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Original research article

Influence of copper (I) nicotinate complex and autophagy modulation on doxorubicin-induced cytotoxicity in HCC1806 breast cancer cells

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ABSTRACT

Purpose: Doxorubicin is regarded as the most therapeutic active agent available for triple-negative breast cancer (TNBC) treatment. However, the development of drug resistance and toxicity limits its effectiveness. Thus, developing novel strategies for TNBC treatment remains a significant challenge and doxorubicin-based combinations either by metal complexes (Copper I nicotinate complex) or with autophagy modulators could provide novel strategies and alternative strategies contributed to cancer cell death pathways, autophagy and apoptosis.
Materials and methods: The viability of HCC1806 TNBC cells and IC_{50} values of Doxorubicin (DOX), Torin-1 (TOR), Chloroquine (CQ) and Copper (I) nicotinate complex (CNC) were assessed by MTT assay. ELISA was used for detecting microtubule-associated protein 1 light chain 3 (LC3) level. Real time PCR was used to determine (*NBR1*) gene expression. Cell cycle analysis and quantitative detection of acid vesicular organelles (AVOs) was performed by flow cytometry. TOR and CQ were used as autophagy modulators for induction and suppression of autophagy, respectively.
Results: The half-maximal inhibition effect of TOR combination with DOX was revealed to the induction of autophagic cell death and apoptotic cell death. On the other hand, combination of CQ with DOX increased the growth inhibitory effect, induced accumulation of AVOs and suppressed apoptotic cell death. However, combination of CNC with DOX inhibited autophagy and induced cell cycle arrest.
Conclusion: Doxorubicin drug based combinations either with TOR, CQ or CNC could positively affect DOX effectiveness and reduce DOX doses applied on HCC1806 cells through modulation of autophagy.

1. Introduction

Triple-negative breast cancer (TNBC) is a diverse subtype of cancer that is frequently described by aggressiveness [1], and represents 12–20% of all breast cancers [2]. It is characterized by poor expression of three target receptors: ER, PR and Her-2, leading to the lack of targeted therapies and poor clinical outcome [3]. Nevertheless, chemotherapeutic agents are considered as the mainstay of TNBC therapy [4]. Currently, doxorubicin- anthracycline chemotherapeutic drug is regarded as the most therapeutic active agent available for TNBC treatment [5,6]. TNBC could be chemo-sensitive particularly to cytotoxic agents, but once the chemo-resistance developed, the cells became more aggressive and metastatic [1]. The metastasis and chemo-resistance of TNBC were the most common causes leading to the treatment failure, disease recurrence and eventual death in clinic [1]. Chemo-resistance can be attributable to the fact that although cytotoxic chemotherapy aims to kill cancer cells through apoptosis, tumor cells have the ability to maintain viability following chemotherapeutic exposure by undergoing alternative cellular fates such as cellular senescence, therapeutic induced senescence (TIS) and autophagy [7].

Autophagy (type II cell death), is a conserved catabolic process whereby cytoplasmic constituents and organelles are engulfed by autophagosomes, and degraded after fusion with lysosomes [8]. The formation of autophagosome is a multistep process that includes the biogenesis of the phagophore, followed by its elongation and closure. More than 15 autophagy related (ATG) proteins, as well as class III phosphoinositide 3-kinases (PI3Ks; also known as PIK3C3 or VPS34), are required to construct the autophagosome. The protein ATG8 - commonly called (LC3) - appears to have multiple functions in autophagy [7]. In addition to its proposed roles in the expansion and fusion of phagophore edges, LC3 can function as an adaptor protein to recruit selective cargo to the autophagosome via interaction with cargo

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receptors, such as *P62* and *NBR1*, leading to their degradation. Therefore, *NBR1* is used as an index for the autophagic flux measurement [9]. In addition, LC3 is considered as the most widely used autophagosome marker because the amount of LC3 reflects the number of autophagosomes and autophagy-related structures [10,11]. Although autophagy can be induced by chemotherapeutic agents [12], the impact of autophagy on chemotherapy-induced cytotoxicity is still unclear [13]. Inconsistent reports regarding the association between autophagy and cytotoxic effects of drugs have been described. In their review, Sui et al. [14], have shown that autophagy augments the cytotoxic effect of drugs and constitutes a potential target for cancer therapy. On the contrary, others have proposed that autophagy is induced for sustaining the viability of cancer cells against these drugs [15,16]. Interestingly, pharmacological modulation of autophagy by induction or inhibition was reported to produce effective innovation strategies to augment therapeutic activity [7].

Chemo-resistance development due to doxorubicin-induced autophagy is not the only side effect developed by autophagy, there are also cardiac associated side effects. The probability of developing cardiomyopathy is largely dose-dependent [16]. Many studies focus on the mechanisms involved in doxorubicin-induced cardio-toxicity, including: cardiac oxidative stress, reactive oxygen species (ROS) accumulation, lipid peroxidation, mitochondrial dysfunction and autophagy, to minimize compilations while maintaining antitumor efficacy [17]. These studies include loading doxorubicin (DOX) into nanogel or nanoparticles [18–20], amidization of DOX [21], complexation with metals (Mg, Mn, Cu, Zn) [22], or combination with other metal-based drugs [17].

Metal-based drugs have emerged as potential cancer fighters to overcome chemo-resistance [23]. Among metal-based antitumor drugs, copper complexes have played a relevant role in chemotherapy [24]. Moreover, nicotinic acid has been widely used for preventing hyperlipidemia and significantly lowering the risk of cardiovascular disease by about 25% [25]. The current interest in copper complexes e.g., copper (I)-nicotinate complex (CNC) originated from their expanded spectrum of activity as antimicrobial, antiviral, anti-inflammatory and antitumor agents [26]. In addition, CNC has been known to exert a superoxide dismutase (SOD) mimic activity and to reduce oxidative stress [25]. Copper complexes frequently targeted apoptosis as a mechanism of cell death. Additionally, the interplay between autophagy and apoptosis in relation to the antitumor activity of copper complexes was reported in several studies [27–29].

Accordingly, the present study highlights the side effects caused by autophagy due to DOX treatment and aims to present different therapeutic combinations that affect DOX- induced autophagy and lower DOX-dose used without diminishing its anti-tumor activity. Modulation of autophagy was studied either by the combination with torin-1 (TOR) autophagy inducer), or by chloroquine (CQ) (autophagy inhibitor). Moreover, this study aimed to investigate the effect of CNC and/or its combination with DOX on autophagy and their role as possible therapeutic regimens for TNBC treatment.

2. Materials and methods

2.1. Reagents and chemicals

TOR, CQ and DOX were purchased from Tocris Bioscience (Ellisville, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Acridine orange (AO) and propidium iodide (PI) were purchased from (Sigma-Aldrich Co., USA).

2.2. Cell culture

Human breast cancer cell line HCC1806 (ATCC®CRL-2335™) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained at 37 °C, 5% CO_2 in 10% DMEM (LanzaBioproduct, Belgium) supplemented with 10% fetal bovine serum (SeraLab, UK).

2.3. Preparation of copper (I)-nicotinate complex (CNC)

CNC was synthesized as described by Gohar and Dratoviscky in 1975 [30]. This method is based on the reaction of nicotinic acid, $CuCl_2$-$2H_2O$ and L^+-ascorbic acid to form [Cu (I)-(nicotinic acid)$_2$Cl.$2H_2O$] complex.

2.4. MTT cytotoxic assay

Proliferation of HCC1806 cells under different conditions was determined using the MTT cytotoxic assay. Cells were allowed to attach overnight and were then treated with CNC, DOX, TOR and CQ alone or in combination at varying concentrations. Each well was treated with 20 μL of MTT reagent, then cells were incubated for 2 h at 37 °C in 5% CO_2, the formed crystals were dissolved by adding 20μL DMSO to each well. The absorbance was read at 490 nm in plate reader (BioTek Company). All experiments were performed in triplicate. IC_{50} concentration represents a half maximal inhibitory concentration.

The combination index (CI) method was used to examine the impact of combined treatments on HCC1806 cells [31]. Dilution ratios were prepared for combinations of two drugs with regard to their IC_{50} concentrations. In addition, their effect on growth inhibition was determined using the MTT assay. Mutually exclusive equation was used to calculate CI values [32].

2.5. Drugs treated groups

Untreated HCC1806 cells represent untreated control group. Cells were treated with IC_{50} concentrations of DOX, CNC, TOR and CQ. Combination treatment groups include: TOR/DOX (0.0041:0.026 M), CQ/DOX (0.02:0.026 M) and CNC/DOX (0.1:0.071 M) groups as indicated in Table 1.

2.6. Cell cycle distribution analysis

Following treatment, cells were harvested, fixed, pelleted by centrifugation, washed and then resuspended in PBS solution containing 250 U/ml RNase for 20 min at room temperature and stained with 100 μg/ml propidium iodide (PI). FACSCalibur (Becton Dickinson, San Jose, USA) was used to examine the distribution of cell cycle.

2.7. Quantification of acidic vesicular organelles (AVOs)

Cells were seeded into 6-well plates and after treatment for 24 h cells were incubated with acridine orange (1 μg/mL) for 15 min. Total cells were collected by trypsinization and analyzed by flow cytometry utilizing a FACSCalibur from Becton Dickinson [33], and cells containing AVOs were identified in the Q2 quadrant of FL1, FL3 histograms [34].

Table 1
Drug concentrations used in TNBC treated groups.

Groups	Concentrations (M)
Doxorubicin (DOX)	0.1
Copper (I) nicotinate complex (CNC)	0.3
Torin-1 (TOR)	0.016
Chloroquine (CQ)	0.02
TOR/DOX	0.0041 : 0.026
CQ/DOX	0.02 : 0.026
CNC/DOX	0.1 : 0.071

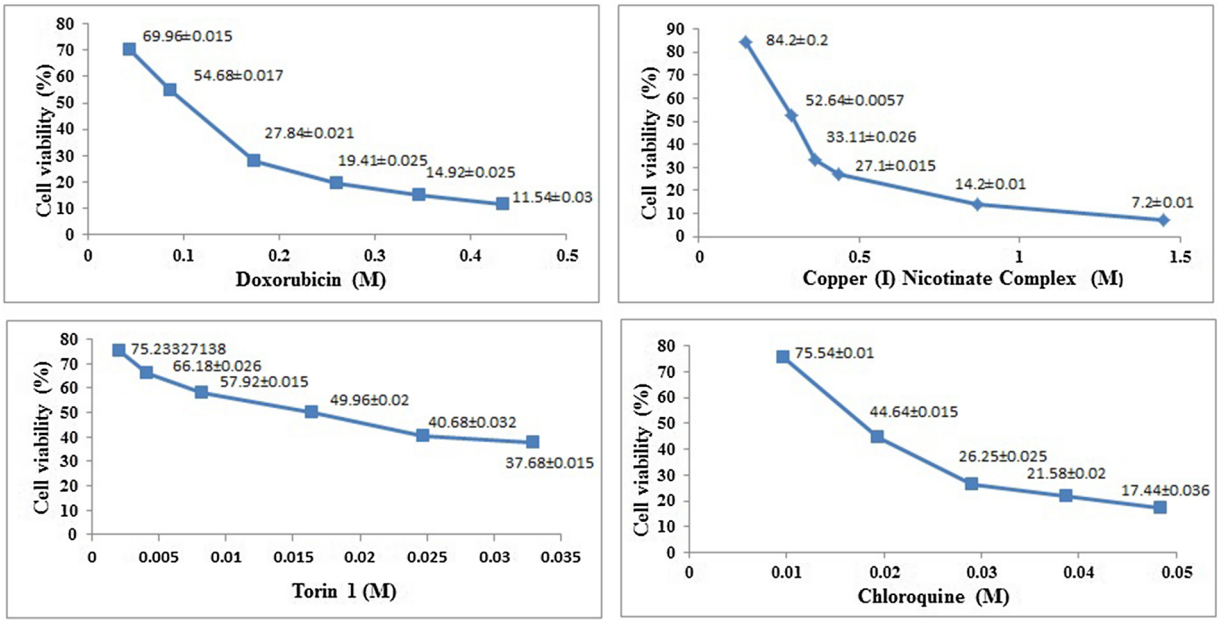

Fig. 1. The effect of Doxorubicin (DOX), Copper (I)-nicotinate complex (CNC), Torin-1 (TOR) and Chloroquine (CQ) on the viability of TNBC cells. TNBC cells were treated with varying concentrations of DOX, ranging from 43 to 430 M; CNC at concentrations ranging from 140 to 1400 M; TOR at concentrations ranging from 4 to 30 M; CQ at concentrations ranging from 9.6 to 48 M for 24 h. The viability of the cells was determined by MTT assay.

2.8. Quantitative real-time PCR for NBR1 gene expression

Real-time PCR was performed in triplicate for quantitative analysis of *NBR1* gene expression by iCycler-iQ Optical System Software version 3.0 A (Bio-Rad). Total RNA Extraction Kit (iNtRON, Korea) was used for RNA extraction. 1.0 μg of RNA using TIANScript RT Kit (Tiangen, Shanghai) was used for cDNA reverse transcription. The following primers were used for the quantitative real-time PCR analysis:

For *NBR1* gene:

5-TCGACGCAATCTCCACCAAT-3′ (forward)

5-GCA GGA CGT GGT TAG TGT CA-3′ (reverse)

For *β-actin*:

5-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′ (forward)

5-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′ (reverse)

RealMOD™ Green FAST qPCR Master Mix (S) (Tiangen, Shanghai) was used for PCR amplification in triplicate. The PCR conditions were as follows: 1 cycle at 95 °C for 30 s, 40 cycles at 95 °C for 5 s, 1 cycle at 95 °C for 5 s [35]. The relative mRNA expression levels of NBR1 gene were calculated using the comparative (Ct) method ($2^{-\Delta CT}$ equation) where, ΔCT equals (CT of gene of interest - CT of control gene) [36].

2.9. Quantitative analysis of LC3 protein by ELISA

Cells were seeded into 6-well plates and after treatment for 24 h cells suspension was prepared. Human LC3 ELISA Kit (Sun Red Biotechnology, Shanghai) was used for detection of LC3 levels according to the manufacturer's instructions. The absorbance was read at 450 nm on a Synergy™ HTMulti-Mode Microplate Reader (Bio-Tek). Data were represented as mean ng/mL of LC3 ± S.D of 6 experiments.

2.10. Statistical analysis

Statistical analysis was performed with SPSS 17.0 for Windows. For comparison of the quantitative variables, one way ANOVA was used to compare between different studied groups. Generally, p values were considered statistically significant at level < 0.05.

2.11. Ethical issues

All procedures were authenticated and approved by the Committee of Medical Research Institute, Alexandria University, Alexandria, Egypt (approval number: IORG0008812).

3. Results

3.1. Cytotoxicty

MTT assay was used to determine the effect of different treatments on proliferation of HCC1806 cells. Cell proliferation decreased following treatment of cells with CNC, TOR, CQ [37], and DOX in a concentration-dependent manner (Fig. 1). The IC_{50} values observed at 0.3, 0.016, 0.02 after 24 h of treatment and 0.1 M, respectively.

Based on the law of large numbers theory and the median-effect equation, the Chou-Talalay analysis [31] is widely used to analyze the interaction ways between drugs; CI < 0.9 indicates synergism, CI = 0.9–1.1 indicates an additive effect and CI > 1.1 indicates antagonism. The CI values for combination treatment TOR/DOX, CQ/DOX and CNC/DOX after 24 h were representing synergism, antagonism and additive effect respectively (0.58, 1.5 and 0.988, respectively). The rate of growth inhibition for all combinations showed more cytotoxic effect on cell growth than the same doses of each drug used in this combination (Fig. 2a–d).

3.2. Cell cycle distribution

In untreated control cells, 59.67% of cells were in G0/G1 phase and 8.21% of cells were in the sub G1 peak. Treatment of HCC1806 cells with CNC, DOX, TOR and CQ either alone or in combination on cell cycle distribution showed alterations in G0/G1, S, and G2/M phases of cell cycle compared with untreated cells (Fig. 3a and b). Treatment with CNC induced an increase of cells in sub G1 fraction (25.81%) and G2/M cell cycle arrest (31.69%). Compared with untreated controls, treatment with DOX induced cell accumulation at the sub G fraction and reduction in the number of cells in the other cell cycle phases. On the other hand, treatment with TOR caused decrease of cells in the apoptotic peak (3.91%) and induced accumulation in G0/G1 phase (69.08%).

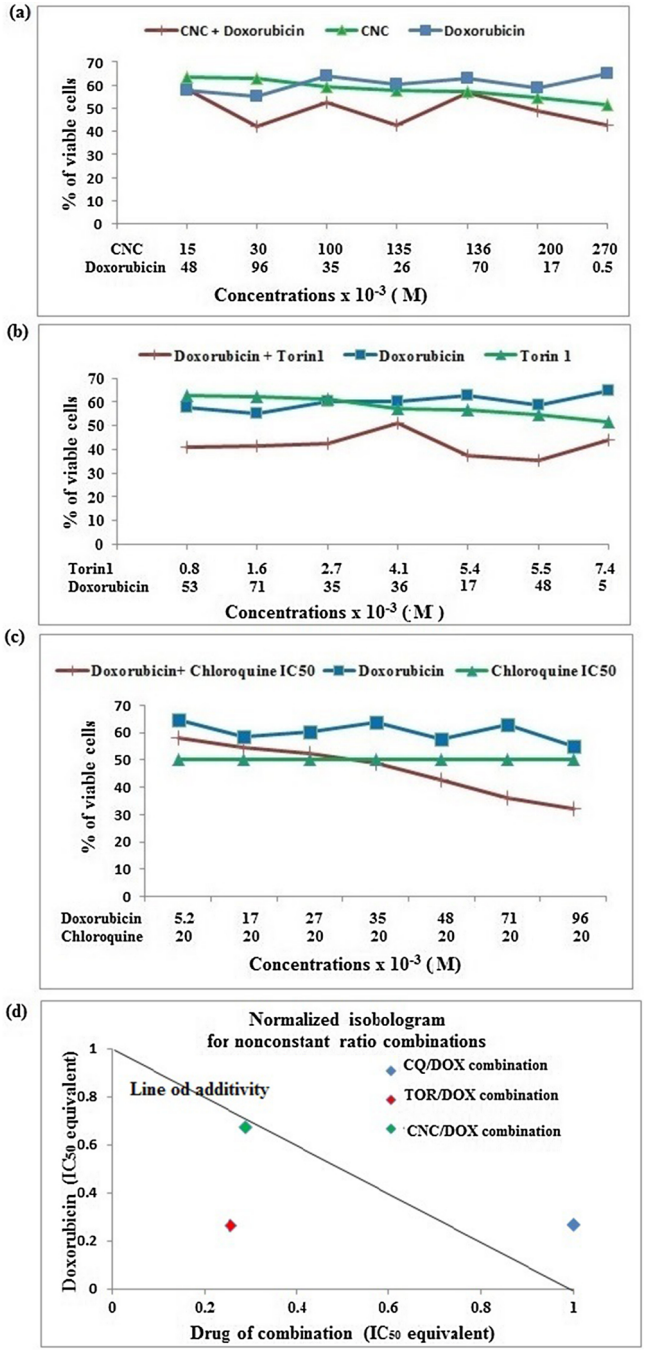

Fig. 2. The impact of combination treatment on cell viability of HCC1806 TNBC cells after 24 h of treatment. (a) Combination of CNC with DOX. (b) Combination of TOR with DOX (c) Combination of CQ with DOX. (d) Normalized isobologram for non-constant ratio combination design. The concentration in IC_{50} equivalent was calculated by dividing its actual concentration used in the combination treatment from its corresponding single-agent IC_{50} value. The line of additivity on the isobologram represents the 50% effect level of each drug. Synergy, additivity, or antagonism effects are indicated below, on, or above the line of additivity, respectively. CI > 1.1 is antagonism; CI = 0.9–1.1 is additivity; CI < 0.9 is synergy.

Compared to untreated control cells, the increase in G2/M arrest was observed in CQ/DOX treated cells by 29.36% with a decrease in the sub G1 fraction to 2.51%. Meanwhile, CNC/DOX treated cells showed a slight increase of cells at G0/G1 phase (60.96%) and sub G1 population (8.75%) compared to untreated cells. In addition, the increase of cells population in sub G1 phase was observed in CQ and TOR/DOX treated cell by 92.61% and 72.94%, respectively, versus 8.21% of the untreated controls.

3.3. LC3 protein levels

Treatment of HCC1806 cells with DOX caused a statistically significant increase of the level of LC3 protein ($p < 0.0001$) while TOR and CQ treated cells showed insignificant increase compared with untreated control ($p = 0.892$ and $p = 0.343$, respectively). However, TOR/DOX treatment caused a statistically significant increase of LC3 level ($p < 0.0001$) compared with untreated cells and TOR treated cells as shown in Table 2. Moreover, CQ/DOX treatment caused a statistically significant increase ($p = 0.008$) of LC3 level compared with untreated control. On the other hand, treatment of HCC1806 cells with CNC and CNC/DOX combination caused a statistically significant decrease of LC3 level ($p = 0.02$ and $p = 0.004$, respectively) compared with untreated cells.

3.4. NBR1 gene expression

It was observed that treatment with either DOX, TOR solely or with their combination caused insignificant suppression of *NBR1* gene expression while insignificant up-regulation was detected on treatment with CQ and CQ/DOX (Table 2). On the other hand, treatment with CNC alone or with CNC/DOX combination showed a statistically significant up-regulation of *NBR1* gene expression compared with untreated cells (= 0.001 and $p = 0.037$, respectively).

3.5. Development of acid vesicular organelles

Treatment of HCC1806 cells with DOX, TOR (1.6 mM) or CQ induced development of AVOS by 96.42, 95.85 and 95.37%, respectively, versus 90.51% at untreated control as indicated in Fig. 4. Moreover, the increase of intensity of the red fluorescence as an indication of AVOs development was observed in cells treated with either TOR/DOX or CQ/DOX (97.25 and 99.33%, respectively, compared with untreated control). In contrast, CNC and CNC/DOX treated cells showed inhibition of the development of AVOs by 72.73 and 83.1%, respectively, compared with untreated cells.

4. Discussion

Autophagy could potentially be suppressed at any stage of autophagic flux [38]. If any step upstream of autophagosome formation is blocked, the numbers of autophagic structures decrease. In contrast, the blockade of any step downstream of autophagosome formation increases the number of autophagosomes [39]. Therefore, it is important to discriminate between blocking autophagy at early or later steps because differences in outcome for the cell have been noted [38]. In the present study, the decrease of AVOs, the significant reduction in the LC3 level and over-expression of *NBR1* gene may reflect down-regulation of autophagy at early steps of autophagosome formation. It was reported that, suppressing autophagy at early stages, could be achieved genetically by knockdown of *ATG* genes or pharmacologically by inhibition of type III PI3 kinase complex (this point is under investigation) giving a suggestion to the probable mechanism that mediate inhibition of autophagy by CNC in TNBC cells [39]. Moreover, flow cytometric analysis showed that CNC treatment resulted in inhibition of cell growth, arrest in cell cycle progression and increase of cells in Sub G1 reflecting its pro-apoptotic effect. Thus, the results of the present study may point to anti-autophagy and pro-apoptotic effects of CNC that may contribute to anti-tumor activity of Cu-complexes reviewed previously [24,37].

In the same context, the cytotoxicity of DOX includes free radical generation, DNA damage, triggering of apoptosis and induction of autophagy [40]. In the present study, treatment of HCC1806 cells with

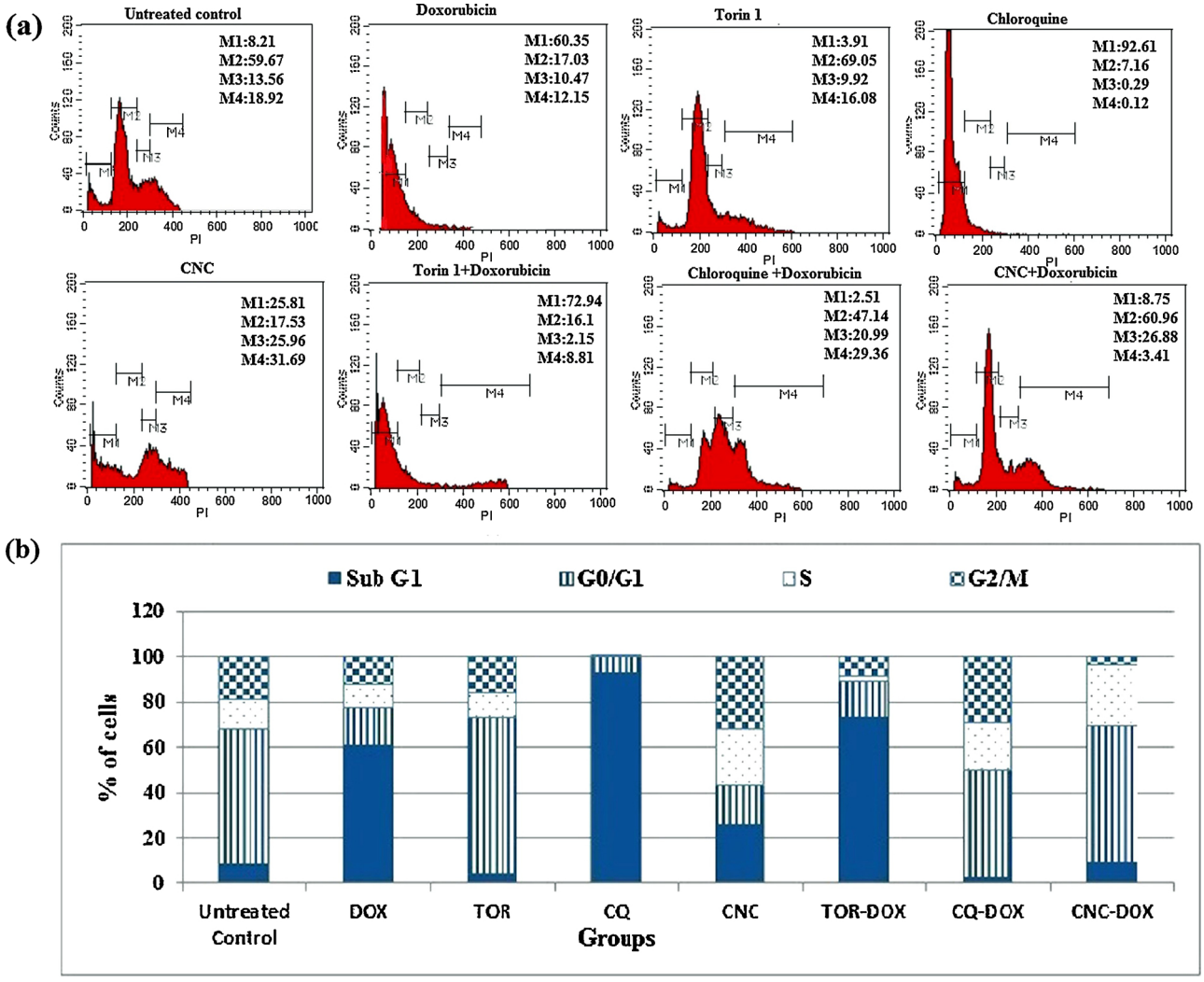

Fig. 3. The impact of treatment with Doxorubicin (DOX), Torin-1 (TOR), Chloroquine (CQ), Copper (I) nicotinate complex (CNC) and their corresponding combinations on the cell cycle distribution in HCC1806 TNBC cells. Cells were treated with DOX at concentration of 100 mM, CNC at concentration of 300 mM, TOR at concentration of 1.6 mM, CQ at concentration of 20 mM and their corresponding combinations for 24 h. Then, cells were subject to flow cytometry analysis. **(a)** Histographs of cell cycle distribution of cells after 24 h treatment. M1 represents sub G population, M2 represents G0/G1phase, M3 represents S phase and M4 represents G2/M phase. (**b**) Bar graphs showing the percentage of HCC1806 cells in sub G1 population, G0/G1, S, and G2/M phases after treatment for 24 h.

DOX showed an increase in the number of cells at the apoptotic peak, indicating triggering of apoptosis. In addition, treatment of HCC1806 cells with DOX activated the autophagy machinery indicated by the development of acid vascular organelles, increase in LC3 level and suppression of NBR1 gene expression. DOX mediated autophagy activation could be through the depletion of ATP and NAD^+ as well as activation of nuclear enzyme (poly (ADP-ribose) polymerase-1) (PARP-1) [40]. Thus, DOX-induced autophagy may promote resistance [1], and/or development of cardio-toxicity [41]. Based on a panel of breast cancer cell lines, it has been shown that TNBC cells are more autophagy addicted and more sensitive to autophagy inhibition [42]. On the contrary, it has been illustrated that excessive autophagy may result in autophagic cell death [43]. This contradiction between autophagy-associated cell death versus cell survival highlights the role of autophagy modulation in combination with DOX in treatment of TNBC [42].

The combination of DOX with CNC could enhance the anti-cancer effects of DOX [44]. In the present study, CNC-DOX combination increased the cytotoxic effect on growth than did each drug alone and their combination described as additive interaction. Moreover, CNC-DOX treatment resulted in a pronounced impairment in AVOs development suggesting a more efficient therapeutic effect of CNC-DOX through autophagy down-regulation. The observed decrease in the level of LC3 protein and the up-regulation of *NBR1* gene expression may support more the down-regulation of autophagy as result of CNC-DOX treatment. In addition, the combination of CNC with DOX, augmented the decrease of DOX dose used for 50% growth inhibition giving a possibility to avoid its dose-dependent side effects.

On the other hand, in the present study, the impact of autophagy modulation on the efficacy of DOX treatment was investigated. TOR was used as an inducer of autophagy that inhibits the mammalian target of rapamycin (mTOR)-containing complexes leading to induction of autophagy [45]. Treatment of HCC1086 cells with TOR (1.6 mM) augmented the development of AVOs, increase in LC3 protein level and the suppression of *NBR1* gene expression, suggesting autophagy up-regulation. Meanwhile, the activation of mTOR signaling contributes to cancer development [46]; its inhibition has a great impact on increasing chemo-sensitivity in a variety of tumors [47]. In the present study, the combination of TOR as (mTor inhibitor) with DOX had greater cytotoxic effect on HCC1086 cells than did each drug alone and their combination described as synergistic interaction. Alongside, treatment of HCC1086 cells with the TOR/DOX combination resulted in an increase in cells at the apoptotic peak and the induction of autophagy. This was supported through the development of AVOs, the increase of LC3 level and suppression of NBR1 in HCC1086 cells. It was

Table 2
Involvement of LC3 protein level and NBR1 gene expression in TNBC cell line treated with Doxorubicin (DOX), Torin-1 (TOR), Chloroquine (CQ), Copper (I) nicotinate complex (CNC) and their combinations.

	LC 3 protein level (ng/mL)	Relative expression of NBR1 gene
Untreated control		
Min – Max	13.6 – 13.8	3.90 – 4.18
$\bar{x}$ ± SD	13.7 ± 0.09	4.05 ± 0.14
DOX		
Min – Max	13.93–16.11	2.94–4.34
$\bar{x}$ ± SD	15.44 ± 0.79[a]	3.82 ± 0.768
TOR		
Min – Max	13.6 – 15.0	3.75 – 4.16
$\bar{x}$ ± SD	14.1 ± 0.61[b]	3.91 ± 0.22
TOR/DOX		
Min – Max	15.12 – 16.51	3.02 – 4.11
$\bar{x}$ ± SD	15.73 ± 0.5[a,c]	3.55 ± 0.54
CQ		
Min – Max	13.05 – 15.94	4.7 – 5.44
$v\bar{x}$ ± SD	14.16 ± 0.36[b,d]	5.07 ± 0.36
CQ/DOX		
Min – Max	14.43 – 15.30	3 – 7.55
$\bar{x}$ ± SD	14.88 ± 0.36[a,c,d]	4.64 ± 2.52
CNC		
Min – Max	12.26 – 13.76	8.06 – 15.89
$v\bar{x}$ ± SD	12.75 ± 0.68[a,b,c,d,e]	10.97 ± 4.2[a,b,c,d,e]
CNC/DOX		
Min – Max	12.14 – 12.91	9.1 – 9.48
$\bar{x}$ ± SD	12.51 ± 0.3[a,b,c,d,f]	9.3 ± 0.19[a,b,c,d,f]

[g]Statistically significant when compared to CQ/DOX group.
(*p*) values were considered significant at level ≤ 0.05.
[a] Statistically significant when compared to untreated control group.
[b] Statistically significant when compared to DOX group.
[c] Statistically significant when compared to TOR group.
[d] Statistically significant when compared to TOR/DOX group.
[e] Statistically significant when compared to CQ group.
[f] Statistically significant when compared to CNC group.

demonstrated that, induction of autophagy by rapamycin (an mTOR inhibitor) in combination with DOX provides a cardio-protective role against DOX-induced cardiotoxicity [48]. Moreover, the activation of autophagy upon drug treatments can induce autophagic cell death independent of or in parallel with apoptosis and necrosis [49]. Thus, it could be concluded that, the increase of cytotoxicity of TOR/DOX combination could be mediated through the interplay between cell death pathways apoptosis and autophagy. The hallmark of this combination included lowering of the DOX dose used compared with its IC_{50} concentration, which may help to attenuate its relative side effects.

Paradoxically, CQ is a well-known lysosomotropic agent [50], which inhibits autophagy in its late phase, leading to accumulation of autophagic vacuoles and LC3 [51], and activates other cell death pathways [42]. CQ is often used in combination with chemotherapeutic drugs because it has been shown to enhance their efficiency [52]. In the present study, combination of CQ enhanced the growth inhibitory effect of DOX in HCC1806 cells at low DOX concentration and their combination was described as antagonistic interaction. Additionally, the accumulation of AVOs and LC3 protein level as well as up-regulation of *NBR1* gene expression suggested the impairment of autophagy degradation and thus nutrient shortage [42]. Meanwhile, the continued DOX-induced autophagy, which is accompanied by blocking degradation of AVOs, may direct cells towards apoptotic pathway [50]. In the present study, treatment of CQ/DOX combination resulted in diminishment of their apoptotic-induced activity reflected by the decrease in apoptotic peak. The delay of the apoptotic cell death induced by each drug alone gives suggestion that the combination of CQ/DOX may direct cells to undergo alternative cell death pathways since CQ has the ability to activate both apoptotic and non-apoptotic death pathways [50]. Autosis is an autophagy-dependent non-apoptotic form of cell death, which is induced by autophagy-inducing peptides, starvation, and hypoxia [53]. The unique feature of autosis is that, it is considered as a type of cell death which occurs in starved cells that are incapable of undergoing apoptosis or necrosis [54]. In vitro, autosis occurs in a subpopulation of cells that undergo the highest levels of autophagy and become substrate-adherent during nutrient starvation [53]. The exposure of cancer cells to specific stimuli, when the pharmacological or genetic inhibition of the autophagic machinery delays (rather than accelerates) cell death, the term 'autophagic cell death' should be employed to indicate this specific cell death subroutine [55].

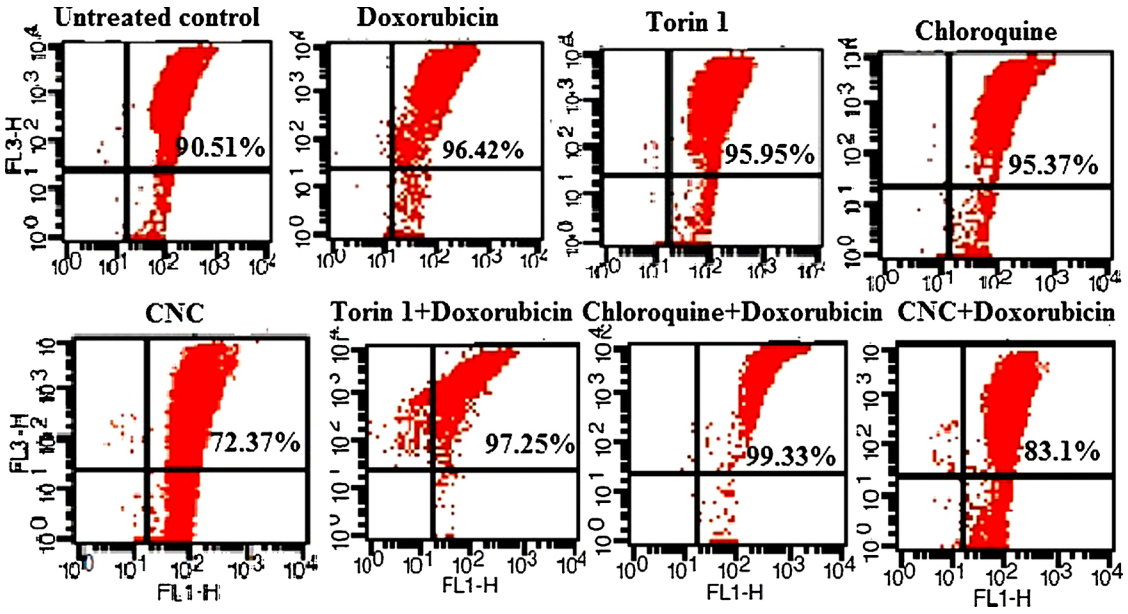

Fig. 4. The impact of treatment with Doxorubicin (DOX), Torin-1 (TOR), Chloroquine (CQ), Copper (I) nicotinate complex (CNC) and their corresponding combinations on the development of AVOs in HCC1806 treated cells. Flow cytometric quantification of AVOs formation in HCC1806 treated cells after stained with acridine orange, the red fluorescence of acridine orange was quantified by flow cytometer, FL1-H indicates green color intensity, while FL3-H shows red color intensity.

5. Conclusion

The findings of the present study may reveal that the autophagic flux is involved in DOX-combination strategies. Combination of TOR/DOX showed a synergistic interaction (CI = 0.58) reflected by increase of its cytotoxic effect on the growth of HCC1086 cells. Generally, autophagy is known to act as a pro-survival pathway in TNBC cells leading to cell survival. However, the synergistic interaction between DOX and TOR as autophagy inducers stimulates the induction of autophagic cell death in parallel with apoptotic cell death. On the other hand, combination treatment with autophagy inhibitors such as CQ potentiates the effects of several anti-cancer therapeutic agents. However, CQ/DOX combination showed more cytotoxic effect on cell viability; the combination index value (1.5) indicates an antagonistic interaction. This antagonism may be a result of different therapeutic targets of both drugs as CQ acts through blocking DOX-induced autophagy at late stage leading to autophagosome accumulation. In addition, the antagonistic effect of this combination leads to deactivation of the apoptotic pathway and activation of possible autophagy-dependent non-apoptotic pathway of cell death called autosis. In the same context, DOX and CNC act through different therapeutic targets, but CNC inhibits DOX-induced autophagy during early stages of formation and this could explain the additive interaction (CI = 0.988) shown by CNC/DOX combination. The hallmark of those combinations included lowering of the dose of DOX used while maintaining its anti-tumor activity. Therefore, understanding the features of the current combination therapies with DOX may provide a new design of autophagy-based cancer therapy for TNBC.

Conflict of interests

The authors declare no conflict of interests.

Financial disclosure

The authors have no funding to disclose.

The author contribution

Study design: Mohamed A. Abdel-Mohsen.
Data collection: Eman S. El- Shafey.
Statistical analysis: Mohamed A. Abdel-Mohsen.
Data interpretation: Eman S. El- Shafey.
Manuscript preparation: Eman S. El- Shafey.
Literature search: Camelia A. Abdel Malak.
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