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RESEARCH PAPER

Improved anticancer efficiency of Mitoxantrone by Curcumin loaded PLGA nanoparticles targeted with AS1411 aptamer

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ABSTRACT

Objective(s): Mitoxantrone (MTX) is one of the most commonly used chemotherapeutic agents for treatment of different cancers. However, prolonged treatment with MTX results in unwanted side effects and drug resistant cancer cells. Combination therapies and exploiting of targeted nanoparticles have the potential of improving the efficiency of drug treatment as well as reducing the side effects. Curcumin (CUR) is a biological molecules with anticancer property. In this study, we investigated whether targeted PLGA (Poly Lactic-co-Glycolic Acid)–CUR nanoparticles (NPs) can reinforce the effect of MTX on breast cancer cells.

Materials and Methods: PLGA NPs containing CUR targeted with AS1411 aptamer were prepared by single emulsion evaporation method. Physicochemical properties of NPs were investigated. The cytotoxicity of non-targeted and targeted NPs along with MTX was evaluated on MCF7, 4T1 and L929 cell lines.

Results: The results showed that PLGA-CUR NPs were synthetized with an average encapsulation efficiency of 66% with a mean size of 186±3.2 nm. The drug release of curcumin from these NPs within 72h was about 59% in neutral medium and 90% in acidic medium. Interestingly, the combined treatment with PLGA-CUR-Apt and MTX inhibited the cancer cell's proliferation significantly more than the non-targeted nanoparticles, CUR and MTX-treated group alone.

Conclusion: These results suggest that targeted PLGA-CUR nanoparticles may consider as a potential therapeutic contender in improving the efficacy of MTX in Breast cancer therapy.

Keywords: *AS1411 aptamer, Breast cancer, Curcumin, Mitoxantrone, Polymeric nanoparticles*

How to cite this article

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INTRODUCTION

Breast cancer is one of the most common malignant tumors and the second leading cause of deaths among women in the world. Among usual anti-cancer approaches, chemotherapy remains as the most effective methods [1]. Mitoxantrone (MTX) a type II topoisomerase inhibitor, as an antitumor antibiotic can inhibit DNA synthesis and DNA repair via intercalation between DNA bases causing DNA strand breaks as well as DNA aggregation both in healthy and cancer cells. MTX based chemotherapy was broadly used for the treatment of breast cancer. Anyway, the chemotherapy may yield poor efficacy result because of drug resistance and high toxicity. Curcumin (CUR) is a natural phytochemical compound isolated from dried root and rhizome of Turmeric (Curcuma longa). It has been used for a long time in medicine and responsible for the wide range of pharmacological activity of

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turmeric [2]. It has been documented that CUR exerts anticancer effects through multiple targets such as tumor necrosis factor-alpha (TNFα), NFkB, TrxR, epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) and modulate numerous cell signaling pathways involved in apoptosis, proliferation, angiogenesis as well as inflammatory pathways [3-5]. In vivo and in vitro studies has further highlighted that CUR has ability to prevent the proliferation and survival of HER2-positive breast cancer cells [6-8]. However extremely rapid metabolism, limited water solubility and poor absorption of this nutraceutical agent harshly decrease its bioavailability. Nanotechnology, a vigorous research field, is an alternative way to overcome many barriers for efficient drug delivery to attain better and safer treatment outcomes. It is well established that entrapping of CUR in poly (lactic-co-glycolic acid) (PLGA) nanoparticles as the controlled release polymer could enhance the bioavailability [9-11]. Targeted drug delivery using nanoparticles (NP) is a favorable and most extensively studied approach to improve the selectivity and specificity of different drugs or therapeutic genes for malignant cells [12-14]. Aptamers are a class of artificial single-stranded DNA or RNA oligonucleotide generated by in vitro process that bind to multiple targets with high affinity and specificity [15-17]. This key property of aptamers creates them as an alternative opportunity for targeted carriers to transfer specific cargoes into tumor cells [18]. AS1411, a guanine (G)-rich aptamer with anti-proliferative activity, specifically binds to nucleolin protein that highly expressed on the plasma membrane of cancer cells. The uptake of AS1411 aptamer occurring by macropinocytosis pathways through a nucleolin-dependent mechanism leading to therapeutic agents becoming trapped inside tumor cells [19, 20].

This aptamer has been effectively used as a targeting ligand in multiple nanoparticles [21].

The goal of this study is to elucidate whether PLGA-CUR functionalized with AS1411 aptamer could augment the efficacy of MTX treatment in breast cancer cell lines by targeting nucleolin molecules.

MATERIALS AND METHODS

Materials

PLGA (Mw: 7000–17.000;lactide: glycolide = 50:50), CUR,N-hydroxysulfosuccinimide(NHS),1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), MTT and were prepared from Sigma Aldrich. RPMI 1640 medium, fetal bovine serum (FBS), penicillin–streptomycin and trypsin (0.025%) were bought from Gibco (Darmstadt, Germany). The AS1411 DNA aptamer (5′-GGTGGTGGTGGTTGTGGTGGTGGT GG-3′ amino) was synthesized by Microsynth AG (Balgach, Switzerland). Polyvinyl alcohol (PVA, 87–89% hydrolyzed, average Mw = 88,000–97,000) and all other chemical reagents were commercially available.

CUR-Loaded PLGA nanoparticle preparation

CUR loaded PLGA NPs were prepared by single emulsion evaporation method as described previously [22]. Briefly, 20 mg of PLGA and 0.2 mg CUR were dissolved in 1 mL dichloromethane (DCM). Prepared solution was then added to PVA (5% w/v) as an aqueous phase under sonication on ice (amplitude 80%, 10 min) using a probe sonicator (Fisons Instruments Ltd., Crawley, UK). The prepared emulsion was added to 10 ml of PVA 0.1% dropwise. The reaction process was continued while stirring overnight in order to evaporate the organic solvent. The NPs as final products were obtained by centrifugation (18000 rpm, 20 min) followed by washing to remove extreme surfactant and finally lyophilized to get powder form [23].

Particle size and zeta potential measurement

Particle size and zeta potential of PLGA NPs were analyzed with Malvern NanoZS instrument and DTS software (Zetasizer Nano Z, Malvern Instruments Ltd, Malvern, Worcestershire, UK). For this purpose, 1 mg of the synthesized NPs was suspended in 1ml double-distilled water and sonicated to form a homogeneous suspension. Then, three independent measurements were performed.

Drug loading content (LC %) and encapsulation efficiency (EE %)

To determine the percentage of CUR encapsulated in PLGA NPs, 1 mg of PLGA–CUR NPs freeze-dried powder dissolved in dimethyl sulfoxide (DMSO) and then sonicated. The quantity of CUR was measured by spectrophotometer (λ max: 435 nm). Then a standard curve was plotted with a series of different concentration of CUR. The

encapsulation efficiency EE (%) and drug loading content (LC %) of PLGA NPs loaded with CUR were obtained from following Equations.

$$\text{Drug loading content (\%)} = \frac{\text{Weight of the drug in nanoparticles}}{\text{Weight of the nanoparticles}} \times 100$$

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Weight of the drug in nanoparticles}}{\text{Weight of the feeding drugs}} \times 100$$

In vitro release study

The release of CUR from PLGA NPs was determined using the dialysis membrane technique by measuring the cumulative amount of CUR released from the PLGA NPs over a periodic time intervals. Lyophilized PLGA–CUR NPs (1 mg) is placed into a dialysis bags (MWCO: 10 kDa) and then sealed and immersed into 50 ml release media containing phosphate buffer saline (PBS, pH 7.4) and citrate buffer (0.1 M, pH 5.5) containing 0.1% tween 80 at 37 °C under constant shaking using a magnetic stirrer. At fixed times, small amounts of samples (1 mL) were removed and The same volume of fresh release medium was added back to mimic a physiological sink condition [24].

Conjugation of AS1411 on the surface of nanoparticles

Approximately 1 ml (10 mg/mL) of PLGA–CUR NPs suspension in DNase/RNasee free water incubated with 400 mmol/L of EDC and 100 mmol/L of NHS for 1 hour at room temperature with gentle shaking. The resulting NHS-activated particles were washed with DNase/RNaseefree water to remove any remaining NHS or EDC by means of an ultrafiltration device (15 min, 1500g, MWCO: 30 kDa) (Millipore). The activated PLGA–CUR NPs covalently linked to 100µl of 3' NH2 modified AS1411 Apt (50 µM) at room temperature for 18 h with constant mixing. To remove any free aptamers a centrifugation step was applied at 1500g for 15 min (MWCO: 30 kDa; Millipore) [25].

Cell culture

Human breast adenocarcinoma cell lines MCF-7 (C135), Mouse mammary gland 4T1 (C604) and Mouse fibroblast cell lineL929 (C200) were cultured in RPMI (supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) and kept at 37 °C in a humidified 5% CO2 atmosphere.

Cytotoxicity assay

MCF-7, 4T1 and L929 cells were seeded in 96-well cell culture microplates at a density of 7 × 103 cells/100 µl. After 24 h, cells were treated with a serial concentration of free MTX (0.03-5 µg/ml), CUR (1.25-40 µg/ml), PLGA–CUR NPs and PLGA–CUR-Apt NPs alone or in combination with MTX (0.1 µg/ml) incubated for 48h. At the end of experiment, 10 µl of fresh MTT reagent, made up in medium, (final concentration 0.5 mg/ml) was added into each well and incubated for 4 h. The MTT reagent was then discard and the purple formazan crystals were solubilized in DMSO. The absorbance of the sample was measure at 570/630 nm by a microplate (ELISA) reader (BioTek).

Determination of synergistic activity

Drug combination effects were measured using the following formula:

(CI) = (D)1 /(Dχ)1 + (D)2/(Dχ)2

In this formula, (Dχ)1 and (Dχ)2 indicate concentrations of each drug alone to show χ% effect, and (D)1 and (D)2 represent concentrations of drugs in combination to produce the same outcome. Combination index (CI) values less than 1 represents synergy, a CI value of 1 shows additive and finally a CI value more than 1 indicates antagonism effects [26, 27].

Statistical analysis

The unpaired t-tests and ANOVA was used to determine the statistical differences. The obtained results are expressed as standard errors of means (±SEM). A p values below 0.05 were considered to be statistically significant.

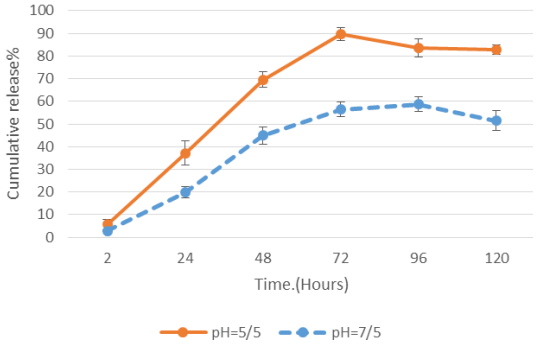

Fig 1. Release profiles of CUR–PLGA nanoparticles in PBS (pH 7.4) and citrate buffer (pH 5.5)

RESULTS

Preparation and characterization of PLGA- CUR NP

PLGA–CUR NPs were formulated using emulsification–solvent evaporation approach. The

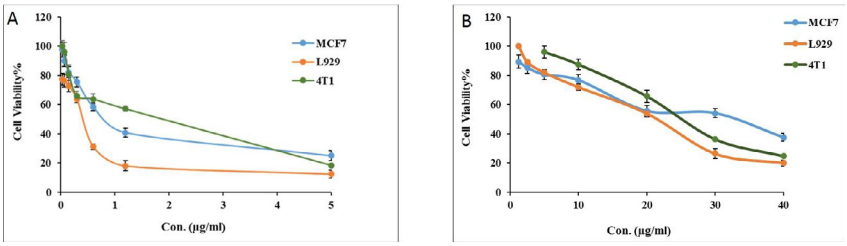

Fig 3. Cytotoxicity of free MTX (A) and CUR (B) on the MCF7, L929 and 4T1 cell lines after 48 h. (error bars represent standard deviation).). Cytotoxicity is given as the percentage of viable cells remaining after treatment. Each point represents the mean ± S.E.M. (n = 3)

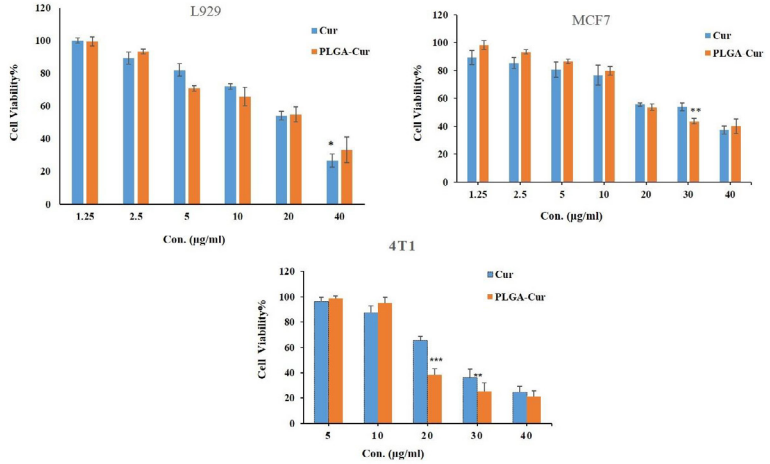

Fig 4. Cytotoxicity of cells treated with free CUR and PLGA-CUR NPs after 48 h exposure determined by MTT assays. Cytotoxicity is given as the percentage of viable cells remaining after treatment. Each point represents the mean ± S.E.M. (n = 3)

particle size of prepared PLGA-CUR NPs was around 186 nm in diameter with a narrow polydispersity index (PDI) of 0.041. The zeta potential was a little anionic and was in the range−13.8±0.75 mV as measured by the dynamic light scattering method. The drug entrapment efficiency was about 66% and the LC was also calculated to be 6%.

In Vitro Curcumin release profile

The in vitro CUR release from PLGA into PBS (pH 7.4) and citrate buffer (pH 5.5) over a period of 100 h is shown in Fig 1. At pH=5.5 an initial burst drug release was occurred in the first 24 hr of incubation and after that release values increased up to 90% f within the following 72 h. At pH 7.4, the release of CUR was about 50% after day 5 and then, the release curve slope lessened to reach a plateau. The results showed that during 100 hours, CUR was released more at pH of 5.5 corresponding to lysosomal pH.

Coupling of AS1411 Apt to PLGA-CUR nanoparticles

The 3'-NH2-modified AS1411 Aptamer (26 bp) was grafted onto the surface of PLGA-CUR using EDC/NHS chemistry to form a targeted delivery system. The Aptamer conjugation was visualized by the agarose gel electrophoresis.

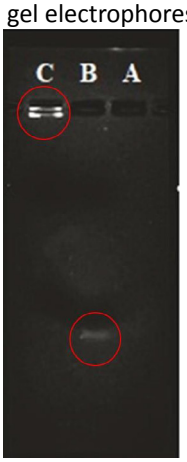

Fig 2. Agarose gel electrophoresis (2.5%). From left to right: lane A: PLGA-CUR NPs; lane B: free AS1411 aptamer; Lane C: PLGA-CUR-Apt NP

As illustrated in Fig 2, the free Aptamer showed a bright band on the gel while PLGA-CUR-Apt did

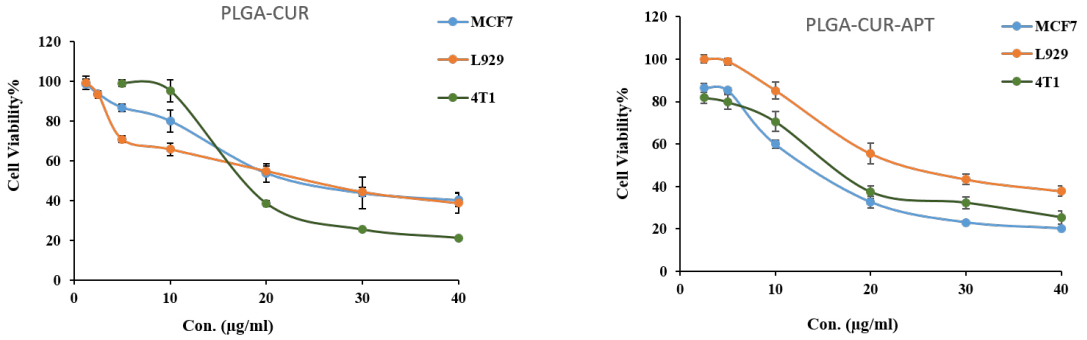

Fig 5. The in vitro cytotoxicity graph of normal L929 cells, MCF7 and 4T1 after incubation with PLGA-CUR and PLGA-CUR-Apt for 48 h determined by MTT assays. Cytotoxicity is given as the percentage of viable cells remaining after treatment. Each point represents the mean ± S.E.M. (n = 3)

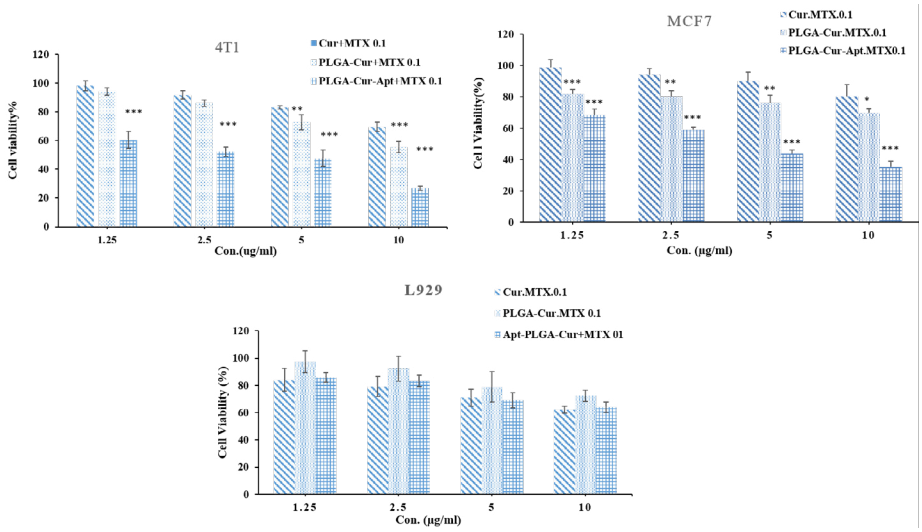

Fig 6. The in vitro cytotoxicity graph of normal L929 cells, MCF7 and 4T1 after incubation with CUR+ MTX (0.1), PLGA-CUR +MTX (0.1) and PLGA-CUR-Apt + MTX (0.1) for 48 h determined by MTT assays. Cytotoxicity is given as the percentage of viable cells remaining after treatment. Each point represents the mean ± S.E.M (n = 3)

not move on the gel. The band observed at the loading site ascertained the successful Aptamer conjugation on to the surface of the PLGA-CUR which support targeted drug delivery.

Cell experiments

The growth inhibitory effects of free MTX, free CUR, targeted/non-targeted PLGA–CUR NPs alone or in combination with MTX were evaluated in three cell lines: L929 cell line (as negative control), MCF7and 4T1 using MTT assay following 48 h exposure. In the first step, the 50% inhibitory concentration (IC50) value of free MTX and free CUR in treated cells were calculated (fig 3 A, B).

Fig 4 illustrated the toxicity effects of CUR and PLGA-CUR NPs against these cells. The PLGA-CUR NPs exhibited toxicity in normal cells (L929) as well as in tumor cells (MCF7).However, in 4T1, PLGA-CUR NPs was more toxic in comparison to free CUR.

Table 1. The amount of IC50 in different treated groups

Cell line	IC 50(µg/ml)				
	MTX	CUR	PLGA- CUR	PLGA- CUR- Apt	PLGA- CUR- Apt +MTX(0.1µg/ml)
MCF7	1.01	32.75	27.77	12.55	2.82
4T1	1.13	23.1	18.72	14.61	0.408
L929	0.32	21.97	21.11	26.75	1.24

The calculated IC50 values for MCF-7 and 4T1 cells treated with PLGA-CUR-Apt NPs were 2.2 and 1.3 fold lower than those for cancer cells treated with non-targeted NPs, respectively (Fig 5).In table 1, the amount of IC50 in treated groups have been compared to each other. Upon in combination treatment with MTX, the cytotoxicity was significantly increased for

PLGA-CUR-Apt+MTX NPs treatment in breast cancer cells. The IC50 values for MCF-7 and 4T1 cells treated with PLGA-CUR-Apt+ MTX were reduced to 2.82 and 0.408 µg/mL compared with 12.55 and 14.61 µg/mL obtained for the same formulation without MTX, respectively (Fig 6).

Combination index

To assess the synergy of CUR with MTX, PLGA-CUR+MTX (0.1) and PLGA- CUR-Apt +MTX, the combination index method was carried out. The calculated CI values in both MCF-7 and 4T1 cells was less 0.9 indicated the synergetic effect of MTX with various CUR compounds, especially targeted nanoparticles.

Table 2. The results of the combination index of the CUR in different formulation with MTX

Compounds	CI / 4T1	CI / MCF7	CI / L929
Co-delivery CUR with MTX	0.941	0.878	1.123
Co-delivery PLGA - CUR with MTX	0.726	0.730	1.961
Co-delivery PLGA-CUR-Apt with MTX	0.281	0.408	1.24

However, in L929 cell line, the CI value were greater than 1, indicating that in combination of MTX with CUR and PLGA-CUR NPs has reduced the therapeutic efficiency of MTX in this cell line (table 2).

DISCUSSION

The combination therapy with natural compounds is a novel aspect in the research and therapy of cancers. Natural compounds reinforce the drug effective concentration while decreasing of adverse effects attributed with conventional chemotherapy [28]. CUR acts as anti-cancer through multiple oncogenic molecules related to the cell proliferation and apoptosis, angiogenesis invasion, transformation as well as chemoresistance [29-33]. In different clinical trials the safety and tolerability of free CUR or in combination with anticancer drugs have been reported [34, 35]. Previous studies have demonstrated that CUR improved treatment efficacy of doxorubicin in tumor due to its efflux inhibitory effect [36, 37]. Combination of CUR and doxorubicin was shown stronger additive effect by reducing Hodgkin lymphoma cell growth compared to doxorubicin alone [38]. Some evidences documented that CUR enhanced the efficacy of methotrexate treatment in different cancers [39, 40]. However, its utility significantly restricted due to its insolubility in water [41]. In different studies, loading of CUR into targeted NPs have been carried out to improve its anticancer efficiency [42, 43]. The present study demonstrated the combination of CUR-loaded targeted PLGA NPs and MTX could be useful for blocking breast cancer cell proliferation. In order to achieve water soluble form of CUR and improve its therapeutic effects in cancer therapy, we developed an effective CUR encapsulated PLGA NPs formulations via single emulsion method. The release patterns of CUR from PLGA NP demonstrated a greater rate at a pH of 5.5 compare to pH 7.4. It was documented that hydrolysis of PLGA augmented by acidic pH [44, 45]. Then, it is expected that at 37˚C and at pH of 5.5, corresponding to lysosomal pH, the structure of PLGA NPs hydrolyzed, therefore, the rate of CUR release increased [46, 47]. Delivery of the therapeutic agent to the right target within the body, increases the therapeutic efficacy and diminishes the side effects related to drug [48, 49]. The targeting of the NPs with AS1411 enhances the uptake by tumor cells which over expresses nucleolin receptors and reduces tumor cell growth [21, 50]. Guo et al have reported the successful delivery of PEG–PLGA NP functionalized with AS141 into C6 glioma cells [51]. In another study, PLGA-lecithin-PEG-AS1411 Apt have been exploited for delivery of Paclitaxel to MCF7 cells [52]. Here, As1411 Apt was successfully attached to the PLGA-CUR nanoparticles by EDC-NHS coupling reaction. Our result confirmed that the AS1411 aptamer played an important role in selective delivery of CUR.

In the present study, we evaluated the cytotoxic effect of MTX and CUR alone or various concentrations of targeted/non targeted PLGA-CUR (1.25-10 µg/ml) in combination with MTX (IC10= 0.1 µg/ml) against MCF7 and 4T1 cells by MTT assay. Cytotoxicity results confirmed that PLGA-CUR NPS was more efficient compare to free CUR against 4T1 cells. It seems that free CUR has more potent activity than PLGA-CUR NPS in MCF7 and L929 after 48 h incubation which may indicate 48 h incubation, was not adequate period for the release of CUR from the PLGA within these cells [53]. Obtained results revealed that the IC50 value for PLGA-CUR-Apt against MCF7 and 4T1 was much lower than PLGA-CUR NPS ($p < 0.05$), whereas the IC50 values for PLGA-CUR-Apt NPs and PLGA-CUR NPS in L929 was similar ($p > 0.05$).

These results are consistent with several studies in which have shown targeted NPs intensified the

cytotoxicity of encapsulated drug into nucleolin-overexpressing tumor cells [54-56].

The results obtained from this study, suggested that AS1411 antinucleolin aptamer –decorated PLGA may successfully enhance the transfer of CUR into breast cancer cells. Furthermore, combination study revealed that PLGA-CUR NPS not only synergized with MTX to enhance the cell death but also reduced the effective concentration of MTX (tenfold lower) in both MCF7 and 4T1 cells that these findings are in agreement with several studies [57, 58].

CONCLUSION

In summary, our data demonstrated that targeted PLGA-CUR enhanced the therapeutic efficacy of MTX by inhibiting proliferation in MCF7 and 4T1. Furthermore, in vitro and in vivo exploring synergy mechanism between PLGA-CUR-Apt and MTX may provide a novel therapeutic approach with low side effects for breast cancer treatment.

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