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Research Article

The Effectiveness of Raloxifene-Loaded Liposomes and Cochleates in Breast Cancer Therapy

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Abstract. Liposome (spherical vesicles) and cochleate (multilayer crystalline, spiral structure) formulations containing raloxifene have been developed having dimethyl-β-cyclodextrin (DM-β-CD) or sodium taurocholate (NaTC). Raloxifene was approved initially for the treatment of osteoporosis but it is also effective on breast tissue and endometrial cells. Raloxifene inhibits matrix metalloproteinase-2 (MMP-2) enzyme, which is known to be responsible for tumor invasion and the initiation of angiogenesis during the tumor growth. Therefore, raloxifene was selected as a model drug. A series of raloxifene-loaded liposome and cochleate formulations were prepared. *In vitro* release studies and *in vivo* tests were performed. Breast cancer cell lines (MCF-7) were also used to find the most effective formulation. Highest antitumor activity was observed, and MMP-2 enzyme was also found to be inhibited with raloxifene-loaded cochleates containing DM-β-CD. These developed formulations can be helpful for further treatment alternatives and new strategies for cancer therapy.

KEY WORDS: cochleates; dimethyl-β-cyclodextrin; liposomes; MCF-7; MMP-2; raloxifene; sodium taurocholate.

INTRODUCTION

Cancer is known to be a major cause of mortality and more than ten million people are diagnosed with this disease annually (1). Breast cancer currently shows the highest incidence of cancer-related deaths in women after lung cancer (2). Over a third of women with breast cancer are considered to develop a metastatic disease, and the average survival time from diagnosis to recurrence for cancer patients is reported to be between 18 and 30 months (3).

There are two main strategies in drug treatment of breast cancer: chemotherapy (doxorubicin, methotrexate, *etc.*) and hormonal therapy (aromatase inhibitors, estrogen receptor antagonists) (4). The selective estrogen receptor modulators (SERM) group is relatively new and a different group from conventional estrogens and estrogen receptor antagonists. The basic mechanism of action of the SERM group is known to prevent binding of estrogen to the receptor by competition (5). Raloxifene is one of the members of the SERM group and approved by FDA for the treatment of osteoporosis (6,7). It is reported in the literature that raloxifene has a binding effect to estrogen receptors on breast tissue and endometrial cells but unlike tamoxifen it has no proliferation effect on the endometrial cells (7–9).

Nanotechnology has been gaining a significant momentum in recent years especially for the treatment of cancer. The main applications and research targets of nanomedicines are drug screening, effective drug delivery, gene delivery, detection (imaging), diagnosis, and monitoring (10). Related to cancer and its pathophysiology, some new and important details have been published. MMP enzymes and their role have been reported to be important in cancer treatment. Matrix metalloproteinase-2 (MMP-2) and MMP-9 are considered to be responsible for the tumor invasion, progression, and metastasis (11). It has been reported to be effective at the earliest phase of tumorogenesis, at the onset and during the development of breast cancer by interacting with oncogenes and tumor suppressors (12).

Raloxifene is a highly hydrophobic molecule and representing quite poor bioavailability in patients. Thus, the formulation development especially for the oral application appears to be an important stage. Therefore, effective and orally applicable formulation for raloxifene needs to be developed.

Liposomes are known as spherical vesicular lipid-based carrier systems, and they have been used to increase permeability and bioavailability because of structural similarities with biological cell membranes. Cochleates are a relatively new type of crystalline particles having a large and continuous lipid bilayer sheets rolled up in a spiral structure with no large internal aqueous phases (13,14).

Cochleates represent many advantages. They have a non-aqueous structure, and they are more stable because of the

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less oxidation potential of lipids. Cochleates can be stored by freeze drying, which provides a potential to be stored for longer periods of time at room temperature which would be an extra advantage for worldwide distribution and storage prior to administration. They can keep their structure even after lyophilisation where the structure of the liposome can be destroyed by lyophilisation. Cochleates exhibit efficient incorporations of hydrophobic drug molecules into the lipid layers in the structure, and they have another potential for slow release of drug molecules *in vivo* as cochleates dissociate by the time. Lipid layers of cochleates are accepted to be nontoxic. Cochleates can be produced easily and safely; they do not have any negative effect on the health. Many other small particles are reported to be toxic (15–17).

There is a general need to develop an effective delivery system, which could facilitate diffusion of the drugs across the cell membrane (18). In the literature, it has been shown that cochleates loaded with amphotericin B showed significantly improved oral absorption and anti-fungal activity as compared to solution (19,20). This enhanced activity may be attributed to the membrane fusion and penetration capability of cochleates. It has been reported that the high tension at the bilayer edges of cochleates (19) caused the creation of a kind of driving force for drug molecules to penetrate faster and it should also be taken into consideration that these cochleates can also be targeted (21).

When liposomes or cochleates containing active molecules are given orally, they are preferentially taken up by macrophages in the GI track and they can go through lymphatic vessels or they can even deliver drug molecules to the site of action effectively (22). Therefore, these systems were developed for oral administrations. Caco-2 cell line has been used as a model in absorption studies (23). Therefore, permeability experiments were performed using caco-2 cell lines to understand how formulations delivered raloxifene through biological membranes. All formulations were then tested on MCF-7 cell lines and they were finally given once a week to tumor-bearing female rats orally for 8 weeks and the changes in the tumor size were observed. MMP-2 enzyme was found to be inhibited with raloxifene-loaded cochleates containing dimethyl-β-cyclodextrin (DM-β-CD), and this was found to be the most effective formulation.

MATERIAL AND METHODS

Materials

Raloxifene (Sigma, USA) was used as a drug model. Dipalmitoil phosphatidyl choline (DPPC), dioctyl phosphatidyl choline (DOPS) (Avanti Polar Lipids Inc., USA), and calcium chloride (Merck, Germany) were used for the preparation of cochleates. Dimethyl-β-cyclodextrin (DM-β-CD) and sodium taurocholate (NaTC) (Sigma, USA) were used as penetration enhancers. MCF-7 was purchased from ATCC, USA. The ELH-MMP2-001 Elisa kit was provided by RayBio, Germany. Caco-2 cells were provided from Food and Mouth Diseases Institute. All other chemicals and solvents were of analytical grade.

Preparation of Liposomes

Raloxifene and DPPC were dissolved in methanol-ethyl acetate (1:9) mixture in a round-bottom flask. NaTC or DM-β-CD was also added to the flask and dissolved in ultrasonic bath. 1 mg/ml chitosan solution was prepared in 0.02 M acetate buffer/0.1 M NaCl (pH 4.5). 100 μl of prepared chitosan solution was added to the round-bottom flask in ultrasonic bath. After the formation of reverse micelles, methanol and ethyl acetate were evaporated at 30–35°C. The resulting gel-like lipid film was hydrated during magnetic stirring, and liposome suspension was formed (24). It was centrifuged for 15 min at 20,000*g* and the supernatant was separated, its volume was measured. All liposomes were precipitated after the centrifuge and they were washed two times and filtered through membrane filter. The amount of raloxifene was analyzed from the supernatant by HPLC, and it is used for the calculation of encapsulation efficiency (25–27).

Preparation of Cochleates

DOPS was dissolved in chloroform in a round-bottom flask. Raloxifene solution in methanol was added to the flask. NaTC or DM-β-CD was also added to the flask and dissolved in ultrasonic bath. Methanol and chloroform (1:1) was evaporated at 40–42°C. Large unilamellar vesicle (LUV)-type liposomes were obtained and suspended in 40% dextran solution. Liposomes were suspended in dextran and were added dropwise to a PEG 8000 solution (15%) under magnetic stirring for 1 h. 100 μM $CaCl_2$ solution was added dropwise and stirred for two more hours (13,14,28–30). Finally, it was centrifuged at 3000 rpm for 30 min and supernatant was separated and raloxifene analyzed from the supernatant by HPLC for the calculation encapsulation efficiency.

Characterizations of Liposomes and Cochleates

Particle size, polydispersity index, and zeta potentials of liposomes and cochleates were determined by Zetasizer-Nano ZS-Malvern (Germany).

DSC Analysis

Differential scanning calorimetry (DSC) was employed in order to assess by thermal experiments potential interactions between raloxifene and the lipid formulations. DSC thermograms of liposomes, cochleates, and formulation components were obtained by using Schimadzu DSC-DTA 60. DSC was set to 300°C and thermograms were obtained with 10°C/min scan speed. Raloxifene alone and mixtures with other excipients in the formulation were subjected to the analysis with a ratio of 1:1.

Assay

Amounts of raloxifene in samples were determined by HPLC using a UV detector at 287 nm. The analysis method for raloxifene was adopted from the literature. Mobile phase was 50 mM phosphate buffer (pH 3.0): acetonitrile (64:36) and C18 4.6×250 mm column was used (31). The analysis was validated. The method was found to be specific, linear, and reproducible.

Cytotoxixity Studies

Metiltiazol difenil tetrazolium tests (MTT) were carried out on the Caco-2 cell line with raloxifene (900, 450, 120, 60 μg/ml), NaTC (7.5, 3.75, 1.875, 0.005 mM), DM-β-CD (5, 1.5, 0.375, 0.15%), raloxifene-free liposomes (75, 50, 25%), and raloxifene-free cochleates (75, 50, 25%) presence for a 24-h time period to observe their effects on cell viability.

Intestinal Absorption of Raloxifene

Caco-2 cells were provided by the Food and Mouth Diseases Institute. Caco-2 cells (80,000 cells/ml) were seeded on semipermeable polycarbonate filter inserts for 21 days (1.2-cm diameter, 0.4-μm pore size). The transport studies were performed from the apical to the basolateral side of the diffusion cells at 37°C. Penetrated amounts of raloxifene passed through the basolateral side were analyzed with HPLC and apparent permeability coefficient (Papp) values were calculated. Transepithelial electrical resistance (TEER) values were also measured by Evom Voltmeter® for evaluating cell integrity at the initial part and at the end of the experiment.

Antitumor Activity

MCF-7 cells were obtained from the Food and Mouth Diseases Institute. Antitumor activity of liposome and cochleate formulations of raloxifene were investigated on the MCF-7 cell line with the density of 1×10^4 of each well of 96 well plates. Raloxifene formulations were suspended in DMEM and seeded on wells of plates at 37°C and 95% O_2/5%CO_2. At the end of 4 days, 10 μL of MTT (5 mg/ml) was added to the each well and incubated for 4 h and then 100 μL 2-propanol, Triton X-100 (10%) and 0.1 N HCl was added and analyzed by spectrophotometer at 570 nm with Elisa reader. Values of growth inhibitions were calculated as antitumor activity according to the following Eq. 1 and compared to average of optical densities of control wells (32).

$$\text{Inhibition \%} = \frac{\text{Absorbance of controlled wells} - \text{Absorbance of sample wells}}{\text{Absorbance of controlled wells}} \times 100 \quad (1)$$

MMP-2 Enzyme Inhibition

Ray®Biotech Human Elisa Kit was used to determine the amount of MMP-2 enzyme of cultured MCF-7 cells (1×10^4 cells in each well); spectrophotometric analyses were performed at 450 nm.

Animal Experiments

In vivo studies were performed on 300±10 g weighted tumor-bearing Sprague Dawley female rats. Tumors were developed using nitroso methyl urea (NMU) with the dose of 50 mg/kg. Calculated dose was administered through an intraperitoneal route to rats (33–38). Tumor development was observed, and formulations (raloxifene+DM-β-CD liposomes, raloxifene+DM-β-CD cochleates, and raloxifene+DM-β-CD solutions) were applied to tumor-bearing rats orally. Untreated tumor-bearing rats were used as controls. Treatments of 1200 μg/ml doses of raloxifene in formulations were given once per week for 8 weeks; weights and tumor diameters were measured each week. Caliper was used to measure the size of the tumors (considering width and length). Weights of the rats were measured each week. Alteration in tumor areas and rat weights were determined at the end of 8 weeks. Animal study was conducted under the protocol approved by the Animal Care and Use Ethical Committee of Gazi University (G.Ü.ET-10.010/199-20288).

RESULTS

Liposomes and cochleates containing raloxifene were successfully prepared; encapsulations efficiencies were calculated (Table I). Liposomes and cochleates were prepared with raloxifene and dimethyl-β-cyclodextrin (DM-β-CD) or sodium taurocholate (NaTC). Formulations were characterized considering particle size (PS), polydispersity index (PDI) and zeta potential (ZP) measurements, encapsulation efficiencies (EE), and transmission electron microscope (TEM) photographs. The details of characterization studies were given in Table I for all formulations. TEM images for raloxifene-DM-β-CD liposomes (a), raloxifene cochleates (b), and raloxifene-DM-β-CD cochleates (c) were obtained, and their structure was clearly observed (Fig. 1).

Any possible interactions or incompatibilities between active molecule and formulation components were investigated. DSC thermograms of formulation components and raloxifene were obtained. There was no interactions observed and/or no incompatibilities were recorded (Fig. 2).

MTT studies were carried out on Caco-2 cells before performing absorption studies, and maximum amounts of raloxifene, DM-β-CD, and NaTC were determined with the dose of 120 μg/ml, 0.15%, and 1.875 mM, respectively, as safe concentrations without causing any cytotoxicity. Effects of formulations components (bare liposomes or cochleates) on Caco-2 cells were also tested, and no effect was observed on cell viability (Fig. 3). Caco-2 cells (80,000 cells/ml) were seeded on semipermeable polycarbonate filter inserts (1.2-cm diameter, 0.4-μm pore size) and cultivated for 21 days (39–42). Liposome and cochleate formulations were dispersed in PG/DMEM (60/40%) for transport studies. The cumulative amounts of penetrated raloxifene at the end of the 24-h time period were calculated, and Caco-2 cell transportations were plotted (Fig. 4).

Initial TEER value at the beginning of the permeation studies were measured around 245Ω. Papp values were calculated considering the linear part of permeation curve (Table I).

After transport studies, antitumor activities of liposome and cochleate formulations of raloxifene were investigated on MCF-7 cell line. All results were compared with raloxifene-containing solutions (Fig. 5).

MMP-2 inhibition studies were performed for prepared formulations. MCF-7 cells were cultured on the wells and using Ray®Biotech Human Elisa Kit MMP-2 enzyme amounts were determined (Table II).

The results of Caco-2 transportation, antitumor activity, and MMP-2 inhibition studies were considered and DM-β-

Table I. Formulation Characteristics and Measured TEER and Calculated Papp Values, Caco-2 Transportation Studies and at the end Encapsulation Efficiency of for Raloxifene Liposomes and Cochleates

Formulation type	PS (nm)	PDI	ZP (mV)	EE (%)	TEER (Ω)	Papp (cm/h)
Raloxifene liposomes	210±6.3	0.28	7.9±0.1	51.9±5.4	240	1.60±0.08
Raloxifene+ DM-β-CD liposomes	344.6±8.6	0.33	−12.5±−1.3	42.9±1.4	210	4.14±0.12
Raloxifene+ NaTC liposomes	223.6±5.9	0.34	9.4±1.4	48.3±5.5	229	1.61±0.07
Raloxifene cochleates	229.7±15.6	1.00	−26.6±−1.8	43.4±1.5	239	0.159±0.010
Raloxifene+ DM-β-CD cochleates	288.7±2.1	1.00	−37.0±−1.6	36.2±3.7	215	1.37±0.03
Raloxifene+ NaTC cochleates	261±5.7	1.00	−18.0±−0.8	39.4±2.5	224	0.511±0.040

CD-containing formulations (raloxifene+DM-β-CD liposomes, raloxifene+DM-β-CD cochleates, and raloxifene+DM-β-CD solutions) were chosen to apply to tumor-bearing Sprague Dawley-type female rats. Prepared formulations were applied to tumor-bearing rats orally (Fig. 6a). Untreated tumor-bearing rats were used as controls. 1200 μg/ml doses of raloxifene in formulations were given once per week for 8 weeks; weights and tumor diameters were measured each week. Caliper was used to measure the size of the tumors (considering width and length) in rats (Fig. 6b). Experimental evaluation was done through the tumor areas comparing to the control group. In addition, weights of the rats were measured each week. The alteration in tumor areas (Fig. 6c) and rat weights (Fig. 6d) was determined at the end of 8 weeks.

Tissue samples were taken out from tumor areas at the end (Fig. 7a, b) and subjected to histopathological

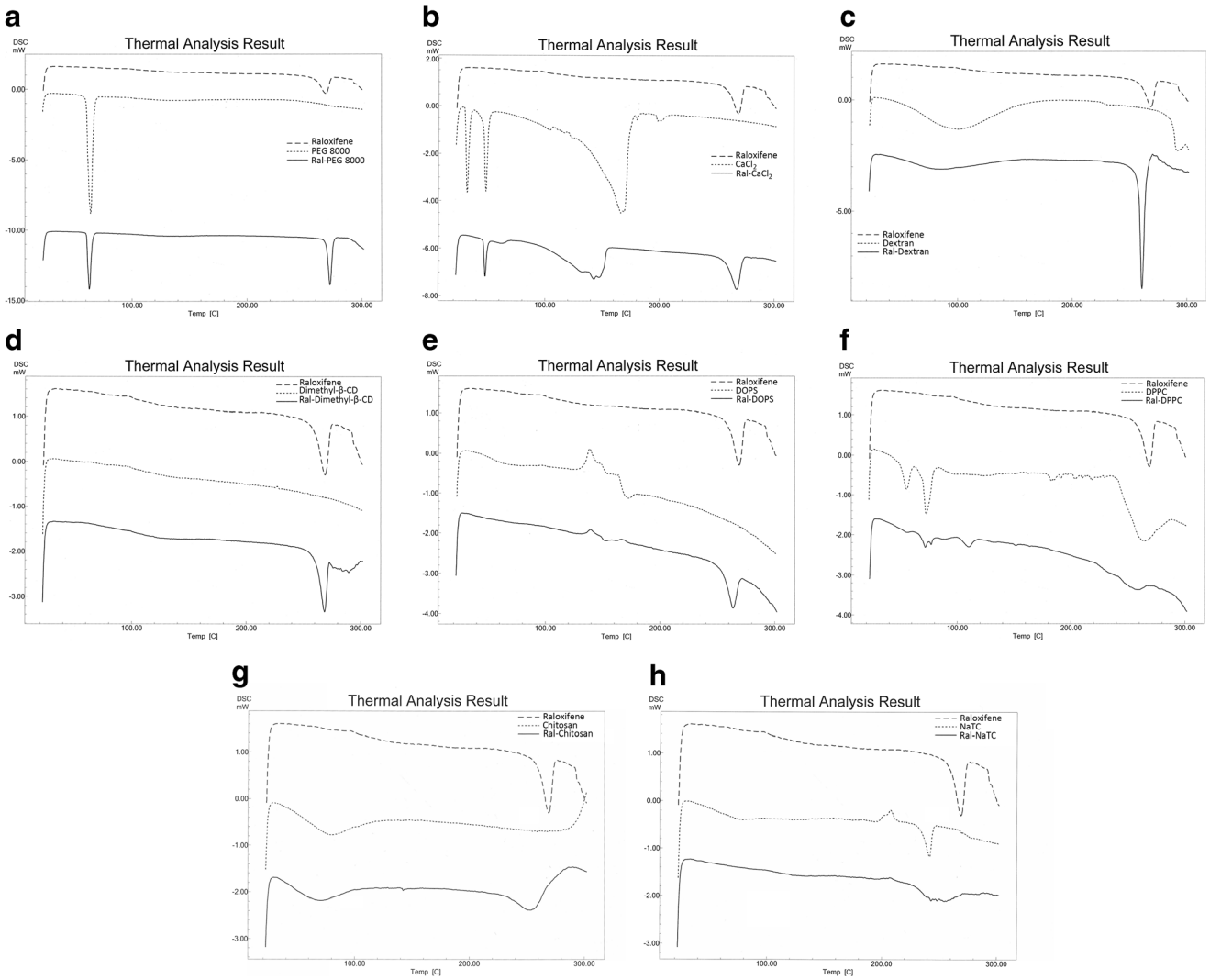

Fig. 1. **a** DSC thermogram of raloxifene and PEG 8000 1:1 mixture. **b** DSC thermogram of raloxifene and $CaCl_2$ 1:1 mixture. **c** DSC thermogram of raloxifene and dextran 1:1 mixture. **d** DSC thermogram of raloxifene and dimethyl-β-cyclodextrin 1:1 mixture. **e** DSC thermogram of raloxifene and DOPS 1:1 mixture. **f** DSC thermogram of raloxifene and DPPC 1:1 mixture. **g** DSC thermogram of raloxifene and chitosan 1:1 mixture. **h** DSC thermogram of raloxifene and sodium taurocholate 1:1 mixture

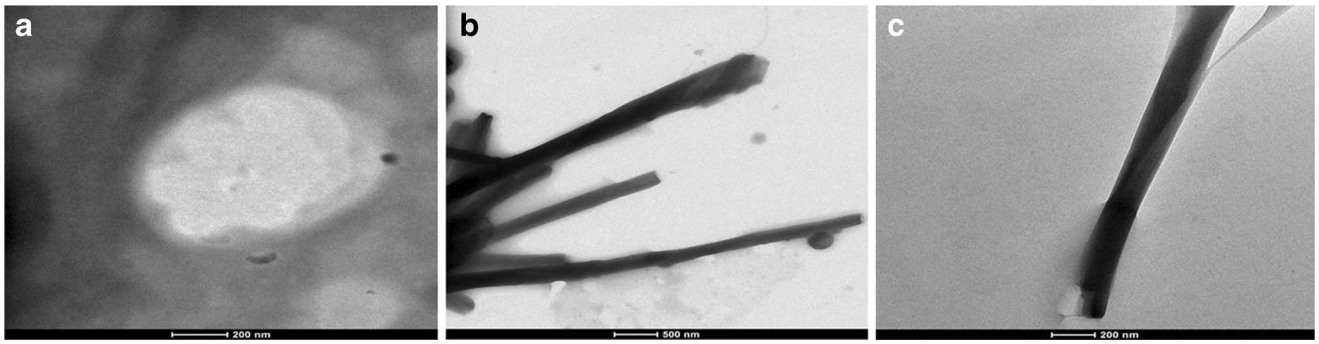

Fig. 2. TEM images for raloxifene-DM-β-CD liposomes (**a**), raloxifene cochleates (**b**), and raloxifene-DM-β-CD cochleates (**c**)

examinations. Tissue samples from the control group and healthy rats were also examined for comparison. Malignancy, the type of malignancy, and response to treatment were assessed by the ratio of fibrosis and necrosis. Photographs of histopathological examinations were given for healthy rats (Fig. 7c), untreated tumor-bearing rats (Fig. 7d), the rats treated with raloxifene+DM-β-CD liposomes (Fig. 7e), and the rats treated with raloxifene+DM-β-CD cochleates (Fig. 7f).

DISCUSSION

DSC thermograms were obtained initially and analyzed entirely to detect any possible interactions between active molecule and formulation components. There was no change or no loss of any peak observed when formulation components and active substance mixtures were subjected to the analysis. There was no interaction, or no incompatibilities were detected.

PDI values of formulations were found to be high for cochleates. The reason of these results were just found to be normal because if the shapes of the cochleates are considered, it can be noticed that they are long in length but they have a very small radius; they are far from the spherical shape. Because of this, PDI values of cochleates were not determined within the small range like liposomes.

Colorimetric MTT assay has demonstrated a potential for chemotherapeutic drug screening (43,44). A better reproducibility with this test can be obtained with many cell lines (44). It has been concluded that nonneoplastic cells can also reduce MTT (45); therefore, MTT assay can be more suitable for MCF-7-type cancerous cells. Many studies in the literature

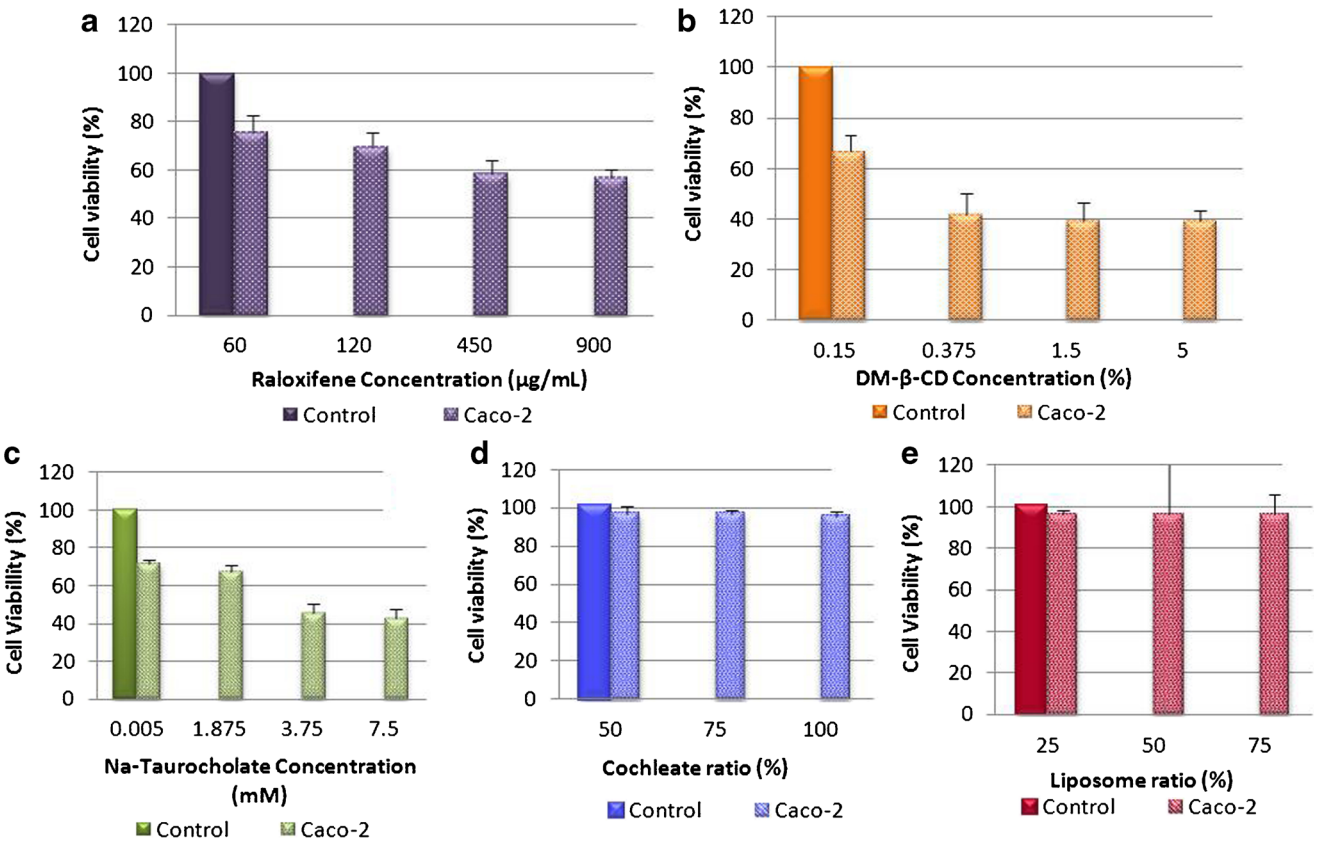

Fig. 3. **a** MTT results of raloxifene solution (900, 450, 120, 60 µg/ml). **b** DM-β-CD (5, 1.5, 0.375, 0.15%). **c** NaTC (7.5, 3.75, 1.875, 0.005 mM). **d** Raloxifene free cochleates (75, 50, 25%). **e** Raloxifene free liposomes (75, 50, 25%)

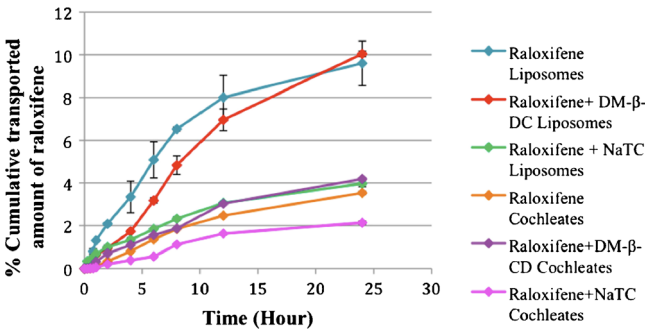

Fig. 4. Mean (±S.D.) cumulative percents of raloxifene transported through Caco-2 cells from various formulations (*error bars* represent standard deviations, n=3)

Table II. The Calculated MMP-2 Enzyme Inhibition Values of Raloxifene Formulations for MCF-7 Cell Line

Formulation type	MMP-2 inhibition (%) (MCF-7)
Raloxifene solution	13.7±2.4
Raloxifene+DM-β CD solution	18.9±2.5
Raloxifene+NaTC solution	17.2±2.4
Raloxifene liposomes	23.4±2.5
Raloxifene+DM-β CD liposomes	32.2±3.3
Raloxifene+NaTC liposomes	28.8±1.7
Raloxifene-free liposomes	8.92±2.66
Raloxifene cochleates	34.6±3.4
Raloxifene+DM-β CD cochleates	51.9±2.6
Raloxifene+NaTC cochleates	39.1±2.6
Raloxifene-free cochleates	13.8±1.5

indicated that MTT test or FACS test can be chosen to study the viability of cells (46–48). FACS test can be done but FACS test is a rather new technique; it especially shows the cell uptake. MTT tests were chosen and have been done in our study and *in vivo* tests were then performed. Prior to cell culture studies, cytotoxic effects of the drug and the other formulation excipients were investigated and their amounts in the formulations were determined. Cytotoxicity test results indicate the cell viabilities should be above 50% (49,50). MTT tests were performed on both Caco-2 and MCF-7 cell lines, and dose of the raloxifene, NaTC, and DM-β-CD were determined as 120 μg/ml, 1.875 mM, and 0.15%, respectively. Liposome and cochleate formulations without having an active substance did not affect cell viabilities in Caco-2 and MCF-7 cell lines significantly at tested concentrations (p<0.01).

Caco-2 cell line is widely used as an *in vitro* model for evaluating oral drug absorption (23,51–54). The medium used in Caco-2 transport in our experiments PG/DMEM (60%/40%) was also tested, and Caco-2 cell viability of the medium was found to be 77.4%. In the literature, it has been reported that the use of propylene glycol as medium for the transport studies of lipophilic drugs would help to get more accurate results (55). Therefore, PG/DMEM (60%/40%) was chosen and used in Caco-2 transport studies. According to the Caco-2 transport study results, raloxifene liposomes showed the highest transports. Liposomes have a similar structure with the cell membranes. Therefore, liposomes can effectively deliver the drug through the biological membranes. Especially in the presence of dimethyl-β-CD in the liposomes, it is considered that dimethyl-β-CD might open tight junctions of the membranes and it can increase the transport of raloxifene through apical to the basolateral side of Caco-2 cells (9.98%) (p<0.001). Sodium taurocholate, a bile salt and acts as an absorption enhancer, may also increase the solubility of raloxifene, but ionized portion raloxifene might not be able to pass the cell membranes with higher rates (3.96%). Data from the cochleate formulations have shown lower raloxifene transports and thus lower permeability coefficients were obtained compared to liposomes. Cochleates have no internal aqueous phase, and they may release the lipophilic drug raloxifene more slowly for a longer time period compared to liposomes. In the literature, in the study of Miclea *et al.*, which has been also done with cochleates containing recombinant factor VIII, cell membrane interactions of cochleates were found to be important for the effect (13). When liposome and cochleate formulations were evaluated individually, prepared formulations with dimethyl-β-CD liposome is observed to have significantly higher permeability coefficients. Raloxifene is a hydrophobic drug thus more soluble in lipids therefore it has higher permeability coefficients when present in the liposomes or cochleates. TEER values were inversely related with permeability coefficients because if the TEER value is small the permeation can take place faster. It means the integrity of the membrane may be destroyed or the membrane is not intact; therefore, it cannot show any barrier properties.

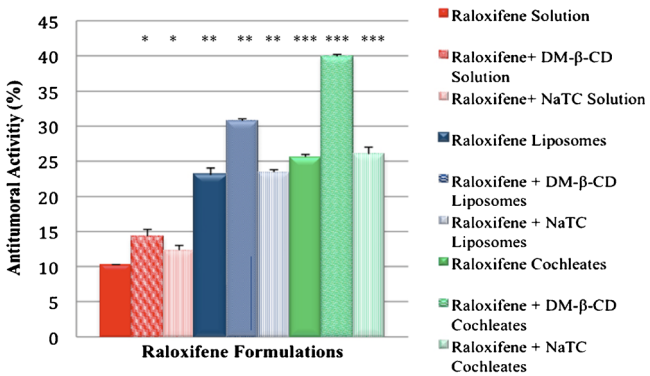

Fig. 5. Antitumoral activity (±S.D.) results of raloxifene solutions, liposomes, and cochleates on MCF-7 cells (n=6), (*p<0.1; **p<0.01; ***p<0.001)

MCF-7 is an estrogen receptor-positive breast cancer cell, and it was selected in order to utilize an antitumor activity and MMP-2 in inhibition tests which is performed frequently in breast cancer studies. In all prepared formulations, the highest antitumor activity was found for dimethyl-β-CD-containing cochleates with the value of 41.6% (p<0.001). Thereafter, the second highest value (28.1%) belongs to dimethyl-β-CD-containing liposomes (p<0.001). Antitumor activity results found to be related to the calculated permeability coefficients from Caco-2 cells. Similarities of the cell membrane structure and lipid natures of the prepared formulations were found to be effective. Significant effect of dimethyl-β-CD on MMP-2 enzyme inhibition of MCF-7 cell line is also found to be noteworthy. Raloxifene- and dimethyl-β-CD-containing cochleates have been shown to cause the maximum MMP-2 enzyme inhibitions with the value of 51.9% (p<0.001).

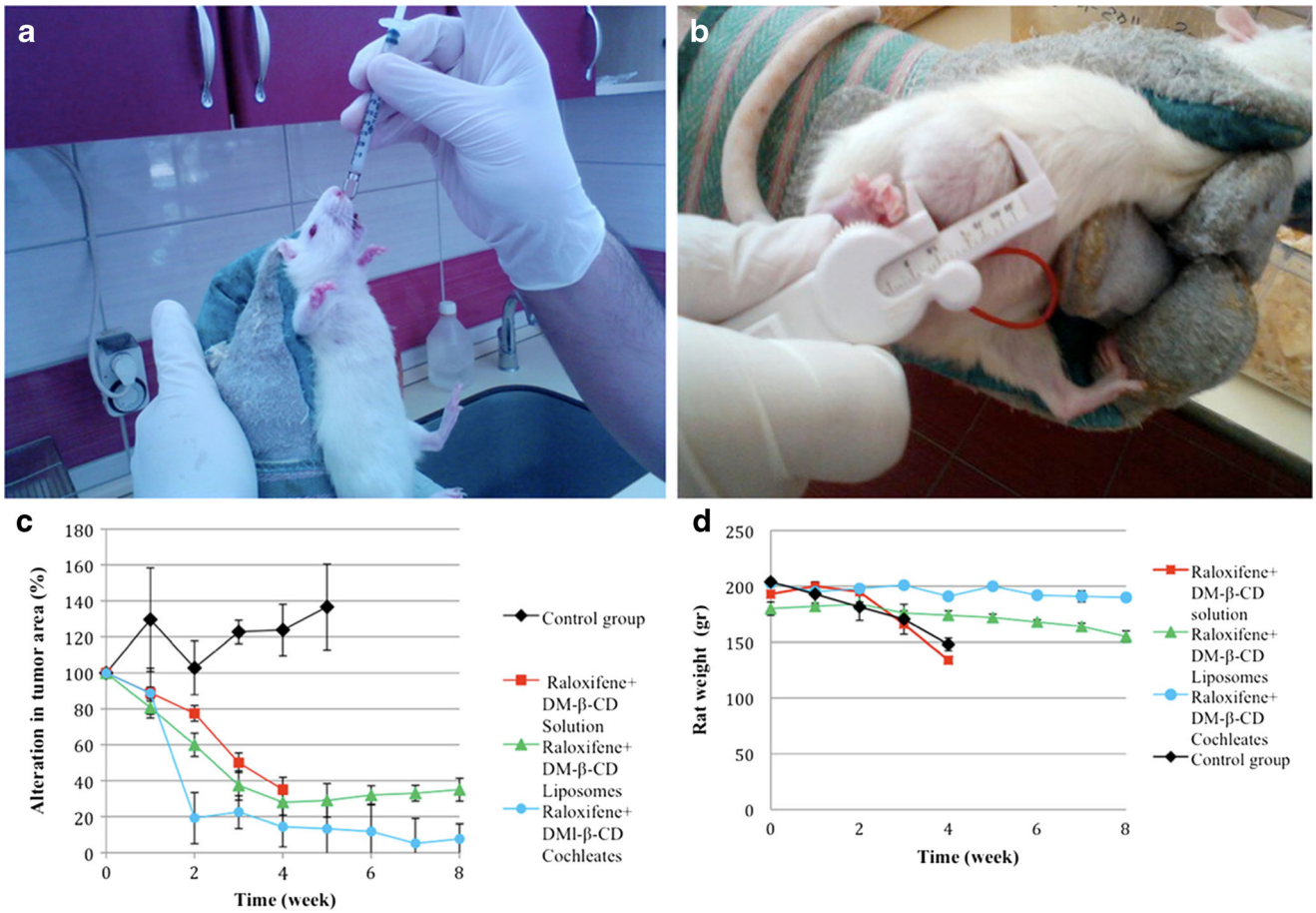

Fig. 6. Studies with tumor-bearing female Sprague Dawley rats. **a** Application of raloxifene formulations to rats. **b** Measurements of tumor length and width by caliper to calculate tumor areas. **c** Alterations in tumor areas (%) for solutions, liposomes, and cochleates comparatively to the control group. **d** Results of rat weight changes weekly (*error bars* represent standard deviations, n=3)

Dimethyl-β-CD-containing liposomes were the second leading group for inhibition of MMP-2 enzyme with the value of 32.2% (p<0.001). Positive effects of lipid containing formulations, especially cochleates and dimethyl-β-CD, have also quite significant effect on MMP-2 enzyme inhibitions for MCF-7 cells.

When liposomes were given orally, they may penetrate and distribute throughout the Peyer's patches, via lymph nodes in the body. When liposomes reach the Peyer's patches, they are considered as antigenic/foreign matter from the reticuloendothelial system and they may be phagocytized. In this manner, active molecule is protected from the acids alkaline and enzymatic degradations. In addition, they can pass further barriers and penetrate faster through membranes because of the lipid character of liposomes and cochleates (56). It has been reported in the literature that cochleates accumulation in the body organs was observed when amphotericin B cochleates were orally administered (27). Likewise, Segarra *et al.* administered amphotericin B cochleates intravenously; the accumulations of cochleates were observed in the lungs, spleen, heart, kidney, and especially in the liver (28). Based on these findings, it can be considered that Peyer's Patches may have an important role. They can accumulate in organs, and the mentioned enterohepatic cycles in these studies may also be valid for liposomes and cochleates.

Raloxifene dimethyl-β-CD liposomes, raloxifene dimethyl-β-CD cochleates, and raloxifene dimethyl-β-CD solutions were finally selected for further *in vivo* studies with female tumor-bearing Sprague Dawley rats. At the end of therapy, the most effective formulation considering tumor size reduction was found to be raloxifene dimethyl-β-CD cochleates (94.8% reduction). In the group, which was treated with raloxifene dimethyl-β-CD cochleates, the less decrease and less fluctuation in rat weights was observed and it was considered as a good general health status and indication of effective therapy. The Control group and solution-treated group significantly lost weights. In histopathological images belonging to dimethyl-β-CD liposomes applied group, malignant changes in stroma were clearly seen. The assessment of the fibro-necrotic areas was expressed as 5%, and this was composed of fibro-necrotic areas. Although the dimethyl-β-CD liposomes formulation group reduced the tumor size to 65%, histopathological findings showed only 5% reduction. This means that the effectiveness of treatment was low (5%) than observed. Measurement of tumor size of this group was calculated to be 65%. The highest permeability coefficient was found to be with raloxifene dimethyl-β-CD liposomes. Performing histopathological examinations are necessary not to interpret results in a wrong direction. Histopathological image of raloxifene dimethyl-β-CD cochleate-treated group

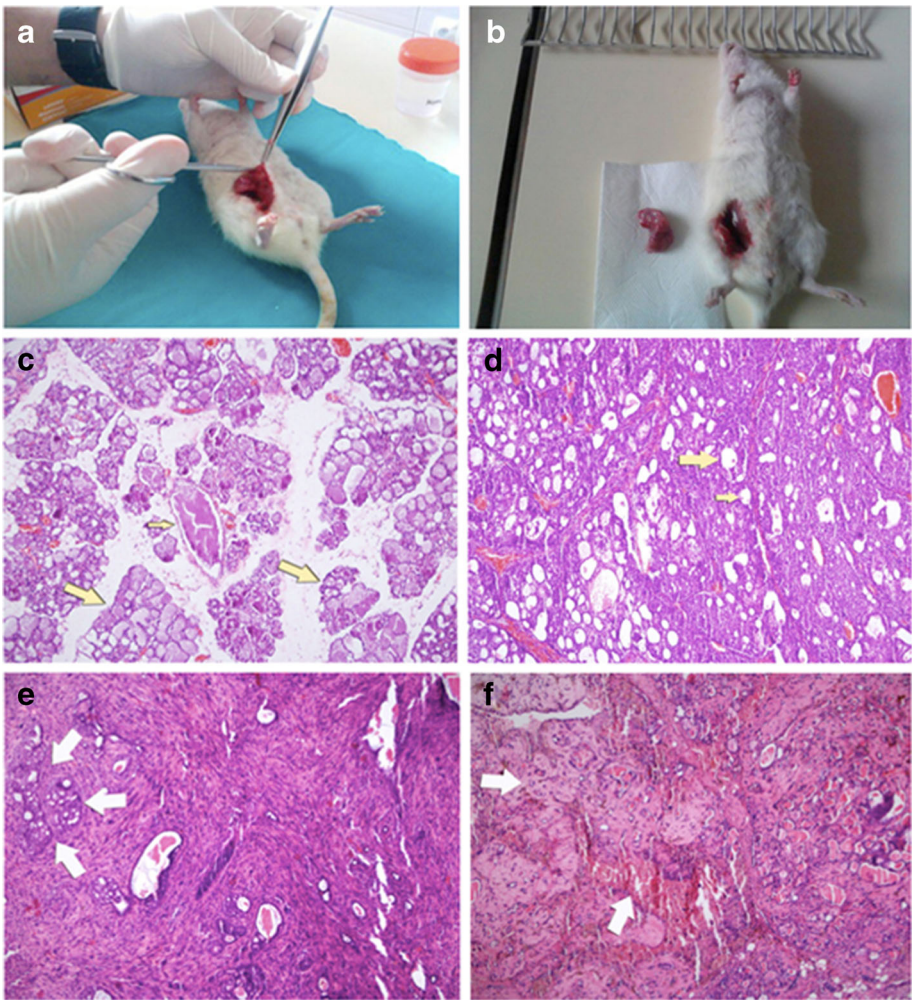

Fig. 7. Summary and results of histopathological studies. **a** Removal of tumor tissue of tumor-bearing rats. **b** A rat with removed tumor tissue after 8 weeks of administration period. **c** Healthy rat breast tissue image (×100) (*long arrows*: normal breast lobules, *short arrow*: ductus). **d** Untreated tumor-bearing rat breast tissue image (×100) (control group) (*arrows*: gland formation). **e** The image of rat breast tissue treated with raloxifene+DM-β-CD liposomes for 8 weeks time period (×100) (*between arrows*: malignant epithelial islands). **f** The image of rat breast tissue treated with raloxifene+DM-β-CD cochleates for 8 weeks time period (×100) (*left arrow*: fibrotic areas, *lower-right arrow*: necrotic areas)

showed the many fibrotic areas, and the ratio of fibrotic/necrotic areas was expressed as 60%. Tumor size reduction was found to be 94%, and this supports the results of the histopathological observations. This formulation provided the highest of treatment efficacy (60%). Cochleates exhibit efficient incorporation of hydrophobic or even hydrophilic drugs into the lipid layers or internal phases, and they were reported to have a potential for slow release for drugs. Therefore, in our experiment results, the reason of observing a better activity is attributed mainly to the sustained release properties of cochleates but targeting or easy penetrating properties of cochleates should also be taken into consideration. Cochleates have spiral lipid layers, and they are in micrometer size. They can prefer to go through the hydrophobic pathway, and this is also a facilitating way for hydrophobic active substance such as raloxifene. They can even go through the lymphatic pathway (22). This enhanced activity may also be attributed to the membrane fusion capability of cochleates and an increased contact by increasing the number of particles due to their size and the high tension at the bilayer edges of cochleates (19). This effect can also create a kind of driving force for the molecule to penetrate efficiently. The interaction with the tissue or cell membrane can be also important; the contact time between cochleates and cell membrane can be also increased because of structure or composition similarities. Penetration to the tumor or enhancing activity at the site of action may also be high because of enhanced permeability and retention (EPR) effect. The effect was found to be high with cochleates than liposomes, and this was attributed to the shape difference. Cochleates has a spiral tubular shape and it may act as micro needle because at the front edge of the cochleates all electrochemical forces come through the longitudinal part and those forces makes the tip of the cochleates very strong and active. When cochleates approach the cell membrane, this force makes the cell

membrane very flexible and unstable; therefore, the surface tension of the cell membrane reduces and the membrane bends spontaneously through the inside. Cochleates can even go further and finally cochleates were engulfed. A kind of insertation/perturbation takes place and conditions at the interior part of the membrane may be more preferable for the active molecules and cochleates may finally release/inject the contents to the interior side. Therefore, the permeability of the active substance can be found high with cochleates. Therefore, the reason for observing a higher effect with cochleates than liposomes was attributed to this phenomenon. This has been explained in detail in the literature for other tubular delivery systems (57,58).

CONCLUSION

When all results were considered and compared, raloxifene dimethyl-β-CD cochleate formulations were found to be successful in reducing breast tumors. The effect of raloxifene is proved and put forward in this study, and the effect of this new formulation is quite noteworthy. The mechanism for this effect was found to be quite complicated. Many factors were found to be incorporated for developed formulation to represent a better activity including slow drug release, longer release time, penetration-enhancing effect of cochleates, similarities of cochleate composition with cell membranes, alteration of distributions, eliminations and half life, *etc.*

Further studies are necessary but these developed cochleate formulations containing dimethyl-β-CD and raloxifene would be promising for the treatment of such disease like breast cancer, which affects women's health seriously.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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