recognize the cascade of events and molecules involved in this necroptosis pathway and its association with apoptosis.

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**Authors' contributions** Conceptualization: PG, AKV. Data analysis: PG, AKV, AKP, AS, PK. Funding: PG. Investigation: PG, AKV, AS, AKP, SK, PK, VP. Experimental studies: PG, VK, AKP, AM, AKV. Validation: PG, AKV, AKP, AS, AM, PK, VP. Manuscript writing & original draft: PG, AKP, AKV, AS. Writing, review & editing: PG, AKV, AS, AKP, SK, AM, VK, VP.

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**Availability of Data and Material** Not applicable.

**Code Availability** Not applicable.

**Declarations**



**Conflict of interest** Authors declare no competing interest.

**Ethics Approval** Study protocol was approved by Institutional Ethics Committee of the institution. Additionally, study was performed in accordance of approved protocol.

**Consent to Participate** Informed written consent was taken from all the participants prior to enrolling in the study.

**Consent for Publication** Written consent was obtained from all the persons prior to publication.

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