DOI: https://doi.org/10.53350/pjmhs2115113313

ORIGINAL ARTICLE

Camel Milk Exosomes Potentiate The Anticancer Effect of Doxorubicin on Multidrug-Resistant Human Leukemia Hl60 Cells in Vitro and in Vivo

RASHAD QASEM ALI OTHMAN[1], ABDELNASER A. BADAWY[2,3], MOHAMMED M. ALRUWAILI[4], MOHAMMED A. EL-MAGD[5]

[1]*Department of Pathology, Faculty of Medicine, Northern Border University, Arar, Saudi Arabia*
[2]*Department of Biochemistry, Faculty of Medicine, Northern Border University, Arar, Saudi Arabia*
[3] *Department of Medical Biochemistry, Faculty of Medicine, Mansoura University, Mansoura, Egypt.*
[4]*Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, Northern Border University, Arar, Saudi Arabia.*
[5]*Department of Anatomy, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt.*
Corresponding author: Rashad Qasem Ali Othman, Email: raqaa76@gmai.com ; ordid id: 0000-0002-6004-4203

ABSTRACT

Background: Multidrug resistance (MDR) is one of the strategies developed by cancer cells to inhibit the anticancer potential of the majority of chemotherapeutic agents and almost results in treatment failure.

Objective: This study aimed to evaluate the therapeutic potential of camel milk exosomes (CME) on multidrug-resistant human acute promyelocytic leukemia HL60 cells (HL60/RS) and to investigate whether this CME could potentiate the anticancer effect of Doxorubicin (DOX) and decrease its side effects.

Results: CME alone or combined with DOX significantly induced HL60/RS cell viability loss, apoptosis, and cell cycle arrest at the G0/G1 phase, and downregulated MDR genes (Abcb1, Abcc1, Abcg2) as compared to cells treated with DOX alone. Additionally, CME and DOX co-treated nude mice had the lowest tumor volume, Abcb1, Abcc1, Abcg2, and Bcl2 expression, and the highest Bax and caspase3 expression in HL60/RS xenografts. This combined therapy also decreased DOX adverse effects as revealed by decreased liver damage enzymes and lipid peroxide (MDA) and increased hepatic antioxidant enzymes (SOD, CAT, GPx).

Conclusion: CME increased sensitivity of HL60/RS to DOX through, at least in part, reduction of MDR genes, induction of apoptosis, and cell cycle arrest. Thus, CME may be used as safe adjuvants to DOX during cancer treatment.

Keywords: Camel milk exosomes; Myeloid leukemia; HL60; Apoptosis; MDR

INTRODUCTION

Acute myeloid leukemia (AML) could aggressive hematologic malignancy characterized by an uncontrollable proliferation of immature myeloid blasts. AML progresses rapidly and is typically fatal within weeks or months if left untreated. Although the newly developed chemotherapeutic agents significantly enhanced the survival rates in AML patients over the past decades, the adverse effects of these agents restricted their wide uses. One of the main strategies by which cancer cells can resist the effect of chemotherapy is the development of multidrug resistance (MDR) which makes the chemotherapy less effective and causes tumor recurrence (Matsumoto et al., 2012; Musgrove and Sutherland, 2009). MDR is associated with overexpression of P-glycoprotein (P-gp), multidrug-resistance-related protein (MRP), and adenosine triphosphate-binding cassette (ABC) transporters (Chen et al., 2018; Gao et al., 2015; Matsumoto et al., 2012). These transporters act as energy-dependent efflux pumps that promote the efflux of anti-cancer drugs out of the cells, thereby leading to minimal concentrations of the anti-cancer drugs within the target cells and therapy failure. P-gp (also known as MDR1) is a member of the ATP-binding cassette transporters family encoded by the Abcb1a and Abcb1b genes (Renaud et al., 2016). The other transporter genes include the Abcc1 gene which encodes MRP1 protein and the Abcg2 gene which encodes BCRP2 protein (Chen et al., 2012). Finding a safer and more effective adjuvant to conventional chemotherapeutics to improve their efficacy and reduce their adverse effects is becoming an urgent need to improve outcomes in AML. Some of these new approaches involved using herbal extracts or natural products with an anticancer drug to improve anticancer potential, inhibit MDR, and decrease toxicity on normal cells (Hwang et al., 2019; Magdy et al., 2020; Shakor et al., 2014; Siveen et al., 2017; Sun et al., 2013).

Regular drinking of camel milk was believed to decrease the risk for cancer in the Middle East. Camel milk has the potential to inhibit the proliferation of a large variety of cancer cells including HepG2, HepaRG, MDA-MB-231 and MCF7, Hepa 1c1c7 cells (Badawy et al., 2018; Badawy et al., 2021; El-kattawy et al., 2021; Homayouni-Tabrizi et al., 2017; Korashy et al., 2012a; Korashy et al., 2012b; Shariatikia et al., 2017). Camel milk contains a large number of extracellular vesicles called exosomes with a nano-size range from 30 to 100 nm (Badawy et al., 2018; Badawy et al., 2021; El-kattawy et al., 2021; Ibrahim et al., 2019). These membranous nanovesicles are very important for cell-to-cell communication through their cargo of proteins, mRNAs, microRNAs, lncRNAs, and metabolites (Alzahrani et al., 2018; Alzahrani et al., 2021). Camel milk exosomes (CME) significantly inhibited the proliferation of MCF7 cells both in vitro and in vivo and their effect mediated by triggering apoptosis and preventing inflammation and metastasis (Badawy et al., 2018). CME also potentiate the anticancer effect of tamoxifen against MCF7 and minimize tamoxifen side effects (Badawy et al., 2021). Moreover, CME have been shown to prevent the proliferation of HepaRG cells by induction of apoptosis and

inhibition of inflammation and angiogenesis (El-kattawy et al., 2021)

To the best of our knowledge, no available data in the literature addressed the effect of CME on HL60/RS cells in vitro or in vivo. Therefore, the present study aimed to investigate CME effect on HL60/RS both in vitro and in vivo and to check whether CME/DOX combined therapy could decrease DOX side effects.

MATERIAL AND METHODS

Isolation and characterization of camel milk exosomes: Milk samples were collected from 5 camels (400 ml/animal) at the mid-lactation stage. Exosomes were isolated from these samples by twice ultracentrifugation (100,000 g at 4 °C for 60 min for each) using a fixed angle rotor Optima L-90K ultracentrifuge (Beckman Coulter, Brea, California) as previously described (Badawy et al., 2021; El-kattawy et al., 2021). The concentration of the obtained exosome samples was determined by a Nanodrop (Q5000, Quawell, USA). For characterization of exosomes, after fixation in 2.5% glutaraldehyde for 1 h, a drop of exosomes (10 µL) was added to a grid and examined using TEM (JEM2100, Joel Inc., Japan) at 80 kV (Badawy et al., 2018).

Cell line and cell culture: Acute promyelocytic leukemia cell line (HL60) was purchased from the German collection of microorganisms and cell cultures (DSMZ, ACC 115, Germany). DOX-resistant HL60 cells (HL60/RS) were obtained by long-term exposure to sequential culturing with increasing concentrations (0.01 - 40 µg/ml) of DOX for 30 days (Chen et al., 2018). The prepared HL60/RS was approved to be resistant also to other chemotherapeutic agents. Cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS and were incubated at 37 °C, 5% CO2, and appropriate humidity.

Measurement of cell proliferation by MTT assay: HL60 and HL60/RS cells were separately seeded at a density of 1×10^4 cells/well in 96-well plates and incubated overnight until 70% confluence. Serial concentrations of exosomes (0, 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 µg/ml) or DOX (0.01, 0.65, 1.25, 2.5, 5, 10, 20, and 40 µg/ml) were added to the media and the cells were re-incubated for 24 h. The media were removed and cells were cultured with 5 mg/ml MTT (Sigma) for 4 h. The viable cells converted MTT to formazan, which generated a blue-purple color after dissolving in 100 µL of DMSO. The absorbance was recorded at 570 nm and IC_{50} was calculated using GraphPad software.

In vitro experimental design: The DOX-resistant human acute promyelocytic leukemia HL60/RS cells were allocated into 4 groups: control (Cnt) group treated with vehicle (DMSO), DOX-treated (40 µg/ml) group, CME group treated with exosomes at a concentration equal to its IC_{50} (47 µg/ml), and DOX+CME group. After all treatments, cells were incubated at 37 °C, 5% CO_2 for 24 h.

Determination of cell cycle by flow cytometry: The harvested HL60/RS cells were fixed in ice-cold 70% ethanol at -20 °C for 24 h after trypsinization. Following twice PBS washes, the cells were stained with 100 µl propidium iodide (PI) and incubated in darkness for 1 h. The stained cells were analyzed by Attune flow cytometer (Applied Bio-system, US).

In vivo xenograft assay: The nude female mice (Swiss Nu/Nu, Swiss, Charles River Laboratories, Paris, France) with an age of 4–6 weeks and bodyweight of 20 - 25 g were housed at optimal conditions (temperature 20ºC - 23ºC, relative humidity 40% - 55%, and light 12h). The animals were housed under specific pathogen-free conditions and were given standard pellet food and water ad libitum. The animals were acclimatized to the housing condition for 7 days before starting the experiment. All experimental procedures were conducted under the guidelines of laboratory animal use and care and were approved by the Animal Ethics Committee of Kafrelsheikh University (license number: KFS127/25).

Thirty mice were randomly allocated into 5 groups (n = 6/group). G1 (normal healthy, Nor) intraperitoneally (i.p.) injected with 0.2 ml phosphate buffer saline (PBS). Xenograft tumors were induced by injecting HL60/RS cells (1×10^7 cells in 200 µl RPMI-1640) subcutaneously on the region behind the right forelimb of mice in G2-G5. When tumor volume reached 60±10 mm^3 (after 2w of induction), G2 (Xen) was administered 0.2 mL PBS, G3 (DOX) was given 4 mg/kg DOX (Bandyopadhyay et al., 2010), G4 (CME) was treated with 1.25 mg/kg exosomes dissolved in 1 ml PBS (Badawy et al., 2021), and G5 (DOX+CME) was cotreated with DOX and CME at doses similar to G3 and G4. All treatments were given i.p. once a week for 2 w from the 3rd w to the 4th w. At day 29, blood samples were collected, sera were obtained by centrifugation, and were stored at -20°C for biochemical assay. Following euthanization, livers and tumors were excised. Tumor volumes were calculated with the equation of $(\text{width} + \text{length}^2) \times 0.5$ in mm^3. Tumors were stored at -80°C for real time PCR.

Biochemical parameters: Liver enzymes [aspartate transaminase (AST), alanine transaminase (ALT)] were determined in serum using commercially available kits (Biodiagnostic, Egypt). Liver homogenates were prepared as previously detailed (El-Magd et al., 2016) and were used to determine lipid peroxide (malondialdehyde, MDA) concentration and antioxidant enzyme [superoxide dismutase (SOD, catalase (CAT) and glutathione peroxidase (GPx)] activities following manufacturer protocol (Biodiagnostics, Egypt).

Determination of gene expression by qPCR: Total RNA was isolated from HL60/RS cells and tumor tissues (xenografts) using a Trizol reagent (Invitrogen). cDNA synthesis was done using Quantiscript reverse transcriptase (Qiagen, #205310) and then amplified by Maxima SYBR Green Master Mix (Thermo Scientific, USA). Primer sequences of the human and mice were shown in Table 1. Quantitative real-time PCR (qPCR) was performed using StepOnePlus real-time PCR system (Applied Biosystem, US) with thermal conditions as previously described (El-Magd et al., 2017b). The expression of each target gene in form of fold change was normalized with β-actin expression and evaluated using the $2^{-\Delta\Delta Ct}$ method. Target genes included apoptosis-related genes (Bax, caspase3, and Bcl2) and MDR-related genes [Abcb1 gene that encoded P-glycoprotein (P-gp) protein, Abcc1 gene that encoded multidrug-resistance-related protein 1 (MRP1), and Abcg2 that encoded breast-cancer-resistance protein 2 (BCRP2)].

Statistical analysis: All data were presented as mean ± standard error of mean (SEM). A comparison of the results was performed with one-way ANOVA and Tukey's Honestly Significant Difference tests (GraphPad Prism software 7, La Jolla, CA, USA). Statistically significant differences between groups were declared at $P < 0.05$.

RESULTS

Characterization of exosomes: The total weight of the isolated exosomes from each 200 ml camel milk was 74 ± 5.23 mg. The isolation of exosomes derived from camel milk was confirmed using transmission electron microscopy (TEM). They appeared as small sizes vesicles with different diameters ranging from 30 to 100 nm (Fig. 1).

Exosomes induce a cytotoxic effect on HL60 and HL60/RS cells: The effects of camel milk exosomes (CME) on the viability of DOX-sensitive (HL60) and DOX-resistant (HL60/RS) were determined by MTT assay. Treatment of drug-sensitive HL60 cells with CME and DOX alone or in combination (DOX+CME) inhibited cell viability in a dose-dependent manner with IC_{50} values of 35.33±0.81, 12.56±0.35, and 8.06±0.26 µg/ml, respectively (Fig. 2). In contrast, after one month of treatment with DOX, HL60 became resistant (HL60/RS) to 0.01 – 40 µg/ml DOX. Notably, CME induced HL60/RS cell viability loss with IC_{50} values of 47.03±0.95 µg/ml (Fig.2). Surprisingly, cotreatment of HL60/RS cells with a constant concentration of DOX (40 µg/ml) and serial concentrations of CME (0 – 100 µg/ml) reduced the cell viability with IC_{50} values of 17.70±0.37 µg/ml (Fig. 2).

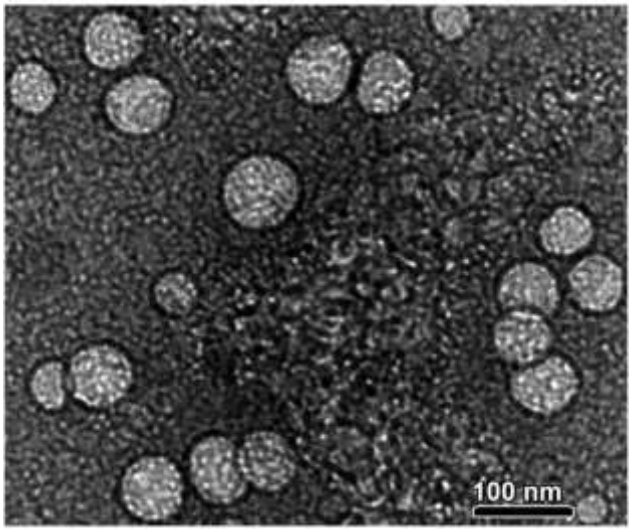
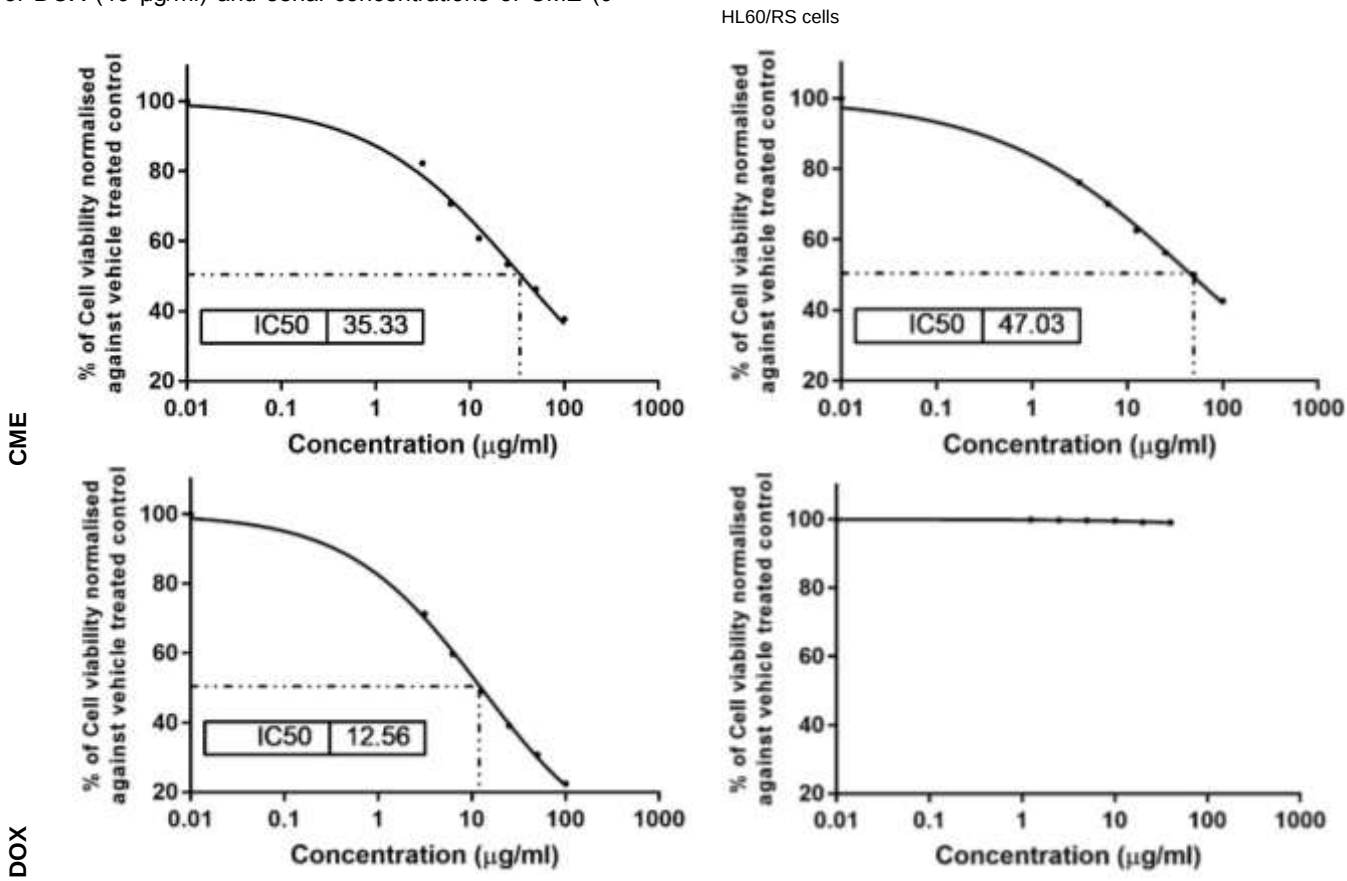


Fig.1. TEM examination of the isolated exosomes from camel milk shows exosomes with a size ranged from 30 to 100 nm.



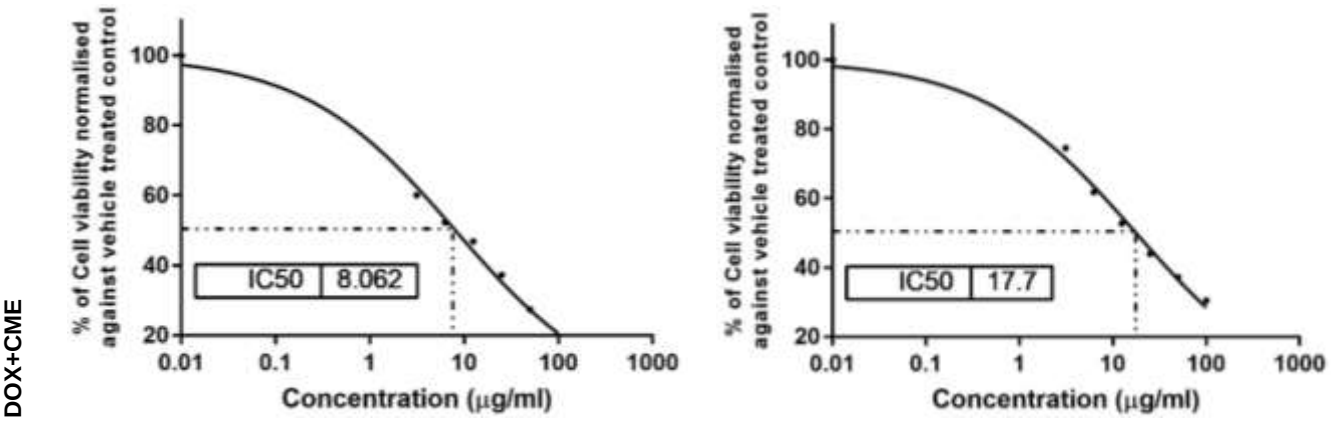


Fig. 2. MTT assay representative images show cytotoxic effects and IC_{50} values of CME and/or DOX on HL60 and HL60/RS cells. The two cell lines were treated either individually with serial concentrations of CME (0, 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 µg/ml) and DOX (0.01, 0.65, 1.25, 2.5, 5, 10, 20, and 40 µg/ml) or cotreated with constant concentration of DOX (40 µg/ml) and serial concentrations of CME and were incubated for 24 h. Each data represents an average of three independent experiments (n = 5).

Effect of exosomes on apoptotic and MDR genes in HL60/RS cells: Treatment of HL60/RS cells with DOX (40 µg/ml) showed no significant changes in the expression of apoptosis-related genes [Bax, caspase 3 (Cas3), and Bcl2] as compared to the control (vehicle-treated) cells (Fig. 3). However, treatment with CME (47 µg/ml) alone or in combination with DOX significantly upregulated the expression of apoptotic genes (Bax and Cas3) and significantly downregulated the expression of the antiapoptotic gene (Bcl2) in HL60/RS cells relative to control and DOX-treated cells (Fig. 3). The combined treated cells showed the highest Bax and Cas3 and the lowest Bcl2 expression. These results along with MTT outputs infer the ability of CME to increase the sensitivity of HL60/RS cells to DOX.

Control and DOX-treated cells exhibited significantly higher expression of MDR genes [Abcb1 gene encoding P-glycoprotein (P-gp), Abcc1 gene encoding multidrug-resistance-related protein 1 (MRP1), and Abcg2 encoding breast-cancer-resistance protein 2 (BCRP2)] than cells treated with CME alone or in combination with DOX (Fig.3). The cotreated cells showed the lowest Abcb1, Abcc1, Abcg2 expression. This suggests that CME could increase HL60/RS sensitivity to DOX through the downregulation of MDR genes.

Effect of exosomes on cell cycle of HL60/RS cells: The effect of CME on the cell cycle of HL60/RS cells was determined by flow cytometry using propidium iodide and the obtained results were presented in figure 4. There were no significant differences in the cell number in the three phases of the cell cycle (G0/G1, S, and G2/M) in HL60/RS cells following treatment with either vehicle (56 ± 1.13, 15 ±1.00, and 29±2.92%, respectively) or DOX alone (58 ± 1.15, 16± 1.12, and 27.72±2.6%, respectively). However, treatment with CME alone or in combination with DOX resulted in a significant increase in the number of cells in the G0/G1 phase with a subsequent cell cycle arrest in this phase (64 ± 1.12 and 71±1.25%, respectively) as compared to control cells and cells treated with DOX alone. On the other hand, treatment with CME or CME+DOX did not significantly change the number of cells in S (14 ± 1.80 and 13±2.28%, respectively) or G2/M (22 ± 1.93 and 20.74±2.16%, respectively) phases as compared to control and DOX-treated cells.

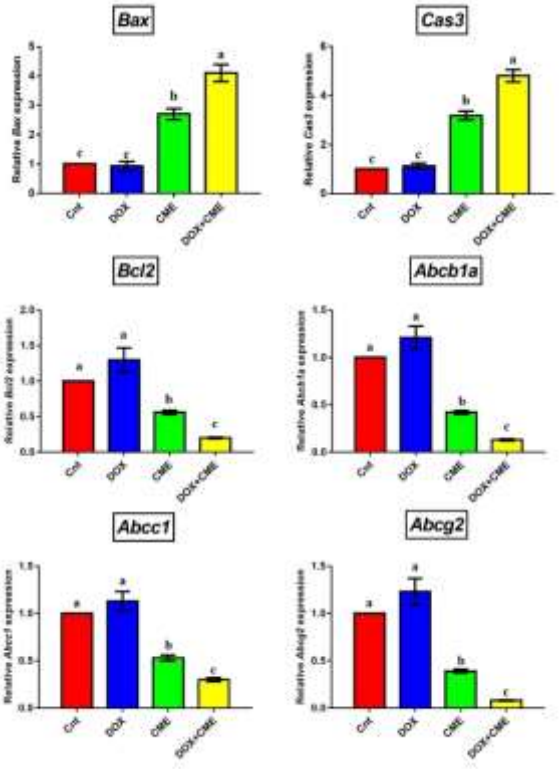


Fig. 3. Camel milk exosomes triggered HL60/RS cells apoptosis (upregulation of Bax, caspase3 and downregulation of Bcl2) and downregulated the expression of MDR (Abcb1, Abcc1, Abcg2) genes as revealed by qPCR. Cells were treated with DOX (40 µg/ml) and CME (47 µg/ml) alone or in combination and were incubated for 24 h. Values (columns and error bars) are presented as mean fold change ± SEM, n = 5. Values with different letters [a (highest value) – c (lowest value)] are significantly different ($P \leq 0.05$). Cnt, control group; DOX, DOX-treated group; CME, camel milk exosomes-treated group; and DOX+CME-treated group.

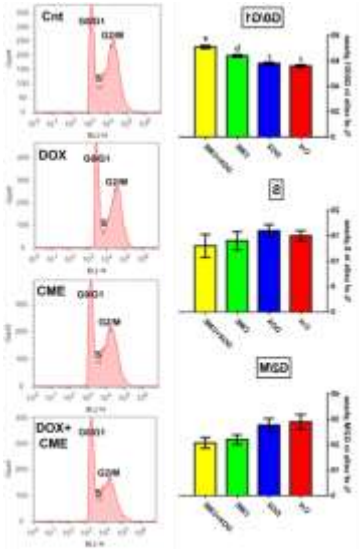


Fig.4. A) Flow cytometry histograms show effect of DOX and/or CME on cell cycle of HL60/RS cells. X-axis reveals PI fluorescence as detected by BL1-H scatter and Y-axis shows the count of cells. B) Statistical graphs show % of cells in G0/G1, S and G2/M phases. Data are presented as mean ± SEM, n = 5. Values with different letters are significantly different (P ≤ 0.05).

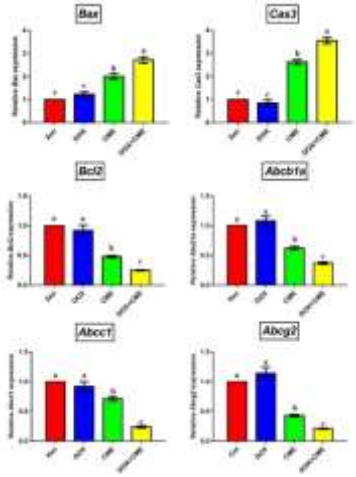


Fig. 5. Effect of CME and/or DOX on the expression of apoptosis (Bax, caspase3, Bcl2) and MDR (Abcb1, Abcc1, Abcg2) genes as detected by qPCR. Values (columns and error bars) are presented as mean fold change ± SEM, n = 5. Values with different letters [a (highest value) – c (lowest value)] are significantly different (P ≤ 0.05). Xen, xenograft group.

Effect of exosomes on the volume of HL60/RS xenograft: Given that CME alone or combined with DOX inhibited the proliferation of HL60/RS cells in vitro, we checked whether similar findings could be translated in vivo. Therefore, we induced a subcutaneous xenograft model using HL60/RS cells in nude mice. Mice treated with CME alone or in combination with DOX showed significantly reduced tumor volumes, with the lowest volume in the CME+DOX group, compared with the mock group (Xen) and DOX-treated group (Table 2).

Table 1: Primers used for real-time PCR.

Species	Gene	Forward (′5 ------ ′3)	Reverse (′5 ------ ′3)
Human	Bax	Cctgtgcaccaaggtgccg gaact	Ccaccctggtcttggat ccagccc
	Caspase3	Tgttgtgccactaattggaag t	Gccctgatctaactgc cacc
	Bcl2	Aggaagtgaacatttcggtg ac	Gctcagttccaggacc aggc
	Abcb1a	Cccatcattgcaatagcagg	Gttcaaacttctgctcct ga
	Abcc1	Tgcagaaggcggggagaa cctc	Gtcgtccgtttccaggt ccacg
	Abcg2	Gctgcaaggaaagatccaa gt	Tagttgttgcaagccg aagag
	B actin	Caccaactgggacgacat	Acagcctggatagca acg
Mouse	Bax	Ggctggacactggacttcct	Ggtgaggactccagc cacaa
	Caspase3	Gaccatacatgggagcaag t	Ccttcatcaccatggct taga
	Bcl2	Ttcgcagagatgtccagtca	Ttcagagacagccag gagaa
	Abcb1a	Gcgactccgatacatggttt	Accctgtagccccttc act
	Abcc1	Agcaactcgtcttcccacag	Acagctgcacctgccc t
	Abcg2	Gcgaatgctgtcctttgct	Gctcacagtggtaacc tgct
	B actin	Actattggcaacgagcggtt	Caggattccataccca agaagga

Table 2. Effect of CME and/or DOX on tumor size, liver function, and oxidant/antioxidant status

Groups	Tumor size (mm^3)	AST (U/l)	ALT (U/l)	MDA (nM/mg tissue)	SOD (U/ mg tissue)	GPx (U/ mg tissue)	CAT (U/ mg tissue)
Nor	-	30.82±1.84^{e}	25.26±1.23^{e}	14.90±0.91 ^{e}	43.21±1.59 ^{a}	77.38±2.61 ^{a}	50.62±2.19 ^{a}
Xen	1.13±0.07^{a}	63.09±2.96^{b}	52.11±2.26 ^{b}	51.28±2.30 ^{b}	22.60±1.00 ^{d}	34.18±1.09 ^{d}	22.05±0.73 ^{d}
DOX	1.22±0.09^{a}	94.45±3.06^{a}	80.35±2.84 ^{a}	75.00±2.72 ^{a}	14.82±0.65 ^{e}	20.77±0.92 ^{e}	14.80±0.49 ^{e}
CME	0.81±0.04^{b}	49.80±2.17^{c}	43.20±2.06^{c}	27.55±1.06 ^{d}	35.38±1.12 ^{b}	69.04±2.17 ^{b}	39.26±1.11 ^{b}
DOX+CME	0.50±0.03^{c}	38.22±1.75^{d}	40.68±1.87^{d}	40.04±1.84 ^{c}	28.06±1.04 ^{c}	58.70±2.09 ^{c}	31.79±1.10 ^{c}

Data (mean ± SEM, n = 5/group) in the same column with different letters were significantly different at P≤ 0.05.

Effect of exosomes on apoptotic and MDR genes in HL60/RS xenograft: Data obtained from qPCR revealed significant upregulation of Abcb1, Abcc1, Abcg2, Bcl2 and significant downregulation of Bax, Cas3 in xenografts of Xen and DOX groups relative to other groups (Fig.5). No statistical differences were noticed in the expression of all 6 genes between Xen and DOX groups. However, tumorous tissues isolated from mice cotreated with CME and DOX showed significantly lower Abcb1, Abcc1, Abcg2, Bcl2, and significantly higher Bax and Cas3 expression than mice treated with CME alone (Fig.5).

Effect of exosomes on liver function and oxidant/antioxidant status: Mice treated with DOX alone exhibited significantly increased ALT and AST compared to

all other groups (Table 2). These enzymes were also significantly higher in the Xen group relative to the normal (Nor) group. However, their levels were significantly decreased after treatment with CME alone or in combination with DOX with lowest level in the CME group.

Treatment with DOX alone (DOX group) resulted in significantly higher hepatic MDA levels and significantly lower activities of antioxidant enzymes (SOD, CAT, GPx) than all other groups (Table 2). Xen group had significantly higher MDA and lower SOD, CAT, GPx than the normal group. These altered levels of lipid peroxide and antioxidant enzymes were restored after treatment with CME alone or in combination with DOX with better effect for CME.

DISCUSSION

In the present study, we investigated the antiproliferative potential of camel milk exosomes (CME) against both DOX-sensitive HL60 and multidrug-resistant HL60/RS human acute promyelocytic leukemic cells. We found a potent inhibitory effect for CME on both cell lines. More interestingly, CME improved the sensitivity of HL60/RS cells to DOX. This conclusion was drawn based on the results of the MTT assay which revealed a higher cytotoxic effect on HL60/RS cells following cotreatment with DOX and CME as compared with individual treatment with DOX which showed a null effect. Inhibition of cell proliferation by CME alone or in combination with DOX was mediated by apoptosis as indicated by increased Bax and Cas3 and decreased Bcl2 expression in HL60/RS cells (in vitro) and HL60/RS xenograft (in vivo). Consistent with our findings, CME alone or in combination with tamoxifen has potent antiproliferative potential against MCF7 cells and this effect was mediated through the induction of apoptosis (Badawy et al., 2018; Badawy et al., 2021). Moreover, CME also inhibited the viability of liver cancer HepaRG cells with a similar mechanism of action (El-kattawy et al., 2021).

Cancer cells induced cell cycle dysregulation to maintain their survival and avoid apoptosis (Abdelwahab et al., 2019). Most anti-cancer agents target cancer cells through induction of cell cycle arrest and/or apoptosis (Awad et al., 2020; Elsayed et al., 2020; Khamis et al., 2018; Mahfouz et al., 2021; Mansour et al., 2021). Herein, CME alone or in combination with DOX induced cell cycle arrest in the G0/G1 phase as compared to control and DOX-treated HL60/RS cells. Taken together, CME alone or combined with DOX inhibited HL60/RS cells by inducing G0/G1 arrest and apoptosis. Recently, El-kattawy et al. (2021) and Ibrahim et al. (2019) reported that CME contained lactoferrin and attributed anticancer and immunomodulatory effects of camel milk to this exosomal lactoferrin. Moreover, cow milk lactoferrin inhibited MCF7 cell viability by triggering cell cycle arrest at the G0/G1 phase (Zhang et al., 2014). Taken together, CME lactoferrin could participate in HL60/RS arrest in the G0/G1 phase. However, further molecular investigations using gain and loss function experiments are needed to validate this hypothesis.

MDR is one of the main hurdles that render the treatment of leukemia ineffective (Chen et al., 2018; Gao et al., 2015). Several researchers have studied MDR associated with leukemia (Chen et al., 2018; Fung and So, 2013; Zhu et al., 2014), however, the underlying mechanisms have not been fully elucidated. Our results revealed downregulated expression of MDR genes [Abcb1 gene encoding P-glycoprotein (P-gp), Abcc1 gene encoding multidrug-resistance-related protein 1 (MRP1), and Abcg2 encoding breast-cancer-resistance protein 2 (BCRP2)] in HL60/RS cells (in vitro) and HL60/RS xenograft (in vivo) following treatment with CME alone or in combination with DOX as compared to those treated with DOX alone. This infers that CME could increase HL60/RS sensitivity to DOX through the downregulation of MDR genes. In agreement with our results, Abcb1, Abcc1, and Abcg2 genes and their encoded proteins were overexpressed in DOX-resistant HL60/RS cells as compared to the DOX-sensitive HL60 cells (Chen et al., 2018). Higher expression of P-gp, MRP1, and BCRP2 proteins and their encoded Abcb1, Abcc1, and Abcg2 genes, respectively increased the resistance to chemotherapy (Schinkel and Jonker, 2003).

To verify our in vitro results, we induced subcutaneous xenografts by injecting HL60/RS cells into nude mice to assess the in vivo anti-cancer potential of CME alone or in combination with DOX. Indeed, mice treated with CME alone or combined with DOX had smaller tumor volumes than mice treated with DOX alone. In agreement, administration of CME alone or combined with hesperidin, and tamoxifen reduced tumor volume in MCF7 xenografts in rats and mice (Badawy et al., 2018; Badawy et al., 2021). CME also decreased DOX adverse effects as revealed by lower serum levels of ALT and AST in mice treated with CME alone or in combination with DOX as compared to those treated with DOX alone. Similar results were reported by Badawy et al. (2021) who found reduced ALT and AST levels in mice bearing tumors (induced by MCF7) after treatment with CME alone or in combination with hesperidin and tamoxifen. Other studies also reported that exosomes could decrease the cancer burden and associated hepatotoxicity (Alzahrani et al., 2018; Ibrahim et al., 2019).

Most adverse effects of anti-cancer drugs are triggered by high oxidative damage to healthy cells due to non-selective targeting (Abu Gazia and El-Magd, 2018; Attia et al., 2020; El-Magd et al., 2017a). In agreement, we found higher hepatic levels of the lipid peroxide MDA and lower activity of antioxidant enzymes (SOD, CAT, GPx) in mice treated with DOX alone. These higher oxidative changes and lower antioxidant capacity were corrected to levels comparable to the normal following treatment with CME. There was also a partial improvement in the CME+DOX combined group. Similarly, CME alone or in combination with anticancer agents exerted notable antioxidant properties in rodents bearing tumors and immunocompromised rats (Badawy et al., 2018; Badawy et al., 2021; Ibrahim et al., 2019). Taken together, CME+DOX co-treatment increased the sensitivity of HL60/RS cells to DOX and reduced DOX side effects. However, further investigations including gain and loss of function experiments in addition to western blot and microarrays are required to explore the actual signaling pathways beyond this effect. It is also important to check whether camel milk exosomes and their cargo can be translated within consumers' cells.

CONCLUSIONS

To the best of our knowledge, this is the first report to demonstrate that CME had a potent inhibitory effect on human leukemic HL60 and HL60/RS cells. CME could also improve the sensitivity of HL60/RS cells (in vitro and in vivo) to DOX through at least in part induction of G0/G1 arrest and apoptosis, and reduction of MDR. They also enhanced antioxidant status and reduced liver damage caused by the tumor and/or DOX. Therefore, CME could be used as adjuvants to DOX during chemotherapy of myeloid leukemia, especially during prolonged exposure to inhibit MDR.

Acknowledgment: This research was funded by the deanship of the scientific research in Northern Border University, Arar city, Saudi Arabia with grant # 7721-MED-2018-3-9-F.

Author contribution: RO, and ME designed the experiments; AB, RO, and ME conducted the experiments; MA, RO, and ME analyzed the data; MA and ME wrote the manuscript, and all authors revised the manuscript.

Declarations: Conflict of interest: Rashad Othman declares that he has no conflict of interest, Abdelnaser Badawy declares that he has no conflict of interest, Mohammed Alruwaili declares that he has no conflict of interest, and Mohammed El-Magd declares that he has no conflict of interest.

Ethical approval: Approval of the present study was obtained by the Ethics Committee of Kafrelsheikh University (license number: KFS127/25).

REFERENCES

1. Abdelwahab, M.A., El-Barbary, A.A., El-Said, K.S., Betiha, M., Elkholy, H.M., Chiellini, E., El-Magd, M.A., 2019. Functionalization of poly(3-hydroxybutyrate) with different thiol compounds inhibits MDM2–p53 interactions in MCF7 cells. Journal of Applied Polymer Science 136, 46924.
2. Abu Gazia, M., El-Magd, M.A., 2018. Ameliorative Effect of Cardamom Aqueous Extract on Doxorubicin-Induced Cardiotoxicity in Rats. Cells, tissues, organs 206, 62-72.
3. Alzahrani, F.A., El-Magd, M.A., Abdelfattah-Hassan, A., Saleh, A.A., Saadeldin, I.M., El-Shetry, E.S., Badawy, A.A., Alkarim, S., 2018. Potential Effect of Exosomes Derived from Cancer Stem Cells and MSCs on Progression of DEN-Induced HCC in Rats. Stem Cells International 2018, 17 https://doi.org/10.1155/2018/8058979.
4. Alzahrani, F.A., Shait Mohammed, M.R., Alkarim, S., Azhar, E.I., El-Magd, M.A., Hawsawi, Y., Abdulaal, W.H., Yusuf, A., Alhatmi, A., Albiheyri, R., Fakhurji, B., Kurdi, B., Madani, T.A., Alguridi, H., Alosaimi, R.S., Khan, M.I., 2021. Untargeted Metabolic Profiling of Extracellular Vesicles of SARS-CoV-2-Infected Patients Shows Presence of Potent Anti-Inflammatory Metabolites. International Journal of Molecular Sciences 22, 10467.
5. Attia, A.M., Khodair, A.I., Gendy, E.A., El-Magd, M.A., Elshaier, Y.A., 2020. New 2-Oxopyridine/2-Thiopyridine Derivatives Tethered to a Benzotriazole with Cytotoxicity on MCF7 Cell Lines and with Antiviral Activities. Letters in Drug Design & Discovery, https://doi.org/10.2174/1570180816666190220123547 17, 124–137.
6. Awad, M.G., Ali, R.A., Abd El-Monem, D.D., El-Magd, M.A., 2020. Graviola leaves extract enhances the anticancer effect of cisplatin on various cancer cell lines. Molecular & Cellular Toxicology. https://doi.org/10.1007/s13273-020-00092-8 16, 385–399.
7. Badawy, A.A., El-Magd, M.A., AlSadrah, S.A., 2018. Therapeutic Effect of Camel Milk and Its Exosomes on MCF7 Cells In Vitro and In Vivo. Integrative Cancer Therapies 7, 1235-1246.
8. Badawy, A.A., Othman, R.Q.A., El-Magd, M.A., 2021. Effect of combined therapy with camel milk-derived exosomes, tamoxifen, and hesperidin on breast cancer. Molecular & Cellular Toxicology, https://doi.org/10.1007/s13273-021-00163-4.
9. Bandyopadhyay, A., Wang, L., Agyin, J., Tang, Y., Lin, S., Yeh, I.T., De, K., Sun, L.-Z., 2010. Doxorubicin in Combination with a Small TGFβ Inhibitor: A Potential Novel Therapy for Metastatic Breast Cancer in Mouse Models. PLOS ONE 5, e10365.
10. Chen, J., Wei, H., Cheng, J., Xie, B., Wang, B., Yi, J., Tian, B., Liu, Z., Wang, F., Zhang, Z., 2018. Characteristics of doxorubicin-selected multidrug-resistant human leukemia HL-60 cells with tolerance to arsenic trioxide and contribution of leukemia stem cells. Oncol Lett 15, 1255-1262.
11. Chen, J., Wei, H., Xie, B., Wang, B., Cheng, J., Cheng, J., 2012. Endoplasmic reticulum stress contributes to arsenic trioxide-induced apoptosis in drug-sensitive and-resistant leukemia cells. Leukemia research 36, 1526-1535.
12. El-kattawy, A.M., Algezawy, O., Alfaifi, M.Y., Noseer, E.A., Hawsawi, Y.M., Alzahrani, O.R., Algarni, A., Kahilo, K.A., El-Magd, M.A., 2021. Therapeutic potential of camel milk exosomes against HepaRG cells with potent apoptotic, anti-inflammatory, and anti-angiogenesis effects for colostrum exosomes. Biomedicine & Pharmacotherapy 143, 112220.
13. El-Magd, M.A., Kahilo, K.A., Nasr, N.E., Kamal, T., Shukry, M., Saleh, A.A., 2016. A potential mechanism associated with lead-induced testicular toxicity in rats. Andrologia 49, e12750.
14. El-Magd, M.A., Khamis, A., Nasr Eldeen, S.K., Ibrahim, W.M., Salama, A.F., 2017a. Trehalose enhances the antitumor potential of methotrexate against mice bearing Ehrlich ascites carcinoma. Biomedicine & Pharmacotherapy 92, 870-878.
15. El-Magd, M.A., Khamis, A., Nasr Eldeen, S.K., Ibrahim, W.M., Salama, A.F., 2017b. Trehalose enhances the antitumor potential of methotrexate against mice bearing Ehrlich ascites carcinoma. Biomed Pharmacother 92, 870-878.
16. Elsayed, S.A., Harrypersad, S., Sahyon, H.A., El-Magd, M.A., Walsby, C.J., 2020. Ruthenium(II)/(III) DMSO-Based Complexes of 2-Aminophenyl Benzimidazole with In Vitro and In Vivo Anticancer Activity. Molecules 25, 4284.
17. Fung, T.K., So, C.W.E., 2013. Overcoming treatment resistance in acute promyelocytic leukemia and beyond. Oncotarget 4, 1128.
18. Gao, F., Dong, W., Yang, W., Liu, J., Zheng, Z., Sun, K., 2015. Expression of P-gp in acute myeloid leukemia and the reversal function of As2O3 on drug resistance. Oncol Lett 9, 177-182.
19. Homayouni-Tabrizi, M., Asoodeh, A., Soltani, M., 2017. Cytotoxic and antioxidant capacity of camel milk peptides: Effects of isolated peptide on superoxide dismutase and catalase gene expression. Journal of food and drug analysis 25, 567-575.
20. Hwang, D., Kim, M., Park, H., Jeong, M.I., Jung, W., Kim, B., 2019. Natural Products and Acute Myeloid Leukemia: A Review Highlighting Mechanisms of Action. Nutrients 11.
21. Ibrahim, H.M., Mohammed-Geba, K., Tawfic, A.A., El-Magd, M.A., 2019. Camel milk exosomes modulate cyclophosphamide-induced oxidative stress and immunotoxicity in rats. Food & Function 10, 7523-7532.
22. Khamis, A.A.A., Ali, E.M.M., El-Moneim, M.A.A., Abd-Alhaseeb, M.M., El-Magd, M.A., Salim, E.I., 2018. Hesperidin, piperine and bee venom synergistically potentiate the anticancer effect of tamoxifen against breast

cancer cells. Biomedicine & Pharmacotherapy 105, 1335-1343.
23. Korashy, H.M., El Gendy, M.A., Alhaider, A.A., El-Kadi, A.O., 2012a. Camel milk modulates the expression of aryl hydrocarbon receptor-regulated genes, Cyp1a1, Nqo1, and Gsta1, in murine hepatoma Hepa 1c1c7 cells. Journal of biomedicine & biotechnology 2012, 782642.
24. Korashy, H.M., Maayah, Z.H., Abd-Allah, A.R., El-Kadi, A.O., Alhaider, A.A., 2012b. Camel milk triggers apoptotic signaling pathways in human hepatoma HepG2 and breast cancer MCF7 cell lines through transcriptional mechanism. Journal of biomedicine & biotechnology 2012, 593195.
25. Magdy, A., Sadaka, E., Hanafy, N., El-Magd, M.A., Allahloubi, N., El Kemary, M., 2020. Green tea ameliorates the side effects of the silver nanoparticles treatment of Ehrlich ascites tumor in mice. Molecular & Cellular Toxicology 16, 271–282.
26. Mahfouz, D.H., EL-Magd, M.A., Mansour, G.H., Abdel Wahab, A.H., Abdelhamid, I.A., Elzayat, E., 2021. Therapeutic potential of snake venom, l-amino oxidase and sorafenib in hepatocellular carcinoma. Molecular and Cellular Toxicology, DOI: 10.1007/s13273-021-00151-8.
27. Mansour, G.H., El-Magd, M.A., Mahfouz, D.H., Abdelhamid, I.A., Mohamed, M.F., Ibrahim, N.S., Hady A. Abdel Wahab, A., Elzayat, E.M., 2021. Bee venom and its active component Melittin synergistically potentiate the anticancer effect of Sorafenib against HepG2 cells. Bioorganic Chemistry. https://doi.org/10.1016/j.bioorg.2021.105329, 105329.
28. Matsumoto, T., Jimi, S., Hara, S., Takamatsu, Y., Suzumiya, J., Tamura, K., 2012. Importance of inducible multidrug resistance 1 expression in HL-60 cells resistant to gemtuzumab ozogamicin. Leukemia & lymphoma 53, 1399-1405.
29. Musgrove, E.A., Sutherland, R.L., 2009. Biological determinants of endocrine resistance in breast cancer. Nature Reviews Cancer 9, 631-643.
30. Renaud, H.J., Klaassen, C.D., Csanaky, I.L., 2016. Calorie Restriction Increases P-Glycoprotein and Decreases Intestinal Absorption of Digoxin in Mice. Drug metabolism and disposition: the biological fate of chemicals 44, 366-369.
31. Schinkel, A.H., Jonker, J.W., 2003. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Advanced Drug Delivery Reviews 55, 3-29.
32. Shakor, A.B., Atia, M., Ismail, I.A., Alshehri, A., El-Refaey, H., Kwiatkowska, K., Sobota, A., 2014. Curcumin induces apoptosis of multidrug-resistant human leukemia HL60 cells by complex pathways leading to ceramide accumulation. Biochimica et biophysica acta 1841, 1672-1682.
33. Shariatikia, M., Behbahani, M., Mohabatkar, H., 2017. Anticancer activity of cow, sheep, goat, mare, donkey and camel milks and their caseins and whey proteins and in silico comparison of the caseins. Molecular biology research communications 6, 57-64.
34. Siveen, K.S., Uddin, S., Mohammad, R.M., 2017. Targeting acute myeloid leukemia stem cell signaling by natural products. Molecular Cancer 16, 13.
35. Sun, Y., Xu, H.-J., Zhao, Y.-X., Wang, L.-Z., Sun, L.-R., Wang, Z., Sun, X.-F., 2013. Crocin Exhibits Antitumor Effects on Human Leukemia HL-60 Cells In Vitro and In Vivo. Evidence-Based Complementary and Alternative Medicine 2013, 690164.
36. Zhang, Y., Nicolau, A., Lima, C.F., Rodrigues, L.R., 2014. Bovine Lactoferrin Induces Cell Cycle Arrest and Inhibits Mtor Signaling in Breast Cancer Cells. Nutrition and Cancer 66, 1371-1385.
37. Zhu, H.-H., Qin, Y.-Z., Huang, X.-J., 2014. Resistance to arsenic therapy in acute promyelocytic leukemia. The New England journal of medicine 370, 1864-1866.