

The synergistic effect and mechanism of doxorubicin-ZnO nanocomplexes as a multimodal agent integrating diverse anticancer therapeutics

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Background: Nanomaterials have emerged as ideal multimodal nanomedicine platforms, each one combining different designs and therapeutic approaches in a single system against cancer. The aim of the current study was to explore the synergistic effect and mechanism of a doxorubicin (Dox)-ZnO nanocomplex as a multimodal drug delivery system, integrating Dox chemotherapy and ZnO-mediated photodynamic therapy, in anticancer therapeutics.

Methods: Dox was loaded onto ZnO nanomaterials, forming complexes with the transition metal Zn to yield the Dox-ZnO nanocomplexes. After culture with SMMC-7721 hepatocarcinoma cells, the cellular uptake was quantitatively detected by flow cytometry and visualized by fluorescence microscopy. The synergistic effects of the different anticancer therapeutic modalities on the proliferation of SMMC-7721 hepatocarcinoma cells were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The expression of B-cell lymphoma 2 protein (Bcl-2), Bcl-2 associated X protein (Bax), caspase 9, and caspase 3 were examined by Western blot, to elucidate the possible molecular mechanisms involved.

Results: Our observations demonstrated that Dox-ZnO nanocomplexes could act as an efficient drug delivery system for importing Dox into SMMC-7721 cells, enhancing its potential chemotherapy efficiency by increasing the intracellular concentration of Dox. With the addition of ultraviolet (UV) illumination, the ZnO nanomaterials showed excellent photodynamic therapeutic properties, attacking the cancer cells further. Thus the caspase-dependent apoptosis was synergistically induced, resulting in distinct improvement in anticancer activity.

Conclusion: The Dox-ZnO nanocomplex presents a promising multimodal agent for comprehensive cancer treatment.

Keywords: nanomedicine, nanocomplexes, drug delivery system, doxorubicin, photodynamic therapy

Introduction

Different strategies, such as surgery, chemotherapy, and radiotherapy, have been employed for the treatment of cancer. However, use of a single modality has not always been sufficiently effective. Today, in the era of synthetic therapies, combined-modality therapies are attracting increasing attention as a strategy for improving the outcome of cancer treatment. For example, hepatocellular carcinoma is the fifth most common cancer and the third leading cause of cancer-related deaths worldwide.¹ Once diagnosed with the disease, only 30%–40% of patients are deemed eligible for curative-intent treatment modalities, including surgical resection, liver transplantation, and chemoembolization.¹ Eventually, most patients will receive some form of chemotherapy, in the hope of prolonging life.¹ Doxorubicin (Dox) has been routinely used as a chemotherapeutic agent for advanced hepatocellular carcinoma but has shown

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inefficacy, with a response rate of about 15%–20%.¹ With that in mind, efforts should be made to develop new strategies integrating diverse anticancer therapeutics to improve the efficiency of this single modality and improve the outcome of antitumor treatment.

As a new modality for cancer therapy, photodynamic therapy (PDT) promises a better selectivity for tumors that are accessible to light, low systemic toxicity, and fewer side effects.^{2,3} In PDT, when photosensitizing agents are exposed to light of specific wavelength, they can generate cytotoxic reactive oxygen species (ROS) to kill tumor cells.^{4,5} Zinc oxide (ZnO) nanomaterials have been regarded as a potential photosensitizer for PDT, due to their unique phototoxic effect upon ultraviolet (UV) illumination and their demonstration of significant cell-killing effect in cancer therapy.^{6–8}

Interesting nanomaterials have emerged as ideal multimodal nanomedicine platforms, combining different designs and therapeutic approaches into a single system against cancer. In these systems, each component has a specially designed function to optimize the specificity and efficiency of the system, and together, offer numerous advantages: surface features for target-specific localization; the opportunity to develop nanocarriers that respond to specific internal or external stimuli; the possibility of delivering multiple therapeutic agents in a single formulation; the possibility of combining imaging and drug therapy for real-time monitoring of effects; and the possibility of combining drugs with energy therapies (heat, light, and sound) for synergized therapeutic effects.⁹

Thus, we were inspired to construct a Dox-ZnO nanocomplex as a prospective multimodal agent for integrated diverse anticancer therapeutics, which offers a promising opportunity for combining Dox chemotherapy and PDT using ZnO, for synergistic therapeutic effects. As a follow-up to the study of ZnO nanomaterial-mediated multimode cancer treatment we reported previously,¹⁰ in this study, we further explored the specific possible molecular mechanisms involved.

Materials and methods

Chemicals and apparatus

Dox was obtained from Hisun Pharmaceuticals Co, Ltd (Taizhou, Zhejiang, People's Republic of China). Monoclonal antibodies of B-cell lymphoma 2 protein (Bcl-2), Bcl-2 associated X protein (Bax), caspase 3, caspase 9, and β -actin, were purchased from Santa Cruz Biotechnology Inc (Dallas, TX, USA). Horseradish peroxidase-conjugated IgG antibody was purchased from Nanjing KeyGen Biotech Co, Ltd (Nanjing,

People's Republic of China). 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St Louis, MO, USA) and stored in the dark. All other reagents were of analytic grade. Transmission electron microscope images were obtained using a JEM-2100 transmission electron microscope (TEM) (JEOL, Tokyo, Japan). The optical density at 492 nm was recorded by a multiwell spectrophotometer reader (DG5033A; Nanjing Huadong Electronics Group Medical Equipment Co, Ltd, Nanjing, People's Republic of China).

Construction of the Dox-ZnO nanocomplexes

ZnO nanomaterials were prepared by solid state reaction at room temperature, according to previous literature.¹⁰ In the following experiments, a 2 mL aqueous solution of Dox (2 mg/mL) was added to a 1 mL aqueous suspension of the obtained ZnO nanomaterials (10 mg/mL). The reaction mixture was kept in the dark overnight to construct the Dox-ZnO nanocomplexes. Dox-ZnO nanocomplexes were then separated from the freestanding drug molecules by centrifugation at 5000 g for 20 minutes, and the supernatant was analyzed using high-pressure liquid chromatography (HPLC) (Shimadzu Corp, Kyoto, Japan), at a detection/excitation wavelength of 488/515 nm, to estimate the drug encapsulation efficiency and loading efficiency.

Cell culture

SMMC-7721 hepatocarcinoma cells were obtained from the Shanghai Institute of Cells (Chinese Academy of Sciences, Beijing, People's Republic of China) and cultured in Roswell Park Memorial Institute (RPMI) medium (Gibco® 1640; Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin (Sigma-Aldrich) at 37°C, in humidified air with 5% CO₂.

Cell internalization studies

The SMMC-7721 hepatocarcinoma cells were treated with either Dox or Dox-ZnO nanocomplexes (Dox concentration 0.5 μ g/mL) for 6 hours. The cells were then observed by fluorescent microscopy (λ_{ex} 488 nm, λ_{em} 515 nm) to visualize the cellular uptake of Dox with the different treatments. Furthermore, Dox in cells was quantitatively detected using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA) to analyze the cellular uptake.

Cell viability assay

The SMMC-7721 cells were seeded, at 1×10^5 cells per mL, in 96-well plates and incubated for 24 hours. Then the cells were separated into different treatment groups: (1) control group with no treatment; (2) ZnO nanomaterials alone; (3) UV irradiation (0.1 mW/cm^2) alone, by a germicidal UV lamp (for the evaluation of the efficiency of PDT in cancer therapy); (4) ZnO nanomaterials plus 3 minutes of UV irradiation administered after 6 hours of cell culture with the ZnO nanomaterials; (5) free Dox ($0.5 \text{ }\mu\text{g/mL}$) (for evaluation of the chemotherapy); (6) free Dox plus UV irradiation; (7) Dox-ZnO nanocomplexes (Dox concentration $0.5 \text{ }\mu\text{g/mL}$); and (8) Dox-ZnO nanocomplexes plus UV irradiation (as multimodal agents of interest, integrating the anticancer therapeutics of chemotherapy and PDT, respectively). Cells were further incubated for 48 hours, and their relative viability was assessed using MTT assays.

Western blot analysis

After the different treatments, the expressions of apoptosis-related proteins, such as Bax, Bcl-2, caspase 9, and caspase 3 were detected by Western blot. In brief, the harvested cells were lysed in lysis buffer for 15 minutes at 4°C , then centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected, and the amount of protein was measured using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). After total protein was isolated, it was subjected to

sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. Once blocked, the membrane was incubated with primary monoclonal antibodies for either Bax, Bcl-2, caspase 3, caspase 9, or β -actin, at 4°C overnight and was subsequently incubated with horseradish peroxidase-conjugated IgG antibody, as the secondary antibody, at room temperature for 1 hour. The protein bands were detected using an enhanced chemiluminescence detection system (Amersham™, GE Healthcare, Little Chalfont, UK). After normalization with the corresponding expression of β -actin, the protein expression of Bax, Bcl-2, caspase 3, and caspase 9 was determined with densitometry scans.

Statistical analysis

All the data are presented as the mean \pm standard deviation. The *F*-test was used for significance testing, and $P < 0.05$ was considered to be statistically significant. All tests were performed using the Statistical Package for Social Sciences (version 13.0; SPSS Inc, IBM Corp, Armonk, NY, USA).

Results and discussion

Drug carrier role of the ZnO nanomaterial

The general TEM image of the ZnO nanomaterials is shown in Figure 1A. As learned from the TEM image, the ZnO nanostructures were shaped as nanorods.

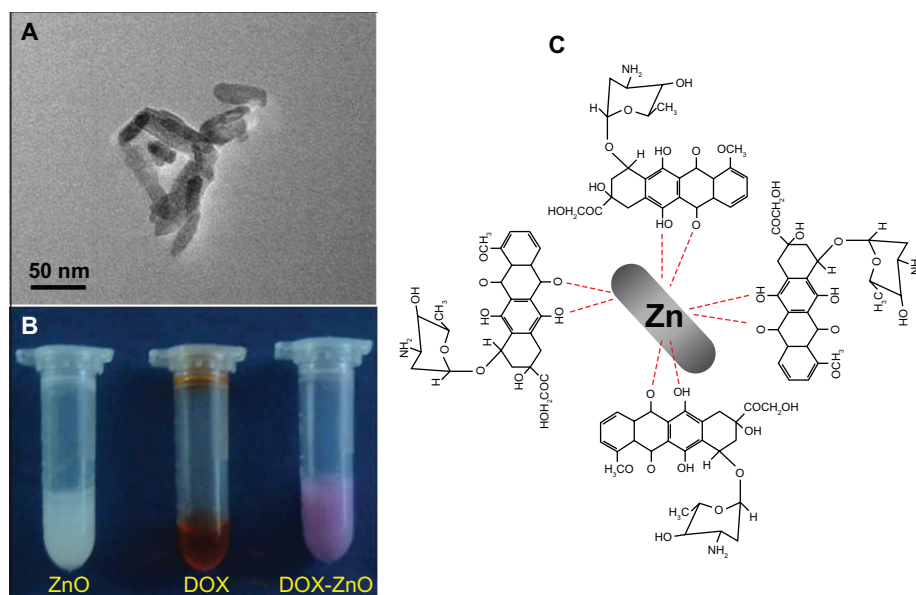


Figure 1 TEM image (A) and photographic image (B) of the ZnO nanomaterials, and the schematic representation (C) of Dox loading onto the ZnO nanomaterial in the formation of a Dox-ZnO nanocomplex.

Abbreviations: Dox, doxorubicin; Dox-ZnO, doxorubicin-ZnO nanocomplexes; TEM, transmission electron microscopy; Zn, zinc; ZnO, zinc oxide.

Dox is one of the most effective anticancer drugs to date, with a wide scope of activity in human cancers, including hepatocellular carcinoma.¹¹ However, its clinical application is limited by its harmful side effects, the most significant of which is its cardiotoxicity.¹² Therefore, efforts have been made to develop new delivery techniques that reduce side effects and improve its therapeutic efficacy. In the present study, the capacity of the ZnO nanomaterials as a drug carrier for loading Dox was explored. As shown in Figure 1B, we observed the color change of pure Dox, from red to purple, during the loading process, indicating that Dox molecules could form a Dox-ZnO nanocomplex drug delivery system (DDS) due to the interaction of Dox with ZnO. Figure 1C shows a proposed schematic representation of Dox loading onto the ZnO nanomaterials. The formation of the complexes is considered to occur at the chelating sites of the quinone and the phenolic oxygen molecules on both sides of the Dox aromatic moiety.¹³ The encapsulation efficiency and loading efficiency of the Dox-ZnO nanocomplexes were assessed and calculated to be $60.63\% \pm 5.78\%$ and $20.36\% \pm 2.85\%$, respectively. The results show that Dox-ZnO nanocomplexes may act efficiently as an anticancer DDS.

Cellular uptake of the drug delivery system of Dox-ZnO nanocomplexes

The fluorescent Dox allowed an easy observation of cellular uptake of the free Dox and Dox-ZnO nanocomplexes by the SMMC-7721 cells by fluorescence microscopy and quantification by flow cytometry without the need for additional markers. The relative intracellular fluorescence intensity of Dox analyzed by flow cytometry permitted a quantitative comparison of the cells treated with free Dox vs Dox-ZnO, which is shown in Figure 2. As learned from Figure 2, for the SMMC-7721 cells treated with equivalent Dox concentration and the same incubation time, the detected fluorescence for the group treated with Dox-ZnO nanocomplexes was of higher intensity than that for the group treated with free Dox ($P < 0.05$), producing an $85.2\% \pm 6.3\%$ enhancement. Further, as shown in the Figure 2 inset, the optical microscopic observations confirmed the results of flow cytometry that after 6 hours of incubation, the internalization of Dox from the Dox-ZnO nanocomplexes (Figure 2 inset, A) was much higher compared with the free Dox (Figure 2 inset, B), resulting in the stronger fluorescence intensity in the SMMC-7721 cells. These results clearly demonstrate that the Dox-ZnO nanocomplex DDS increased the cellular uptake of Dox substantially, which represents a promising approach in cancer therapy.

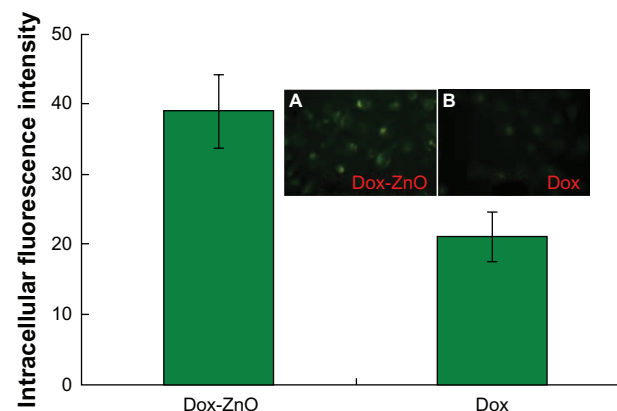


Figure 2 Comparison of the respective average intracellular fluorescence intensity in SMMC-7721 hepatocarcinoma cells treated with free Dox and Dox-ZnO as a DDS. The inset shows fluorescent microscopic images of SMMC-7721 cells treated with Dox-ZnO as a DDS (A) and free Dox (B).

Notes: The concentrations of Dox and ZnO nanomaterials were 0.5 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$, respectively. Data expressed as mean \pm standard deviation ($n = 3$).

Abbreviations: DDS, drug delivery system; Dox, doxorubicin; Dox-ZnO, doxorubicin-ZnO nanocomplexes; ZnO, zinc oxide.

The synergistic effect of Dox-ZnO nanocomplexes as a multimodal agent integrating diverse anticancer therapeutics

Recently, considerable attention has been paid to photoactive nanomaterials that show the ability to produce ROS, for application in biology.^{14,15} Among them, the ZnO nanomaterials are believed to be biologically safe and noncytotoxic, and to have significant photocatalyzed cell-killing effect in vitro resulting from the formation of ROS, which are believed to be responsible for a cascade of cellular and molecular events relevant to tumor destruction.^{6,7,16} As illustrated in Figure 3, when treated with the ZnO nanomaterials, about 95% of the cells survived compared with the control group, suggesting

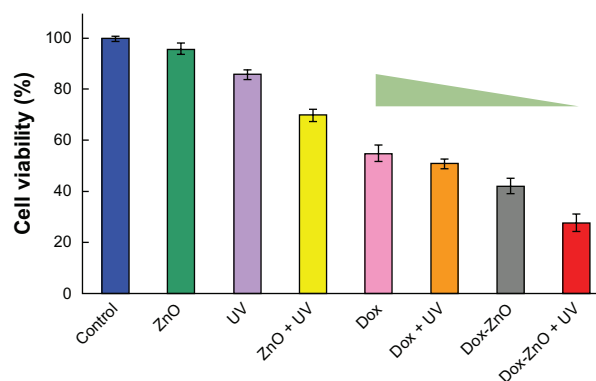


Figure 3 Antiproliferative effect of ZnO, Dox, or Dox-ZnO, in the absence or presence of UV irradiation, against SMMC-7721 cells.

Note: Data are expressed as the mean \pm standard deviation ($n = 3$). The green arrow represents the downward trend of cell viability.

Abbreviations: Dox, doxorubicin; Dox-ZnO, doxorubicin-ZnO nanocomplexes; UV, ultraviolet; Zn, zinc oxide.

the lack of cytotoxicity of the obtained ZnO nanomaterials, thus assuring a wide potential range of applications in the fields of biomedical science and cancer therapy. The UV irradiation itself showed only a small cytotoxic effect on the SMMC-7721 cells, whose surviving fraction was about 80%. The viability of the SMMC-7721 cells treated with ZnO nanomaterials in the presence of UV irradiation decreased remarkably from those treated with either UV irradiation or ZnO nanomaterials alone ($P < 0.05$), which indicates that the photocatalytic activity of ZnO nanomaterials can promote cell death. The increased mortality of the cancer cells indicates that under UV irradiation, ZnO nanomaterials could efficiently induce the formation of ROS and further attack the cell membrane, nucleic acids, and proteins, and therefore could be a promising nanomaterial for PDT in the treatment of cancer. Most photosensitizers, such as ZnO, can effectively generate ROS under PDT, due to their unique phototoxic effect upon UV irradiation; however, the use of UV irradiation is limited by its toxicity and very low tissue-penetration power. Near-infrared (NIR) light has the deepest tissue penetration compared with visible and UV light. It is also safe and causes minimal damage to the biological specimen involved.¹⁷ Indeed, NIR-to-UV upconversion nanoparticles are foreseen to overcome the drawback in the use of low-penetration UV light for PDT. To further explore the use of the Dox-ZnO nanocomplexes as a DDS in multimodal anticancer therapeutics integrating chemotherapy and photodynamic therapy, we cultured SMMC-7721 cells with free Dox (0.5 $\mu\text{g/mL}$) or Dox-ZnO nanocomplexes (with equivalent Dox concentration), with or without UV irradiation, for 48 hours. The cytotoxicity results were estimated by MTT assay and also shown in Figure 3. Compared with those receiving Dox, the viability of the SMMC-7721 cells treated with Dox-ZnO nanocomplexes was obviously decreased ($P < 0.05$). The increased cytotoxicity may have been due to the improved Dox cellular uptake with Dox-ZnO nanocomplexes that was illustrated above. In the current case, the Dox-ZnO nanocomplexes DDS was taken into the cancer cells, providing the further possibility of additional photocatalytic attacks by ZnO inside the cells following drug release and thus, a combined application of Dox with PDT by ZnO nanomaterials, as a strategy for comprehensive cancer therapy. The UV irradiation showed only a small enhancement of effect on SMMC-7721 cells upon incubation with Dox over that had without UV irradiation. As shown in Figure 3, the MTT assay illustrated that UV irradiation clearly decreased the viability of SMMC-7721 cells upon incubation with Dox-ZnO nanocomplexes from that

in cells without UV irradiation ($P < 0.05$), demonstrating that the photocatalytic activity of ZnO nanorods promoted cell death in addition to that induced by Dox. These results demonstrated that the ZnO nanomaterial not only enhanced the intracellular accumulation of Dox, but also caused more cell death via photocatalytic activity. The combination of the nanomaterial and Dox killed more cancer cells, indicating their synergistic effect of anticancer activity.

Potential mechanisms of the synergistic anticancer effect induced by Dox-ZnO nanocomplexes

Next, we investigated the molecular mechanism of anticancer activity induced by the synergistic effect of Dox-ZnO nanocomplexes in the SMMC-7721 cells. Dox can efficiently accumulate in the cell nucleus, intercalate DNA, and act as a cytostatic and apoptotic agent in tumor cells. The production of free radicals and oxidative stress is also closely involved in the action of Dox and ZnO nanomaterials, in terms of both anticancer and toxic effects.^{16,18} Evidence shows that the sensitivity of cells to the apoptotic stimulus is determined by

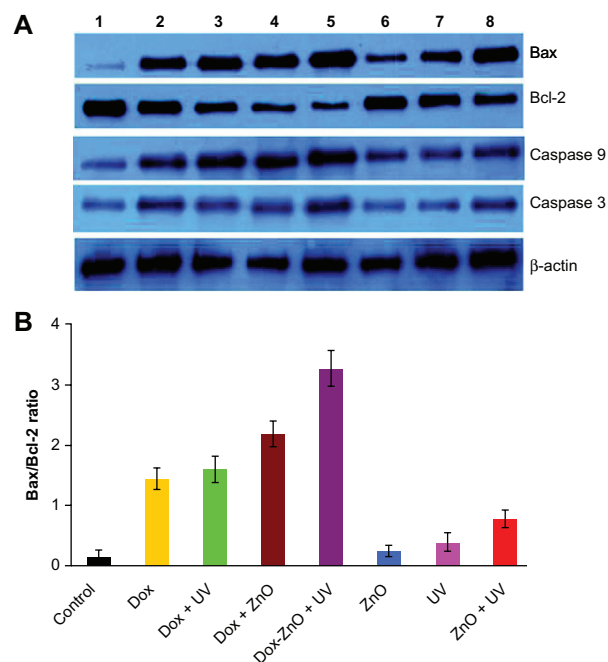


Figure 4 Expression of Bax, Bcl-2, caspase 9, and caspase 3 proteins (**A**) and the Bax/Bcl-2 ratio (**B**) in the SMMC-7721 hepatocarcinoma cells, by Western blot, after different treatments for 48 hours.

Notes: Line 1, control; Line 2, free Dox (0.5 $\mu\text{g/mL}$); Line 3, Dox accompanied with UV irradiation; Line 4, Dox-ZnO; Line 5, Dox-ZnO accompanied with UV irradiation; Line 6, ZnO nanomaterials; Line 7, UV irradiation; Line 8, ZnO nanomaterials accompanied with UV irradiation. Data are expressed as the mean \pm standard deviation ($n = 3$).

Abbreviations: Bax, Bcl-2 associated X protein; Bcl-2, B-cell lymphoma 2 protein; Dox, doxorubicin; Dox-ZnO, doxorubicin-ZnO nanocomplexes; UV, ultraviolet; Zn, zinc oxide.

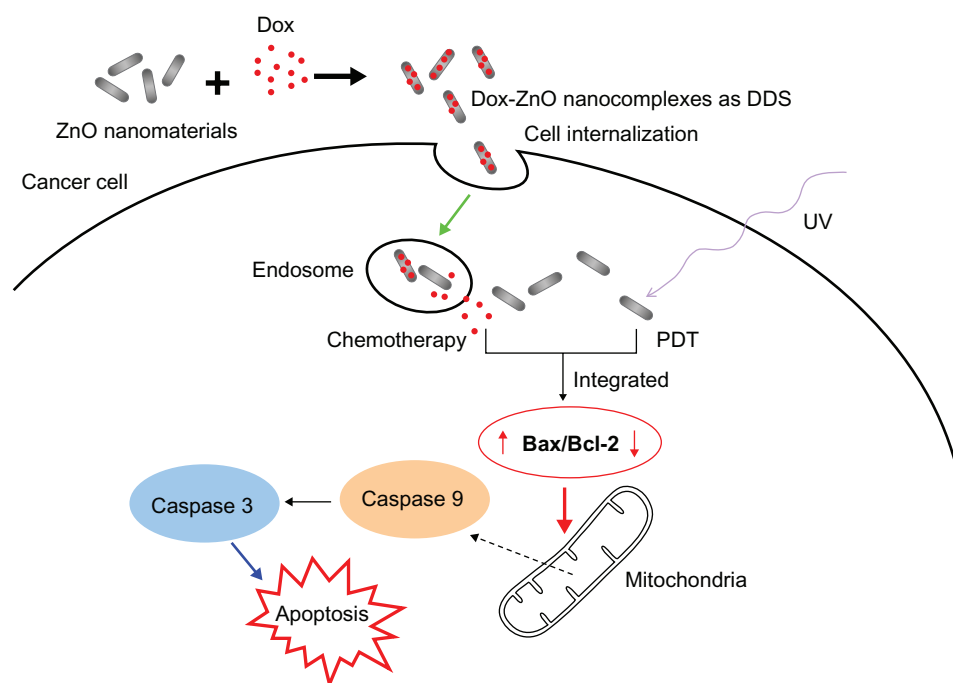


Figure 5 Schematic illustration of the possible process for the synergistic effect and mechanism of the doxorubicin-ZnO nanocomplex as a multimodal agent integrating diverse anticancer therapeutics.

Abbreviations: Bax, Bcl-2 associated X protein; Bcl-2, B-cell lymphoma 2 protein; DDS, drug delivery system; Dox, doxorubicin; Dox-ZnO, doxorubicin-ZnO nanocomplexes; PDT, photodynamic therapy; UV, ultraviolet; ZnO, zinc oxide.

the relative ratio of Bax and Bcl-2, which are the proapoptotic and antiapoptotic members of the Bcl-2 family, ie, the mitochondrial-related death switch.¹⁹ Apoptosis is the consequence of a series of precisely regulated events that are frequently altered in tumor cells, broadly categorized into two pathways, ie, the extrinsic pathway, which involves activation of the tumor necrosis factor (also known as the Fas death receptor family), and the intrinsic pathway, which involves the mitochondria. In both pathways, an apoptotic death stimulus results in the activation of caspases, the major executioners in this process, either directly or via activation of the mitochondrial death program.²⁰ Therefore, we examined the changes in the expression levels of the apoptosis-regulating proteins, including caspase 3, caspase 9, Bax, and Bcl-2 by Western blot to explore the possible signaling pathways through which Dox-ZnO nanocomplexes, as a multimodal anticancer therapeutic agent, induced distinct improvement in anticancer activity. As shown in Figure 4A, when the SMCC-7721 cells were treated by Dox-ZnO nanocomplexes accompanied with UV irradiation for 48 hours, the levels of Bax, caspase 3, and caspase 9 protein were significantly upregulated compared with the control group and other treatments ($P < 0.05$). In contrast, compared with the control group and other treatments, the levels of Bcl-2 protein in cells treated with Dox-ZnO nanocomplexes accompanied with UV irradiation were

significantly downregulated ($P < 0.05$). The ratio of Bax/Bcl-2 protein expression, shown in Figure 4B, increased dramatically with Dox-ZnO nanocomplexes acting as an integrated multimodal anticancer therapeutic agent, which might be a critical factor in the induced cell threshold for undergoing apoptosis. Thus, we deduce that the increased ratio of Bax/Bcl-2 led to a disruption of the integrity of the mitochondrial membrane, stimulating the initiation of apoptosis signaling, and resulting in caspase 9/caspase 3 activation. Caspase activation is generally considered to be a hallmark of apoptosis, and caspase 3 is the main effector caspase that is involved in apoptosis.²¹

Consequently, based on the above studies, Figure 5 schematically illustrates the possible synergizing processes of Dox-ZnO nanocomplexes as an integrated multimodal anticancer therapeutic agent. Firstly, the Dox react with ZnO nanomaterials, forming the Dox-ZnO nanocomplex DDS, which integrates chemotherapy and PDT for the treatment of cancer. The Dox-ZnO nanocomplexes increase the intracellular concentration of Dox dramatically, thus enhancing the suppression of cancer cells. Moreover, the photocatalyzed ZnO nanomaterials further attack the cancer cells, showing excellent prospects for PDT. Thus, the apoptosis, in a caspase-dependent manner, is induced synergistically, resulting in a distinct improvement in anticancer activity.

Conclusion

In summary, in the present study we explored the potential application of Dox-ZnO nanocomplexes as an agent in integrated multimodal anticancer therapeutics. The results demonstrate that the Dox-ZnO nanocomplex can act as a DDS to increase the internalization of the anticancer drug Dox in SMMC-7721 cells. Additionally, with UV irradiation, photocatalyzed ZnO nanorods can further enhance the growth inhibition of cancer cells. The synergistic effect induces a distinct improvement in anticancer activity through caspase-dependent apoptosis. Therefore the Dox-ZnO nanocomplex presents a promising multimodal agent for comprehensive cancer treatment.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31200750), the Natural Science Foundation of Jiangsu Province (BK2012332), and the Natural Science Foundation of Medical School of Southeast University (Qj2012013).

Disclosure

The authors report no conflicts of interest in this work.

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