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RESEARCH ARTICLE

An all-in-one nanoparticle for overcoming drug resistance: doxorubicin and elacridar co-loaded folate receptor targeted PLGA/MSN hybrid nanoparticles

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ABSTRACT

Overexpression of permeability-glycoprotein (P-gp) transporter leads to multidrug resistance (MDR) through cellular exclusion of chemotherapeutics. Co-administration of P-gp inhibitors and chemotherapeutics is a promising approach for improving the efficacy of therapy. Nevertheless, problems in pharmacokinetics, toxicity and solubility limit the application of P-gp inhibitors. Herein, we developed a novel all-in-one hybrid nanoparticle system to overcome MDR in doxorubicin (DOX)-resistant breast cancer. First, folic acid-modified DOX-loaded mesoporous silica nanoparticles (MSNs) were prepared and then loaded into PEGylated poly(lactic-co-glycolic acid) (PLGA) nanoparticles along with a P-gp inhibitor, elacridar. This hybrid nanoparticle system had high drug loading capacity, enabled both passive and active targeting of tumour tissues, and exhibited sequential and pH-triggered release of drugs. *In vitro* and *in vivo* studies in DOX-resistant breast cancer demonstrated the ability of the hybrid nanoparticles to reverse P-gp-mediated drug resistance. The nanoparticles were efficiently taken up by the breast cancer cells and delivered elacridar, *in vitro*. Biodistribution studies demonstrated substantial accumulation of the folate receptor-targeted PLGA/MSN hybrid nanoparticles in tumour-bearing mice. Moreover, deceleration of the tumour growth was remarkable in the animals administered with the DOX and elacridar co-loaded hybrid nanoparticles when compared to those treated with the marketed liposomal DOX (Caelyx®) or its combination with elacridar.

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Hybrid nanoparticles; drug resistance; MDR; P-gp; elacridar; doxorubicin

Introduction

Breast cancer is one of the most common malignant tumours and the leading cause of mortality among women [1]. According to data published by WHO in 2020, there are 2.26 million new cases of breast cancer, surpassing lung cancer with 2.20 million cases, making breast cancer the largest cancer in the world [2]. Among hormonal therapy, radiotherapy and surgery, chemotherapy is an efficient approach to breast cancer treatment and there are several chemotherapeutics using in the clinic including doxorubicin (DOX) [3,4]. Even though patients respond to initial chemotherapy, sensitivity and response rates decline over time due to the occurrence of multidrug resistance (MDR), which can eventually lead to treatment failure.

MDR has a complicated mechanism that can be mediated by a variety of factors including increased activity of drug efflux transporters [5]. The overexpression of permeability-glycoprotein (P-gp), an ATP-binding cassette (ABC) protein, in the cell membrane of tumour cells causes an enhanced cellular efflux of chemotherapeutics, which is one of the main mechanisms underlying the development of MDR [6,7]. Given MDR impairments, strategies involving the use of P-gp inhibitors to block or bypass the drug efflux function and sensitise the P-gp substrate chemotherapeutics against tumours are strongly recommended in the treatment of cancer [8]. Elacridar is a third-generation P-gp inhibitor is relatively more potent and shows higher specificity than first- and second-generation P-gp inhibitors. Elacridar can bind to P-gp non-competitively and acts by inhibiting the ATP hydrolysis by modulating the ATPase activity [9]. Combination therapy of elacridar with a P-gp substrate chemotherapeutics such as DOX holds big potential to improve the overall outcome of the therapy. However, unfavourable pharmacokinetic interactions of P-gp inhibitors with co-administered chemotherapeutics, their inherent toxicity and low water solubility limit their usage in the clinic [10]. To overcome these problems, nanoparticle drug delivery systems come to mind as one of the first options.

Although nanoparticulate drug delivery systems have some drawbacks such as scale-up problems, regulatory challenges and high development costs, nanoparticles have unique advantages. They can be used as drug delivery vehicles to improve the permeability, stability, bioavailability and solubility of the formulated drugs [11]. Moreover, nanoparticles can be passively and actively targeted for the delivery of the drugs to the tumour site [12,13]. The main mechanism of passive targeting mainly relies on the

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size of the nanoparticles and the enhanced permeability and retention (EPR) effect in tumour tissue [14]. Nonetheless, surface modification of nanoparticles with hydrophilic polymers such as polyethylene glycol (PEG) is vital for increasing blood circulation time and improving the overall effect of passive targeting [15]. Although DOX is effectively used in many cancer treatments, side effects such as cardiotoxicity, myelosuppression, vomiting and alopecia limit its usage. Caelyx®, a PEGylated liposomal DOX formulation, provides similar overall survival to DOX in breast cancer treatment with an improved safety profile [16]. Active targeting of nanoparticles is mainly based on the overexpression of various receptors by cells in tumour tissue and using their ligands in the modification of nanoparticles [17,18]. Folate receptors are overexpressed in breast cancer and folic acid modification of nanoparticles can be used for this purpose [19,20].

Although several materials are used to obtain nanoparticles such as polymeric nanoparticles, liposomes, dendrimers or inorganic nanoparticles, none of them are fully superior to each other [21–23]. While each provides a variety of unique advantages, it may also come with some drawbacks. In this point, gathering different types of nanocarriers and obtaining hybrid nanoparticles can not only resolve drawbacks but also gather advantages of both systems [24–28]. Moreover, hybrid nanoparticles can offer some unique advantages such as obtaining sequential release, improving drug loading and inhibiting premature release [29].

In our previous study, we obtained folic acid decorated DOX-loaded mesoporous silica nanoparticles (MSNs) and evaluated their efficacy in breast cancer cells [30]. MSNs offer a wide range of advantages including the possibility of easy surface modification, high colloidal stability, great drug loading capacity (LC), good biocompatibility, tuneable pore diameter and particle size [31,32]. Our results demonstrate that folic acid conjugated DOX-loaded MSNs show pH-dependent drug release and promising cellular uptake and cytotoxic effect in ZR-75-1 and T47-D breast cancer cell lines [30]. In another study of our group, we prepared transferrin receptor targeted elacridar and paclitaxel co-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles and evaluated their efficacy in MDR acquired EMT6/AR1 breast cancer cell line [33]. PLGA nanoparticles are one of the most effective biodegradable polymeric nanoparticle systems due to their controlled/sustained release properties, excellent biocompatibility and low toxicity [34]. The obtained results show that elacridar and the chemotherapeutic combination can be a promising approach to overcoming MDR in breast cancer [33].

Based on these results, in the current study, we aimed to develop a novel all-in-one hybrid nanoparticle drug delivery system to overcome MDR in DOX-resistant breast cancer. In the scope of the study, folic acid decorated DOX-loaded MSNs were loaded to PEGylated PLGA nanoparticles along with the elacridar. In this way, PEGylated PLGA nanoparticles can passively accumulate in tumour tissue and first release the elacridar in the tumour microenvironment. After that, actively targeted DOX-loaded MSNs internalise via folate mediated endocytosis to the P-gp pump disabled breast cancer cells. Lastly, pH-dependent DOX release can occur in acidic media of tumours due to hydrogen bonding between DOX and silica. Obtained hybrid nanoparticle system is characterised by size, morphology and drug release analyses. Their efficacy was evaluated by DOX resistant EMT6/AR1 cell line by *in vitro* cytotoxicity, cellular uptake studies and also *in vivo* breast cancer tumour model.

Materials and methods

Materials

N-cetyltrimethylammonium bromide (CTAB), tetraethylorthosilicate (TEOS), poly(ethylene glycol) methyl ether-block-poly(lactide-co-glycolide) (PEG-PLGA), elacridar, folic acid, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), poly(vinyl alcohol) (PVA), N-hydroxysuccinimide (NHS) and (3-aminopropyl)triethoxysilane (APTS) were obtained from Sigma Aldrich (St. Louis, MO). Foetal bovine serum (FBS), methylthiazolyldiphenyl-tetrazolium bromide (MTT), L-glutamine, penicillin–streptomycin solutions and RPMI 1640 cell medium were obtained from Lonza (Basel, Switzerland). Other than the cell culture consumables, all the reagents used were analytical or reagent grade.

Methods

Preparation of PLGA/MSN hybrid nanoparticles

Briefly to produce PLGA/MSN hybrid nanoparticles, first MSNs were synthesised, folic acid was conjugated and DOX was loaded to obtained MSNs. After that, MSNs loaded to PEGylated PLGA NPs along with elacridar (Figure 1(A,B)).

MSN synthesis. A previously optimised method was used for the synthesis of MSN [35]. Seventy-five milligrams CTAB was dissolved in 30 mL distilled water under a magnetic stirrer at 45 °C and 300 rpm to produce a soft template of nanoparticles. Two hundred and seventy-five microlitres NH_4OH solution was added to this solution to arrange the pH of the system. After that 125 µL TEOS was added all at once as silica precursor. The obtained transparent solution was collected via Amicon® Ultra centrifugal filters. Obtained nanoparticles were transferred to 1% acidic methanol solution at 60 °C for 4 h to remove the CTAB soft template. Lastly, nanoparticles were centrifuged and washed with methanol several times to obtain MSNs.

Folic acid conjugation to MSN. For active targeting of nanoparticles, folic acid was conjugated to MSNs, and the previously characterised nanoparticles' method was used for the folic acid conjugation method [30]. Twenty milligrams MSN was added to 4 mL ethanol solution. Fifty microlitres APTES was added to this solution and stirred overnight for amine functionalisation of MSN. At the same time, 30 mg folic acid was dissolved in a 2.7 mL dimethylformamide/dimethyl sulphoxide (3:1 v/v) solution. 9.3 mg EDC and 7.7 mg NHS were added to this mixture and stirred overnight in dark condition for activation of folic acid. After that, activated folic acid and amine-functionalised MSN solutions were combined and the obtained mixture was stirred overnight in dark conditions for folic acid conjugation to MSN. Lastly, obtained nanoparticles were centrifuged and washed with water and ethanol, and folic acid conjugated MSN was obtained and named as FA-MSN.

Doxorubicin loading to FA-MSN. Twenty millilitres DOX solution (0.5 mg/mL) in phosphate-buffered solution (PBS) was prepared and 20 mg FA-MSN was added to this solution. The mixture was stirred at dark room condition for DOX loading to FA-MSN. Obtained nanoparticles were centrifuged and washed with PBS. Doxorubicin-loaded FA-MSN named as DOX-FA-MSN.

Preparation of elacridar and DOX-FA-MSN-loaded PLGA hybrid nanoparticles (ELC-DOX-HyNP). To obtain the final formulation, DOX-FA-MSN was loaded to PLGA-PEG nanoparticles along with elacridar. Briefly, 50 mg PLGA-PEG and elacridar were dissolved in 1 mL dichloromethane (DCM). 7.5 mg DOX-FA-MSN was dispersed in DCM mixture. Two hundred microlitres distilled water was added to the mixture and sonicated for 30 s (Bandelin SONOPULS HD 2200, Berlin, Germany). After that obtained pre-emulsion was sonicated for 1 min after adding 4 mL 1% PVA solution. Finally,

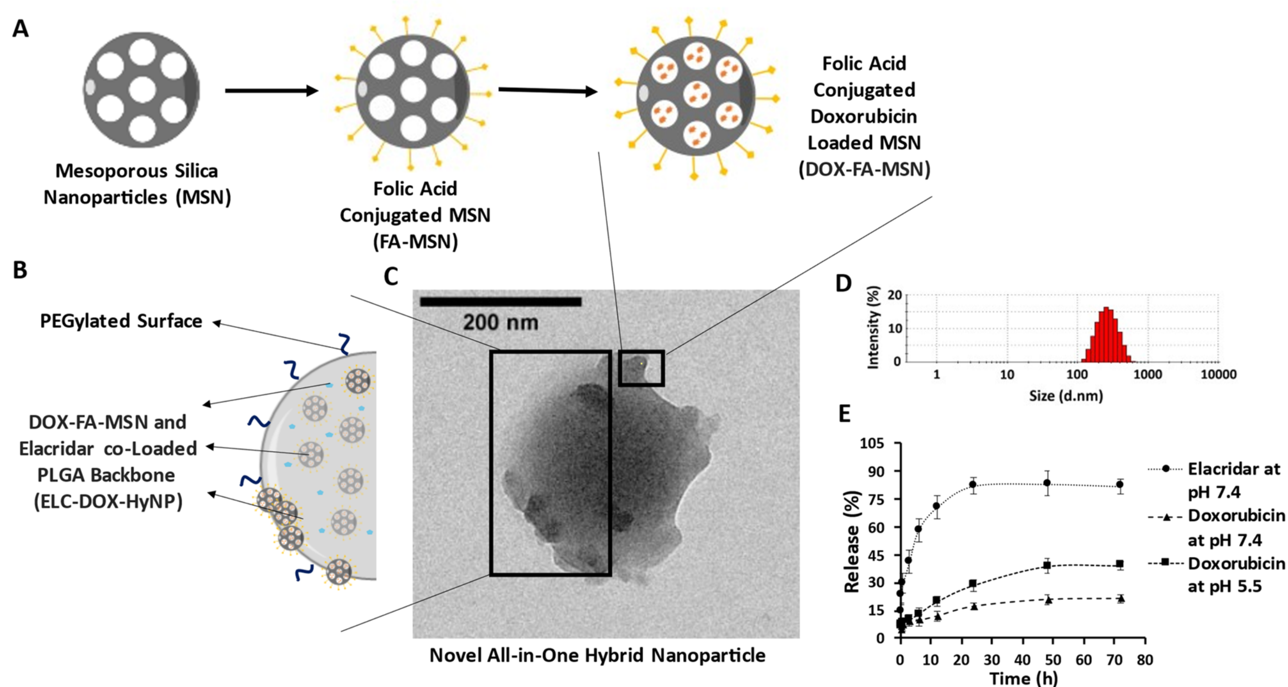

Figure 1. Schematic representation of DOX-FA-MSN preparation. First, MSN was obtained and then folic acid was conjugated to obtain FA-MSN. Second, doxorubicin was loaded to obtained nanoparticle (A). To obtain the final all-in-one nanoparticulate system (ELC-DOX-HyNP), DOX-FA-MSN was co-loaded with elacridar into PEGylate PLGA nanoparticles (B). TEM image (C) and DLS result (D) of the ELC-DOX-HyNP. Doxorubicin and elacridar release from ELC-DOX-HyNP (E).

Table 1. HPLC system condition for quantification of doxorubicin and elacridar.

Condition	Specification
Mobile phase	Acetonitrile:water (38:62) pH: 2.6
Column	C 18 (250 m × 4.6 mm, 5 µm)
Injection volume	50 µL
Flow rate	0.5 mL/min
Column heat	30 °C
Detector	Excitation: 475 nm; emission 555 nm (DOX) Excitation: 265 nm; emission: 485 nm (ELC)

the obtained system was poured into 20 mL 0.3% PVA solution and mixed overnight to evaporate the DCM. Obtained nanoparticles were centrifuged and washed several times. Obtained elacridar and DOX-FA-MSN co-loaded PLGA-PEG hybrid nanoparticles were named as ELC-DOX-HyNP. Only DOX-FA-MSN-loaded PLGA-PEG hybrid nanoparticles were named as DOX-HyNP.

Characterisation of nanoparticles

Average particle size and morphology. The average particle size of nanoparticles was measured by Zetasizer (Malvern Nano ZS, Malvern Instruments, Malvern, UK). Average particle size measurements were performed in ultrapure water at 25 °C and 173° scattering angle.

The morphology of nanoparticles was analysed with transmission electron microscopy (TEM) (FEI Tecnai G2 Sprit Biotwin, Hillsboro, OR) analysis. A drop of nanoparticle suspension was dropped onto a microscope grid and analysed after drying.

Drug loading and drug release. Doxorubicin and elacridar quantifications were made by a fluorescent detector equipped high-performance liquid chromatography (HPLC) system (Shimadzu Prominence Series, Kyoto, Japan). HPLC system condition is given in Table 1.

Drug loading efficiency (LE) and drug LC of nanoparticles were calculated with given HPLC method using the following equation:

$$(\%)\,\text{LE} = \frac{\text{initial amount of drug} - \text{amount of drug in supernatant}}{\text{initial amount of drug}} \times 100$$

$$(\%)\,\text{LC} = \frac{\text{initial amount of drug} - \text{amount of drug in supernatant}}{\text{total weight of nanoparticles}} \times 100$$

The drug release profile of DOX from nanoparticles was analysed in pH 5.5 and 7.4 PBS buffer. Nanoparticles were dispersed in medium containing tubes and maintained at 37 °C while shaking at 100 rpm speed on a reciprocal water bath. Several samples were taken from the tubes at predetermined time points during 72 h and centrifuged. Nanoparticle precipitates were redispersed with the same amount of fresh medium and added to tubes. The obtained supernatant samples were analysed by HPLC.

Cell culture analysis

EMT6/AR1 DOX resistant breast cancer cell line was obtained from the European Collection of Authenticated Cell Cultures (ECACCs) and cultured in RPMI 1640 supplemented with 1% non-essential amino acids (NEAAs) + 1.0 µg/mL DOX + 10% FBS + 1% penicillin–streptomycin and incubated at 37 °C and 5% CO_2 humidified atmosphere.

Cytotoxicity. First, the cytotoxicity of DOX, elacridar and their combination solution was evaluated by MTT assay. Briefly, cells were seeded to 96-well plate and incubated overnight at 37 °C and 5% CO_2 humidified atmosphere. Solutions were applied to the cells with variable concentrations and incubated for 72 h. After the incubation period, 25 µL MTT solution (5 mg/mL) was added

to wells and incubated for 4 h. The cells and formed formazan crystals were dissolved with 25 µL SDS-DMF solution through overnight incubation. Lastly, optical density was measured at 570 nm by a microplate reader. For cytotoxicity analysis of nanoparticles, similar analyses were conducted after the determination of DOX and elacridar loading amount to the nanoparticles and MTT results of their solutions. The same amount of nanoparticles without ELC and DOX was applied as blank nanoparticle groups. The same amount of DOX containing Caelyx® (marketed liposomal DOX formulation) was also applied to the cells.

Cellular uptake. Cellular uptake of nanoparticles was evaluated directly by flow cytometry (FACS Aria II, Becton Dickinson, Franklin Lakes, NJ) and fluorescence microscopy (Olympus America Inc., Center Valley, PA) with the help of the self-fluorescent property of DOX. The median fluorescence intensities (MFIs) of the cells were measured by flow cytometry after formulation was applied to the cells and incubated for 1 h, 4 h and 8 h. In the fluorescence microscopy studies, cytospin preparations were obtained after 8 h incubation period and fixed with 4% paraformaldehyde. Cells were counterstained with DAPI before fluorescence microscopy analysis. The obtained images were processed using the ImageJ software (Bethesda, MD).

Animal studies

Animal studies were performed on 6–8 weeks, 22–25 g female BALB/C mice in accordance with the ethical rules for protection of animals and upon approval by Hacettepe University Animal Experiments Ethical Committee with the approval number 2020/06-05. The animals were maintained under constant room temperature, 50% humidity and filtered air. Mice were allowed free access to food and water.

Breast cancer model. EMT6/AR1 cells were cultured as previously described and prepared for animal study. 2×10^5 cells were subcutaneously implemented in the right flank of mice. Tumour size was checked regularly and when the tumour size reached 5–6 mm, mice were randomly divided into groups and the formulations were injected via i.v. route.

Antitumor efficacy of formulations. Sixty-four mice were divided into eight groups ($n = 8$) as control (PBS), DOX solution, DOX and elacridar combination solution (DOX + ELC), DOX and elacridar unloaded hybrid nanoparticle (blank HyNP), marketed liposomal DOX formulation (Caelyx®), Caelyx® and elacridar combination solution (Caelyx® + ELC), only DOX-MSN-FA-loaded hybrid nanoparticle (DOX-HyNP), elacridar and DOX-MSN-FA-loaded hybrid nanoparticle (ELC-DOX-HyNP). The formulations were i.v. administered every four days at a drug concentration of 5 mg/kg DOX and 5.18 mg/kg elacridar [36]. Mice were weighed, and tumour growth was measured by using a calliper on every formulation administration day and 4 days after from last administration day.

Biodistribution study of hybrid nanoparticles. Biodistribution of hybrid nanoparticles was performed by the i.v. injection of cardiogreen dye-loaded hybrid nanoparticles. For this, cardiogreen was loaded to MSN-FA instead of DOX [37] and then loaded to hybrid nanoparticles along with elacridar. The dye solution was used as a control group ($n = 3$). Mice were monitored at 2, 6 and 12 h after injection using an *in vivo* imaging system (Newton 7.0, Vilber, Collégien, France) under anaesthesia. Mice were sacrificed and organs were dissected at the 12th hour and photographs of organs were taken and the mean fluorescence intensity (MFI) of cardiogreen was calculated.

Statistical analysis

Experiments were performed at least in triplicate with results present as mean ± standard deviation (SD). A one-way ANOVA test was used for multiple comparisons using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA). A value of $p < .05$ was considered statistically significant.

Results and discussion

Physicochemical characterisation of nanoparticles

In our previous studies, we characterised MSN and MSN-FA nanoparticles and results showed that the size of the obtained MSN was about 50 nm with a spherical shape and ready for drug loading. Folic acid conjugation was slightly increased to size around 60 nm and this change was attributed to the presence of folic acid on the surface of the MSNs. Folic acid conjugation was also verified by FTIR in these studies [30,35]. In the current study, obtained DOX-FA-MSN was loaded to PEGylated PLGA nanoparticles along with P-gp inhibitor elacridar. DLS results show that the average particle size of the obtained ELC-DOX-HyNP was 259.1 ± 17.8 nm (Figure 1(D)). Results of the TEM analysis verified the size of the nanoparticles and more importantly, TEM results show that DOX@MSN-FA was successfully incorporated into PLGA nanoparticles (Figure 1(C)). Generally, PLGA nanoparticles have a smooth surface. In the current TEM figure (Figure 1(C)), there are several bulges on the surface. This roughness should be part of the MSN because the size of these is compatible with MSN size.

The LE of DOX to FA-MSN was found as 70.8 ± 5.8% and drug loading was 26 ± 3.1%. The LE of elacridar to ELC-DOX-HyNP was found as 75.1 ± 6.9%. Drug release studies of DOX were conducted at pH 5.5 and 7.4 due to the acidic environment of tumour tissue. In healthy conditions, the pH of the blood and extracellular matrix are consistently at around pH 7.4, whereas the tumour microenvironment has a slightly acidic pH [38]. The pH of the tumour tissues decreases between 6.0 and 7.0 due to high glycolysis rates and high CO_2 levels. The pH value of organelles such as endosomes and lysosomes in tumour tissue becomes more acidic and reduced to below pH 5.5 [39]. pH-dependent drug release of DOX from MSNs can be obtained due to hydrogen bonding between the silica surface and DOX. The hydrogen bond is pH sensitive, and it dissociates in acidic media because of bond energy is weakened by the lower pH [40,41]. As a result, DOX stays in nanoparticles in neutral pH with the help of hydrogen bonding and can freely release from MSN in acidic media. In our previous study, results of the drug release studies show that pH-dependent DOX release from FA-MSN was observed and the release rate and amount increased with the reducing in pH. At the end of 24 h, although less than 20% DOX release from DOX-FA-MSN was observed at pH 7.4, this value increased above 40% at pH 5.5 [30]. In the current study, results show that DOX release from ELC-DOX-HyNP was delayed when compared with DOX-FA-MSN due to the time needed for DOX leakage from PLGA and PLGA degradation time. Doxorubicin release from DOX-FA-MSN reached a plateau after 24 h [30]. Meanwhile, DOX release from ELC-DOX-HyNP reached a plateau after 48 h (Figure 1(E)). Elacridar

cumulative release demonstrated a biphasic pattern of fast initial release within the first 12 h followed by a further slow and continuous release (Figure 1(E)), which is typical of PLGA-based NPs [10]. More important data for the drug release study, sequential release was observed between elacridar and DOX (Figure 1(E)). Almost 75% of elacridar was released in the 12th hour. Meanwhile, only 15% of DOX was released in this time point. Obtaining sequential release is one of the superior effects of hybrid nanoparticles [42]. In this way, elacridar is released first in the tumour environment, blocks the P-gp pump and strengthens the DOX sensitivity to cancer cells. Later, DOX-FA-MSN is internalised by cells via active targeting, and DOX is released due to a decrease in pH without being pumped out from the cells.

Cell culture studies

Cytotoxic effect of HyNP against EMT6/AR1 cell line

In cell culture studies, it was aimed to determine the optimum DOX–elacridar ratio as a beginning step. For this purpose, the cytotoxicity of DOX solution against the EMT6/AR1 cell line was investigated first. Doxorubicin solutions in several concentrations among 200–0.19 µM were applied to cells and incubated for 72 h. Results show that even at the highest concentration, cell viability was around 85% which proves the DOX resistance to the cells. Overexpression of P-gp in EMT6/AR1 cells has been shown to play a major role in the efflux of chemotherapeutics [43–45]. After that several elacridar solution was applied to the cells among 180–40 nM concentrations for the determination of non-toxic elacridar dose (cell viability >80%). This value was calculated as 100 nM and five different DOX concentrations among 50–0.05 µM were applied to cells with non-toxic elacridar dose (100 nM). According to the results, elacridar dramatically sensitised the DOX against the EMT6/AR1 cell line. Although higher than 85% cell viability was observed at 200 µM bolus DOX concentration, complete cell death was observed even at 12.5 µM DOX solution as a synergistic effect when combined with 100 nM elacridar. A similar result was observed in our previous study with paclitaxel and elacridar combination nanoparticle formulation based on transferrin receptor targeted PLGA nanoparticles [33]. In accordance with these results, 3.125, 0.8 and 0.2 µM DOX and 100 nM elacridar-loaded ELC-DOX-HyNP formulations were prepared and applied to the cells. Moreover, the effect of the elacridar encapsulation to HyNP was also investigated and the same amount of elacridar solution was applied with DOX-HyNP. Additionally, the same amount of DOX-loaded Caelyx® was applied to the cells. Results show that the Caelyx® formulation did not have any impact on DOX resistant cell line and its cytotoxicity was not statistically significant compared with the DOX solution. This result correlated with although Caelyx® has a better cardiotoxicity profile in comparison with DOX solution, it has a non-inferior effect instead of a superior effect in breast cancer treatment [16]. HyNPs caused the most cell death among all formulations. Besides, elacridar encapsulation to HyNP formulation provided a higher cytotoxic effect statistically due to probably better cellular internalisation and controlled release of elacridar with a nanoparticulate system (Figure 2).

Cellular uptake of HyNP in EMT6/AR1 cell line

Fluorescence microscope and flow cytometry studies were performed to evaluate the cellular uptake of formulations and analyse the impact of elacridar within the scope of cell culture studies. First, flow cytometry analyses were conducted and time-dependent intracellular delivery of the formulations was evaluated according to the normalised median intensity (nMFI) value of DOX. Doxorubicin (DOX) solution, DOX-FA-MSN and hybrid nanoparticle formulations were applied to the cells. Hybrid nanoparticle formulations were evaluated in three different types as DOX-HyNP, DOX-HyNP + ELC and ELC-DOX-HyNP. In this way, besides the effectiveness of elacridar, the effect of loading into the nanoparticle was also investigated. At the first time point (1 h), the cellular uptake of all formulations was found very similar. nMFI values for elacridar-contained

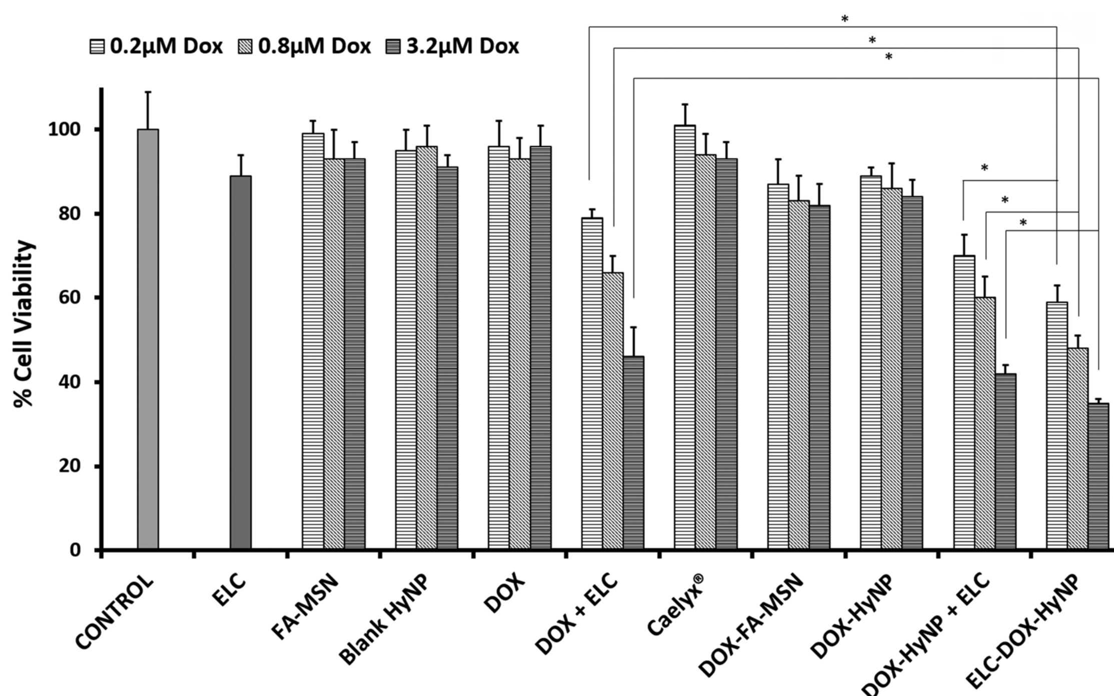

Figure 2. Cytotoxic effect of formulations after 72 h incubation time. Elacridar containing groups contain 100 nM elacridar. A value of $p < .05$ was considered statistically significant and represented by * in the figure.

formulations have become significantly higher than all other formulations at the 4 h time point. After this time point, although nMFI values of non-elacridar containing groups showed a decline, elacridar containing groups continued to increase. These results show that elacridar successfully inhibits the P-gp pump and sensitises the cells to DOX. Moreover, the highest cellular uptake was found in the ELC-DOX-HyNP group (Figure 3(A)). Results of fluorescence microscopy studies were in correlation with flow cytometry results and ELC-DOX-HyNP showed better cellular internalisation than DOX (Figure 3(B)).

Cellular uptake studies revealed that DOX cellular accumulation improved significantly in the presence of elacridar. This suggests that the increased DOX accumulation caused by elacridar-mediated P-gp inhibition may have contributed to the increased cytotoxicity observed with the DOX–elacridar combination. Similar improved cellular accumulation and cytotoxicity of chemotherapeutics in the presence of other P-gp inhibitors such as tariquidar, cyclosporine A and verapamil have been reported previously [46–49].

There are also elacridar and DOX combination studies with different nanoparticulate systems in the literature. Wen et al. prepared nano-gold micelles co-loaded with DOX and elacridar with a spherical shape, uniform particle size distribution and good LE. They concluded that the obtained system significantly improved cell apoptosis, cellular uptake and cytotoxicity of DOX [50]. In another study, Singh and Lamprecht developed DOX and elacridar or verapamil-loaded non-ionic, anionic and cationic surfactant-based nanoparticles and investigated effect of nanocarrier surface charge and formulation parameters for a hydrophilic (verapamil) and lipophilic (elacridar) MDR inhibitor on their ability to reverse drug resistance in ovarian cancer cell lines. Their results show that cationic nanoparticles resulted in higher sensitisation of drug resistant and in comparison to the first generation inhibitor, verapamil hydrochloride based nanoparticles, the third generation inhibitor, elacridar, showed higher encapsulation efficiency, sustained release profiles and higher efficacy in drug accumulation and cytotoxicity experiments [10]. Wong et al. also obtained a DOX and elacridar combination nanoparticulate system with a polymer–lipid hybrid nanoparticle (PLN) system. Anticancer activities of the system were evaluated in a human MDR breast cancer cell line (MDA435/LCC6/MDR1) using trypan blue exclusion and clonogenic assays. Also, cellular uptake and drug distribution within the cells were determined by fluorometry and fluorescence microscopy. They concluded that elacridar and DOX co-loaded PLN system resulted in the highest acute cytotoxicity long-term suppression of cancer cell proliferation, and uptake of DOX by P-gp-overexpressing human breast cancer cells [51]. However, the efficacy and impact of the elacridar were not evaluated with *in vivo* analysis in these studies.

Animal studies

The antitumoural efficacy of formulations was studied in mice by a breast cancer model developed with DOX resistant EMT6/AR1 breast cancer cell line. The antitumor effect of formulations was compared with each other by measuring tumour volume every four days during 24 days. DOX + ELC solution group showed minimum tumour growth at the beginning days and statistically better performance than the ELC-DOX-HyNP group on day 4. Although the DOX-ELC group also showed better performance than the ELC-DOX-HyNP group on days 8 and 12, statistical significance was not determined on these days. Moreover, the ELC-DOX-HyNP group showed a very similar effect compared with the DOX-ELC group on day 16, a better effect on day 20 without statistical significance, and statistically significant lower tumour growth at the end of the therapy. The cause of this delayed action compared with the solution group is probably the slow and controlled release of DOX from nanoparticles. At the end of the treatment period, all of the elacridar-containing groups had a better outcome than any non-elacridar containing group. Additionally, Caelyx® showed similar performance with DOX solution (Figure 4(A)). These results also correlated with a similar effect of Caelyx® when compared with DOX solution at breast cancer treatment [16]. Tumour morphology studies showed that tumour sizes in the non-elacridar contained groups were almost as large as those in the PBS treated group, and the tumour sizes in the ELC-DOX-HyNP group were smaller than all other treatment groups (Figure 4(B)). These results signified that the hybrid nanoparticle mediated combined treatment was superior to solution groups. The possible reason for the superior effect may be attributed to both passive

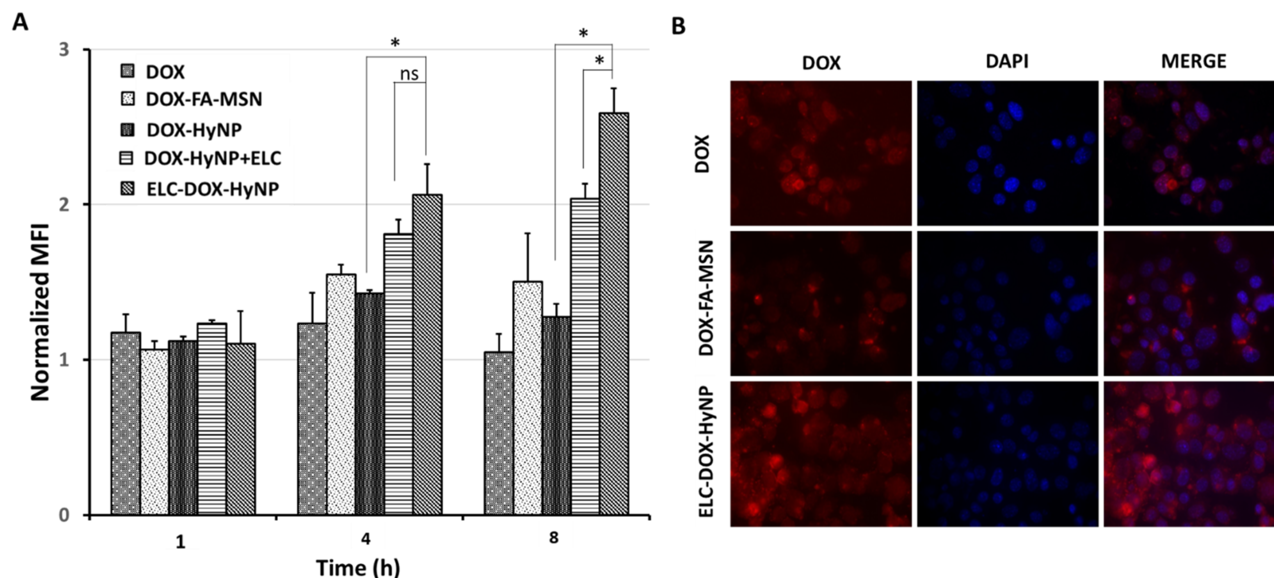

Figure 3. nMFI values of formulation in flow cytometry analysis after 1 h, 4 h and 8 h incubation periods (A), fluorescence microscopy images of formulations after 8 h incubation period (B). A value of $p < .05$ was considered statistically significant and represented by * in the figure, ns: statistically not significant.

and active strategies. Elacridar and DOX-FA-MSN inside the PLGA nanoparticles were able to accumulate in the tumour microenvironment with the help of the EPR effect. After the degradation of PLGA nanoparticles in the microenvironment, elacridar was released first and blocked the P-gp pump and DOX-FA-MSN nanoparticles were able to internalise into tumour cells thanks to folic acid modification and active targeting strategy. As a result, DOX was able to highly accumulate within the tumour cells and showed a better cytotoxic effect.

Biodistribution studies of hybrid nanoparticles were conducted in tumour bearing mice with the help of a fluorescent dye, cardiogreen. After applying the formulations, mice were sacrificed at a predetermined time, and internal organs were analysed. *Ex vivo* images were captured using Vilber Newton 7.0 *in vivo* imaging system and cardiogreen dye in internal organs and tumour tissue were quantified and compared within the HyNP treatment group. The MFI of cardiogreen in the GI segments was analysed using the Fiji® software program. Captured images show that the dye solution was mainly located in the liver and kidneys and most importantly almost none in the tumour. However, dye- and elacridar-loaded HyNPs located in the tumour were almost eight times higher and five times lower in the liver when compared with the dye solution (Figure 4(C,D)).

Body weights of mice were measured every four days in the study. Results show that the change in body weights of mice treated with ELC-DOX-HyNP was similar to control groups with almost a 20% increment. The least increment in body weight was detected in Caelyx® + ELC and DOX + ELC groups with 10% and almost none, respectively (Figure 5(A)). Besides, these two groups were the second and third most effective treatment groups in the antitumor efficacy study (Figure 4(A)). Moreover, the survival rate of the animals at the end of the treatment was found to be 100% only in the ELC-DOX-HyNP group (Figure 5(B)). The biosafety of a formulation is the major problem in the treatment. Although DOX and elacridar showed good antitumoural activity, their effect on the body weight and survival rate showed that they were not well tolerated. These results indicate that safer and more effective drug delivery can be achieved with the obtained novel all-in-one hybrid nanoparticle system.

To the best of our knowledge, two *in vivo* studies were conducted on DOX and elacridar co-delivery with a nanoparticulate system. Chen et al. developed a PLGA nanoparticle system for co-delivery of DOX and elacridar to target both liver cancer stem cells and liver cancer cells. Their drug delivery system is based on standard nanoprecipitation method using TPGS as an emulsifier agent and its potential synergistic effect with DOX. Although their

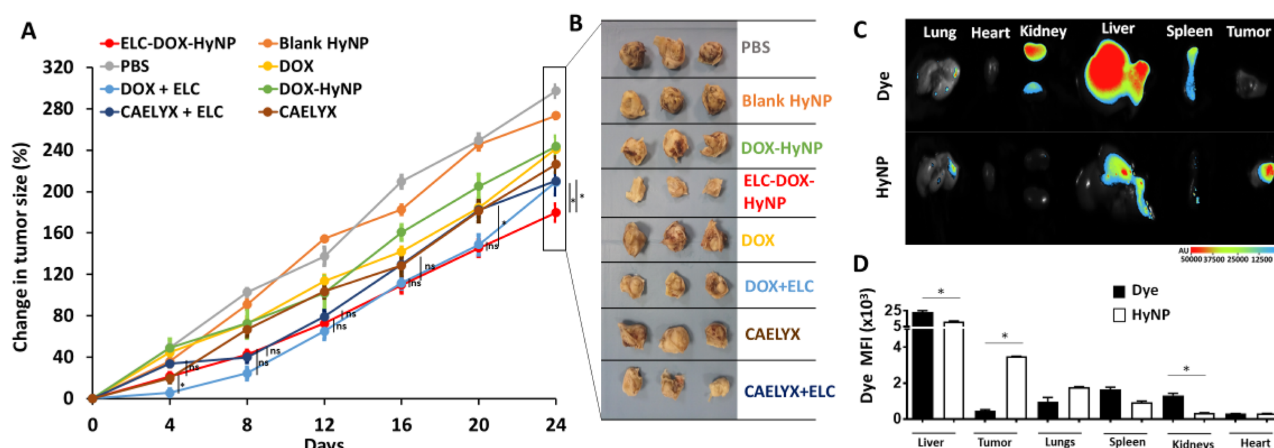

Figure 4. Change in tumour size during the treatment (A) and real image of tumours (three tumours were selected as representative in each group due to three animals alive in a group at the end of treatment) (B). Biodistribution of dye solution and elacridar-loaded hybrid nanoparticle formulation in tumour bearing mice (C). Fluorescence intensity of dye in organs (D). *Statistically significant ($p < .05$), ns: statistically not significant, statistical significances were given for between DOX + ELC vs. ELC-DOX-HyNP and caelyx + ELC vs. ELC-DOX-HyNP groups.

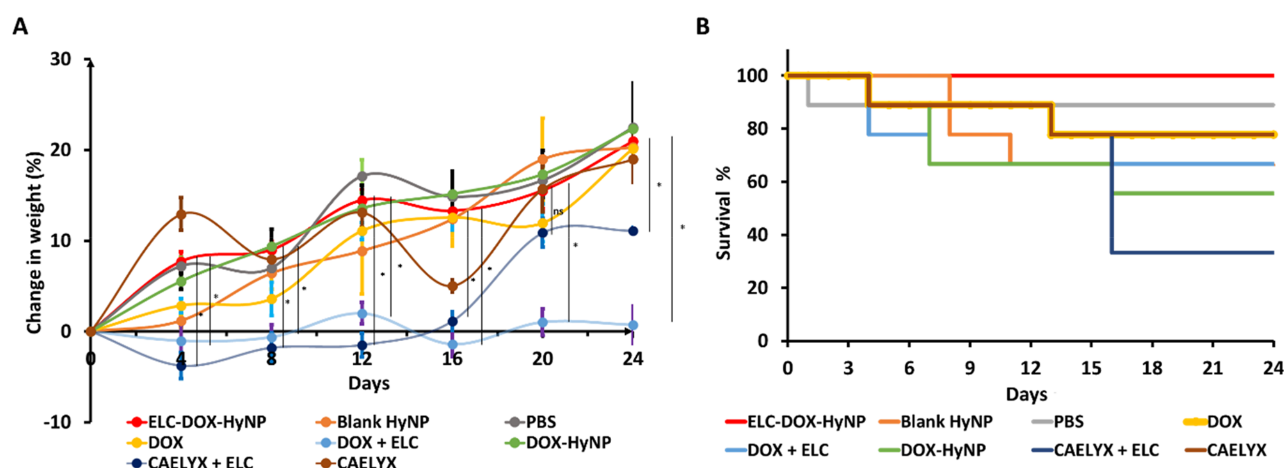

Figure 5. Change in weight of animals upon treatment (A) and survival rate of formulation groups upon treatment (B). *Statistically significant ($p < .05$), ns: statistically not significant, statistical significances were given for between DOX + ELC vs. ELC-DOX-HyNP and Caelyx + ELC vs. ELC-DOX-HyNP groups.

drug delivery system did not contain much novelty, they fully evaluated the effectiveness of the system with *in vitro* cytotoxicity, cellular uptake, colony formation assays, and *in vivo* pharmacokinetic, biodistribution and antitumor effect analyses. Similar to our results, their results also show that the DOX + ELC combination solution exerted systemic toxicity based on the change in body weight. Although obtained system did not actively targeted, their combination with PLGA nanoparticles exhibited better tumour targeting and antitumoural effect *in vivo* [36]. In another study, Xiao et al. synthesised a novel co-polymer methoxy-poly(ethylene glycol)–poly[(N-(6-hydroxyhexyl)-g-doxorubicin-L-aspartamide) -(b-benzyl-L-aspartate)] (mPEG-P[Asp(HPA-g-DOX)-BLA]) and utilised to develop a nanovesicle with DOX elacridar combination. The average particle size of the obtained polymeric vesicle system was 160 nm and it was aimed to accumulate the tumour site via the passive targeting of the EPR effect without active targeting. Similar to previous and our results, combination solution of elacridar and DOX solution leads to systemic toxicity based on weight loss analysis. Overall, obtained polymeric nanovesicle system enhanced the anticancer effect and decreased the systemic toxicity [52]. Based on these studies, elacridar and DOX combination hold big potential to overcome drug resistance.

Conclusions

The overexpression of P-gp is one of the main mechanisms underlying the development of MDR that leads the failure in chemotherapeutic treatment. In this study, a novel elacridar and DOX co-loaded all-in-one hybrid nanoparticle system has developed. The obtained system showed pH-dependent DOX release and highly accumulated in tumour tissue via active and passive targeting strategies. Results show that although elacridar is a potent drug that inhibits the P-gp, systemic toxicity may limit its usage. Obtained novel all-in-one hybrid nanoparticle system improves the elacridar and DOX efficacy and as a result showed better antitumor efficacy and biosafety profile than both DOX/elacridar and Caelyx®/elacridar combinations.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

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