

ORIGINAL ARTICLE

Artemisinin induces selective and potent anticancer effects in drug-resistant breast cancer cells by inducing cellular apoptosis and autophagy and G2/M cell cycle arrest

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Summary

Purpose: To investigate the anticancer properties of a well-known naturally occurring sesquiterpene lactone – artemisinin - against cisplatin resistant human breast carcinoma cells along with examining its effects on apoptosis, autophagy as well as cell cycle phase distribution.

Methods: assay was used to study cytotoxic effects of artemisinin while clonogenic assay analysed its effects on cancer cell colony formation. Fluorescence microscopy using DAPI staining was employed to study apoptotic effects of artemisinin which was followed by annexin V/propidium iodide (PI) assay which quantified the apoptotic effects of artemisinin in cancer cells. Apoptotic effects of artemisinin were finally confirmed by western blot assay by analysing its effects on Bcl-2 and Bax protein expressions. Effects of artemisinin on autophagy were determined by transmission electron microscopy (TEM). Effects on cell cycle were analysed by flow cytometry and western blot.

Results: The results indicated that artemisinin led to considerable and dose-dependent antiproliferative effects on

MDA-MB-231 cisplatin-resistant breast cancer cells with less toxicity in normal cell line, thus exhibiting selectivity. The colony formation decreased significantly as artemisinin dose increased. DAPI and comet assays revealed that artemisinin induced powerful apoptotic effects, triggering significant dose-dependent DNA damage. TEM indicated that artemisinin induced autophagy in MDA-MB-231 cisplatin-resistant human breast cancer cells by creating autophagosomes and autophagic vacuoles. The molecule also targeted G2/M phase cell cycle along with targeting some key cell cycle related proteins including cyclin-B1, cyclin D1 and cyclin E.

Conclusions: The results show that artemisinin showed strong anticancer effects in MDA-MB-231 cisplatin-resistant cancer cells by triggering apoptosis and autophagy and G2/M phase arrest.

Key words: Artemisinin, breast cancer, apoptosis, autophagy, cell cycle

Introduction

Breast cancer (BC) is a malignant tumour prevailing among females globally [1,2]. Countless women suffer from BC each year and in 2012 alone nearly about 1.7 million new cases were reported [3]. Studies from the last decade revealed that BC possesses distinctive tumour subtypes categorized primarily on hormone receptor (HR) and HER2 ex-

pressions: HR and HER2 like luminal (HER2-positive or HER2-negative, HR-positive), HER2 (HR-negative, HER2-positive) and triple-negative (HER2 negative, HR-negative) [4,5]. These subtypes show different pattern of biology, metastasis, treatment strategies and prognosis. Various studies have reported a clear relationship between patterns of re-

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productive behaviour and development of BC like breastfeeding and pregnancy [6-8]. Till today, it remains unclear whether these reproductive factors govern or prevent certain subtypes of BC. A previous meta-analysis reported that young age and parity at first birth reduces the chances of developing HR-positive BC, and likewise breastfeeding reduces the chances of both HR positive and negative BC occurrence [9-12]. At present, different therapeutic agents are used to treat subtypes of BC like tamoxifen for estrogen receptor (ER)-positive BC and trastuzumab for HER2/neu-positive BC [13,14]. Beside the use of these therapeutics, the risk of disease relapse and severe side-effects reduces their efficiency. Current treatment modalities only impacted the morbidity associated with BC and didn't prove fruitful in reducing disease relapse and enhancing overall survival chances. Current operational treatment modalities for BC include hormone therapy, chemotherapy, radiotherapy and surgery. There is a necessity and critical need for developing novel treatment options to treat BC and reduce its occurrence in high risk females. Throughout the last two decades numerous bioactive compounds have been recognized in human diets and plants and are developed as therapeutic drugs for cancer treatment including BC [15-19]. Sesquiterpene lactones are biologically active compounds with remarkable pharmacological potential. These are used as antimigraine, antimicrobial, enhancers of cardiac muscle functions, analgesic, digestives and anti-cancer as well [20-22]. Artemisinin- a well-known naturally occurring sesquiterpene lactone isolated from *Artemisia annua* (traditional Chinese remedy for chills and fever)-a pharmacologically active compound with potential anticancer behaviour [23]. Artemisinin targets a wide range of cellular pathways in cancer cells including NF- κ B, PI3K/Akt, p38-MAPK, Wnt/ β -catenin and Ras pathways. Herein, the present study was designed to unveil the selective and potential anticancer effects of artemisinin in cisplatin-resistant breast cancer cells mediated via induction of cellular apoptosis, autophagy and G2/M cell cycle arrest.

Methods

Estimation of cell proliferation rate

MTT assay was performed to assess *in vitro* cell proliferation rate of artemisinin-treated cancer MDA-MB-123 cell line and normal human mammary MCF10A epithelial cell line. Briefly, cells were collected at exponentially growing phase and seeded into 96-well plates (8×10^3 cells/well) in 200 μ l of RPMI-1604 culture medium. DMSO (0.01%) was taken as blank control. Seeded cells were then incubated with varying doses of ar-

temisinin (0, 5, 25, 50 and 100 μ M) for 48 h. Following artemisinin treatment, each well was supplemented with MTT solution (10 μ l) and further incubated for about 3 h. Finally, absorbance was recorded at 560 and 630 nm with an Envision microplate reader (PerkinElmer, Waltham, MA, United States) for optical density calculation. Independent experiments were repeated three times for each assessment. Untreated cells were considered as 100% viable cells.

Cell colony assay formation determination

Colony formation assay was performed to assess the impact of artemisinin on MDA-MB-123 cells. Artemisinin treatment was performed in 6-well plates (nearly 325 cells/well) at varying doses (0, 25, 50 and 100 μ M) for 24 h. Afterwards, treated cells were washed with phosphate buffered saline (PBS), followed by trypsinization. Trypsinized cells were then incubated for 12 days. Thereafter, cell colonies were fixed with alcohol and stained with crystal violet. Finally, colonies were counted under light microscope.

Apoptosis analysis via DAPI

To monitor apoptosis, DAPI staining was performed. Breast MDA-MB-123 carcinoma cells were cultured (2×10^4 cells/well) in 96-well plates. After culturing, cells were treated and incubated with different doses of artemisinin (0, 25, 50 and 100 μ M) for about 24 h. Afterwards, treated cells were DAPI-stained for 1 h. Stained cells were then washed with PBS and fixed with formaldehyde. Finally, MDA-MB-123 cells were observed for cell morphology analysis under fluorescence microscope.

Quantification of apoptosis

For quantification of apoptosis annexin V-FITC/PI dual staining was performed. MDA-MB-123 cancer cells were cultured with a density of 2.3×10^4 cells/well in 24-well plates. Culturing of cells was performed for 48 h, followed by artemisinin treatment (0, 25, 50 and 100 μ M) for 24 h. Subsequently, treated cells were stained with FITC-conjugated annexin-V/PI for apoptosis quantification through Apoptosis Detection kit (Santa Cruz Biotechnology, Dallas, TX, United States). The manufacturer's instructions were strictly followed and finally cells were analyzed via GUAVA EasyCyte cytometer and data was recorded.

Cell cycle analysis

For assessing the distribution of MDA-MB-123 breast cancer cells in variant phases of cell cycle, flow cytometric analysis was performed. Briefly, test cells were exposed to changing concentrations of artemisinin (0, 25, 50 and 100 μ M) for 24 h. Treated cells were harvested and PBS-washed twice. Next, cells were fixed for 1 h with 70% ethanol and again washed with PBS. Using RNase1 (250 μ g/ml) and PI (50 μ l/ml) cells were resuspended, which was followed by incubation for 30 min at 25°C. Cater-plus cytometer was used in fluorescence-activated cell sorting considering 10,000 cells in each group.

Autophagy assessment via transmission electron microscopy (TEM)

Breast carcinoma MDA-MB-123 cells were harvested at exponentially growing stage and treated with changing artemisinin doses (0, 25, 50 and 100 μM). Drug treatment was followed by fixation with 4% glutaraldehyde in 0.05M sodium cacodylate buffer. Subsequently, OsO_4 (1.5%) was used for post-fixation and dehydration of these post-fixed MDA-MB-123 cells was performed with alcohol. Finally, cells were evenly implanted and organised in Epon 812 for further observation under Zeiss CEM 902 electron microscope.

Western blot

Western blotting analysis was performed to examine the expressions of various cell signalling proteins like Bax, Bcl-2 and Cyclin-B1, -D1 and -E. Artemisinin-treated breast carcinoma MDA-MB-123 cells were lysed using RIPA lysis buffer for collection of protein extracts. From each group equal amounts of protein extracts were run on SDS-PAGE followed by transference to PVDF (polyvinylidene fluoride) membrane. Afterwards, membranes were blocked by using non-fat milk and subjected to incubation for 1 h at room temperature. Thereafter, blocked membranes were exposed to primary antibody (Bax and Bcl-2, Cyclin-B1, -D1 and -E antibodies) treatment, followed by overnight incubation at 4°C. Next, the membranes were washed with PBS and further incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse (secondary antibodies). ECL Advanced Western Blot Detection Kit (GE Healthcare, Little Chalfont, UK) was employed to visualize proteins bands of interest.

Statistics

The experiments were performed in triplicate and the values presented are the mean \pm SD of three experiments. $P < 0.05$ was considered statistically significant. Student's t-test using GraphPad prism 7 software was employed for statistical analyses.

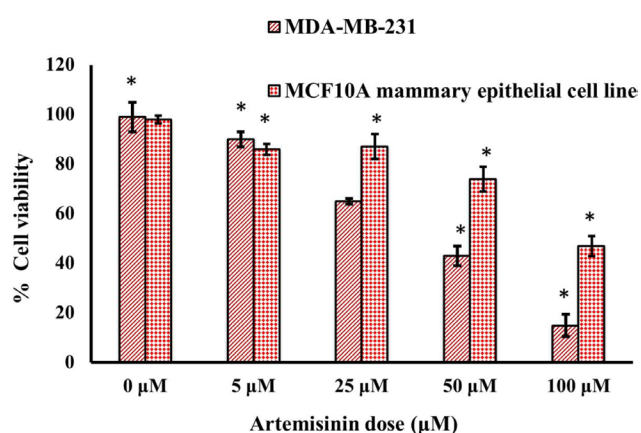


Figure 1. Effect of artemisinin on cell viability of breast cancer MDA-MB-123 cell line and human mammary MCF10A epithelial cell line. Individual experiments were repeated three times and data are presented as mean \pm standard deviation. * $p < 0.05$.

Results

Cell viability inhibition by artemisinin treatment

MTT assay revealed a dose-dependent suppressive effect on the viability of MDA-MB-123 cells. Cell viability decreased from 100% to nearly 90% at 5 μM of drug concentration and reached 45% at 50 μM . At a concentration of 100 μM of drug treatment the viability reduced to nearly 10% (Figure 1). Cell viability of the human mammary MCF10A epithelial cells was also assessed along with cancer cells but the impact of artemisinin treatment was insignificant as their viability didn't change much. Thus from MTT assessment it is clear that artemisinin exhibited dose-dependent and selective anti-proliferative effects.

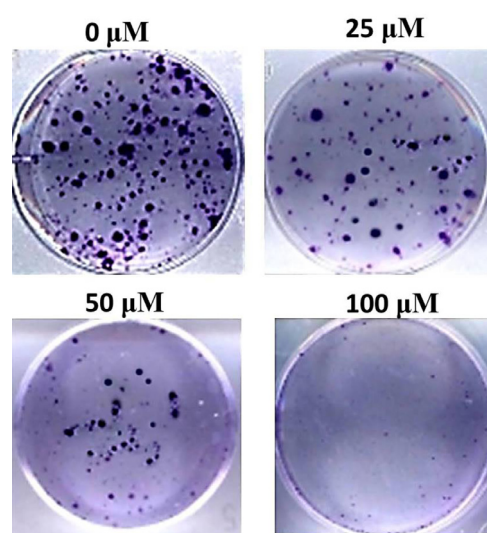


Figure 2. Effect on clonogenic potential of MDA-MB-123 cell line by artemisinin treatment. Individual experiments were repeated three times.

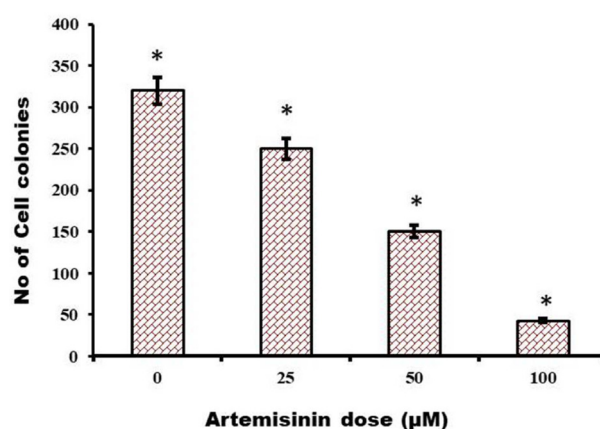


Figure 3. Graphical representation of clonogenic assay indicating dose-dependent colony formation suppressive effects of artemisinin. Individual experiments were repeated three times and data are presented as mean \pm standard deviation. * $p < 0.05$.

Inhibition of cell colony formation by artemisinin treatment

MDA-MB-123 cell colonies were assessed after 10 days of treatment with artemisinin. The clonogenic survival was observed as diminished with increased artemisinin concentrations (Figure 2). The number of MDA-MB-123 cell colonies reduced from nearly 325 to about 250 at 25 μ M of artemisinin dose and to near about 150 at 50 μ M, in contrast with the controls. On further increasing the drug dose to 100 μ M the number of colonies significantly reduced to nearly 40 (Figure 3), thus indicating potential dose-dependent suppression of colony formation by this molecule against MDA-MB-123 cells.

Artemisinin induced apoptotic cell death in MDA-MB-123 breast cancer cells

To establish whether cell viability was suppressed by apoptosis-inducing effect of artemisinin, DAPI staining was performed. Formation of apoptotic crops and blebbing of membrane was elucidated by the results from DAPI (Figure 4). Moreover, the results indicated that apoptosis-inducing potential of artemisinin was dose-dependent, as the percentage of apoptotic cells was found to be remarkably increased with higher molecule concentrations. Furthermore, annexin V-FITC/PI assay depicted increased apoptotic cell population from 5.2% in controls to nearly about 38% at 100 μ M of artemisinin concentration (Figure 5). Thus it may

be concluded from DAPI and annexin V-FITC/PI assay that artemisinin-induced antiproliferative effects in MDA-MB-123 cells are mediated via apoptosis.

Artemisinin treatment altered Bcl-2/Bax ratio in MDA-MB-123 cells

To further strengthen the fact that artemisinin-induced apoptotic cell death in MDA-MB-123 breast cancer cells, western blotting analysis was performed to examine the Bcl-2/Bax ratio. The results revealed that artemisinin treatment downregulated the Bcl-2 expression and upregulated Bax

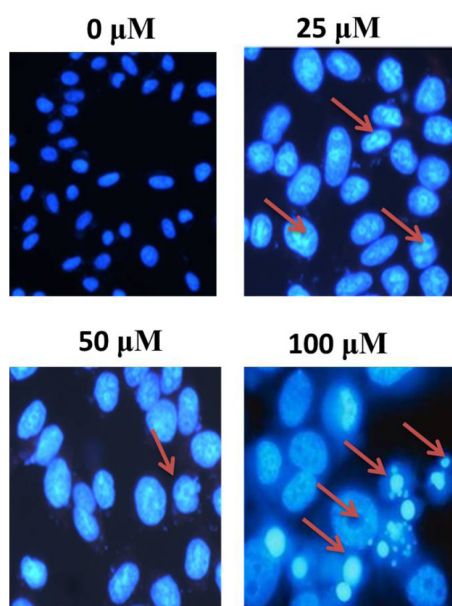


Figure 4. DAPI staining assay showing apoptotic cells with membrane blebbing and apoptotic bodies as signified by arrows. The number of apoptotic cells increased with increasing artemisinin doses. Individual experiments were repeated three times.

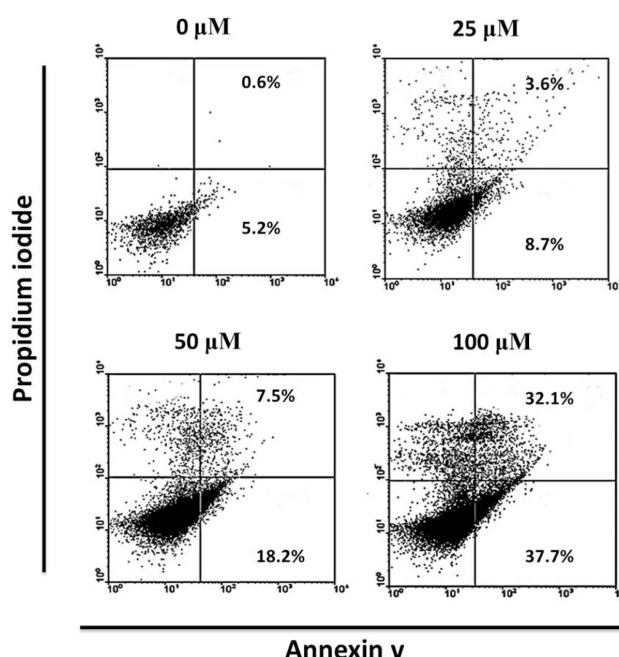


Figure 5. Effect on apoptotic cell population by artemisinin treatment in MDA-MB-123 cell line. Individual experiments were repeated three times. The results show that increased drug doses significantly increased the number of apoptotic cells.

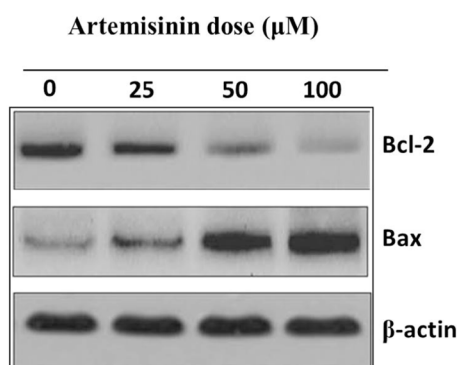


Figure 6. Artemisinin treatment altered Bcl-2 and Bax expressions which altered Bcl-2/Bax ratio as well in breast MDA-MB-123 cancer cells at presented doses. Individual experiments were repeated three times.

expression in a concentration-dependent manner (Figure 6) resulting in lowering Bcl-2/Bax ratio and eventually supported apoptosis.

Artemisinin treatment caused cell cycle arrest in MDA-MB-123 cells

Antiproliferative agents induce their effects via different mechanisms including cell cycle arrest. Hence, the effect of artemisinin on cell cycle phases of MDA-MB-123 cells was investigated. Interestingly, flow cytometry showed significantly increasing frequency of G2/M-phase cells, which ultimately led to arrest of cell cycle at G2/M-phase. The percentage of G2/M cell populations increased to nearly 50% at 100 μ M as compared to nearly

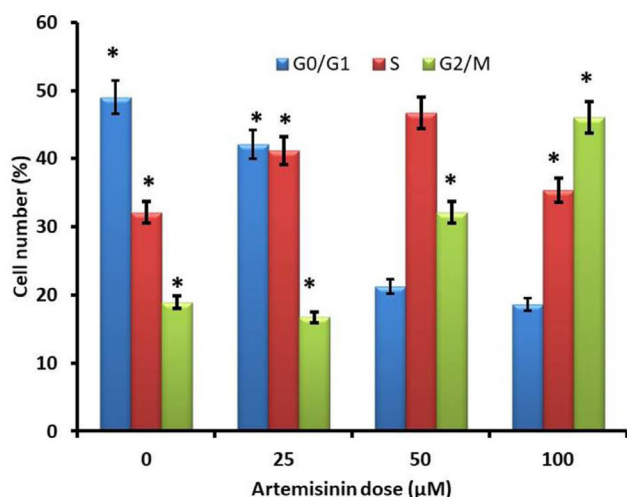


Figure 7. Estimation of cell cycle phases via flow cytometry. Artemisinin induced G2/M-phase cell cycle arrest in a dose-dependent manner. Individual experiments were repeated three times and data are shown as means \pm standard deviation. * $p < 0.05$.

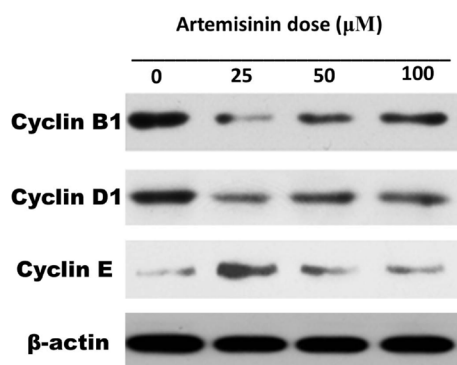


Figure 8. Impact of artemisinin treatment on the expressions of MDA-MB-123 cell cycle-related proteins (Cyclin-B1, -D1 and -E). The results display dose-dependent inhibition of Cyclin-B1 and -D1 and no significant change in Cyclin-E expression. Individual experiments were repeated three times and data are presented as means \pm standard deviation.

20% in controls (Figure 7). Furthermore, the expression of cell cycle associated proteins through western blotting dose-dependent downregulation of Cyclin-B1 and Cyclin-D1 expressions with no apparent impact in Cyclin-E expression (Figure 8). Thus, flow cytometric and western blotting analysis clearly indicate that the antiproliferative effects of artemisinin was dose-dependent G2/M-phase cell cycle arrest.

Artemisinin treatment induced autophagic cell death in MDA-MB-123 breast cancer cells

To elucidate that artemisinin induced autophagic cell death in MDA-MB-123 cells TEM was performed. The results indicated that artemisinin treatment induced remarkable morphological changes in target cells which included agglutinated heterochromatin, degenerated mitochondrial vacuoles, nuclear swelling and declined number of intracellular organelles. A major observation from TEM analysis was the visualization of autophagosomes, a complete hallmark of autophagy (Figure 9).

Discussion

Unfortunately, due to lower efficiency of current treatment modalities BC management remains a challenge for clinicians. BC is an hormone-dependent disease, hence targeting female reproductive hormones through appropriate drugs can prove a leading therapeutic target in BC treatment.

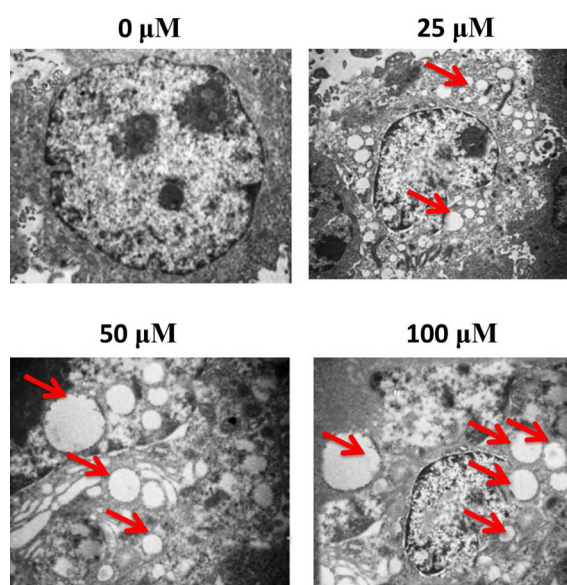


Figure 9. Autophagy analysis via transmission electron microscopy. Artemisinin treatment of MDA-MB-123 cells clearly indicating autophagosomes as marked by the arrows. Individual experiments were repeated three times.

Currently targeted major therapeutic pathways in cancer management include apoptosis, autophagy, necrosis and cell cycle [24]. Apoptosis (type-I) and autophagy (type-II) are phenomena of naturally operating programmed cell death and are activated to combat any type of cellular damage and malfunctioning. Apoptosis and autophagy play a central role in maintaining homeostasis of tissues in response to frequent stimuli. These processes are characterized by different biochemical and morphological changes. Apoptosis is hallmarked by the formation of apoptotic bodies and it is associated with alterations in Bax and Bcl-2 protein expressions. Similarly, autophagy is hallmarked by the formation of autophagosomes and is associated with alterations in LC3B-I, LC3B-II and Beclin-1 expressions. Hence, inducing apoptosis or autophagy or both in cancer cells prevents cancer cell growth and progression. In the recent past artemisinin has revealed significant anticancer effects against different human cancers in addition to its antimalarial property. The anticancer effects of artemisinin are reported to be due to its potential of autophagy and apoptosis induction and targeting multiple signalling pathways which are responsible for cell growth and development. Herein, artemisinin was testified for its selective and potent anticancer effects against cisplatin-resistant breast cancer cells. This molecule was investigated for apoptosis, autophagy and cell cycle analysis against drug-resistant breast MDA-MB-123 cancer cells. The antiproliferative effects of artemisinin were evaluated through MTT assay, and the results revealed that this molecule reduced cell viability significantly and dose-dependently. The viability of normal cells was not altered much by exposure which ultimately elucidated that the antiproliferative effects of artemisinin were breast cancer-se-

lective. Artemisinin resulted in colony formation suppression as evidenced from clonogenic analysis of artemisinin-treated MDA-MB-123 breast cancer cells. Artemisinin-induced apoptotic cell death was assessed through DAPI and annexin V-FITC/PI staining assay. Interestingly, the results revealed that artemisinin induced apoptosis in a dose-dependent manner. Western blotting analysis showed significant alterations in Bcl-2/Bax ratio which further supported apoptosis. Next, flow cytometric analysis was carried out for cell cycle phase determination which clearly indicated increased number of G2/M phase cells (signifying cell cycle arrest) with increasing artemisinin doses. Western blotting showed that the expressions of Cyclin-B1 and -D1 reduced significantly with increasing molecule doses, whereas Cyclin-E expression remained almost unchanged. Finally, TEM analysis for autophagy estimation displayed that artemisinin not only induced apoptotic cell death in MDA-MB-123 cells but dose-dependent autophagic cell death as well.

Conclusion

In conclusion, all these results indicate that artemisinin is a potent anticancer agent in MDA-MB-231 drug-resistant human breast cancer cells. Artemisinin effectively revealed its cancer suppressive effects via induction of apoptosis, autophagy and cell cycle arrest. Hence, this molecule may be considered as a lead drug in breast cancer management, provided further *in vitro* and *in vivo* studies are carried out.

Conflict of interests

The authors declare no conflict of interests.

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