INTRODUCTION

Erlotinib HCl is an epidermal growth factor receptor inhibitor that was approved by the FDA in 2004 for treatment of non-small cell lung cancer and its anticancer effects promised hope in various preclinical models (1). Tablet forms containing Erlotinib HCl (ERLO) are available in the market (2) but when the FDA-Orange Book for USA market or electronic Medicines Compendium (eMC) for EU market were checked, there was no nanosystem with ERLO found. ERLO is slightly soluble in water. Aqueous solubility is dependent on pH and its solubility increases is below pH 5 (2). ERLO has toxic effects such as diarrhea, skin rash and fatigue, as well as toxic effects on pulmonary, hepatic and renal systems (3-6). In order to overcome these toxic effects, it is aimed to load this drug into the nano drug carrier system. In a study on healthy rats in the literature, no toxic effect with ERLO was observed compared to its free form when it was encapsulated in polymeric nanoparticles (7).

Dexketoprofen trometamol (DEX) is a water-soluble but it has also some oil-solubleility; DEX is a salt of the S-isomer of the racemic non-steroidal anti-inflammatory drug ketoprofen (8,9). Although it inhibits cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isoenzymes, it has partially selective activity for COX-1 (10,11). Recent studies with non-steroidal anti-inflammatory drugs have shown that this drug has a protective effect against breast and colorectal cancers, which are frequently observed all over the world (12,13). The underlying mechanism is can be explained with angiogenesis by associated COX-derived prostaglandins (14). Taking all these observations into account, when DEX is used with ERLO, as a combination therapy, more effective cancer treatment can be obtained.

Cochleates are packaged lipidic structures, which are composed of negatively charged phospholipids in the presence of divalent counter ions such as Ca^{+2} and not containing water in the internal phase (15). It is thought that as the mechanism of formation, fusion occurs through Ca^{+2} followed by the leakage of the aqueous phase of the liposome, and the lipid layers are folded on each other to form solid spiral rods (15,16). Proteoliposome derived cochleates are known to exhibit highly immunogenicity when administered by intramuscular, oral, or intranasal routes. Previous studies have also supported the use of these constructs in the design and development of vaccines and adjuvants (17). In addition to this use, they are particularly effective in the oral use of hydrophobic drugs. Unlike liposomes, water is not present in their internal phases and they have a solid rod structure. Due to these constructions, they can protect molecules that are trapped against harsh environmental conditions such as pH, lipase degradation and temperature. They are also resistant to lyophilization. Cochleates include phosphotidyl serine (PS), dioleoylphosphatidylserine (DOPS), phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylglycerol (PC) as soy based phospholipids either alone or as mixtures (16).

The aim of this study is to load NSAIDs in combination with an anticancer drug to nanocochleate delivery systems, which is a new approach for cancer treatment. In this way, by targeting anticancer drug delivery systems directly to the tumor tissues, side effects will be reduced, a low dose will provide more effective treatment, and combined drug administration will enhance the treatment. Furthermore, the combination of an NSAID and an anticancer drug, which are currently used separately will more convenient for patients. Therefore it has been aimed to load ERLO (which is a hydrophobic drug) and DEX (which is a hydrophilic auxiliary drug) into the nanocochleates and to characterize the system.

***Correspondence:** E-mail:zdegim@gmail.com

MATERIALS AND METHODS

Materials

DOPS and methoxy-poly(ethyleneglycol)2000-distearoylphosphatidylethanolamine (DSPE-PEG_{2000}) were purchased from Avanti Polar Lipids, USA. Folic acid (FA), sodium acetate trihydrate, chloroform and ethanol were from Sigma-Aldrich, Germany. Erlotinib HCl were from Biotang, USA. Dexketoprofen trometamol and calcium chloride dihydrate ($CaCl_2.2H_2O$) were purchased from Sigma. Acetic acid glacial was purchased from Fisher Scientific, UK. All chemicals were analytical grade and were used without further purification. Dialysis membrane (cellulose acetate molecular weight cut-off (MVCO) 12000 Da) was obtained from Sigma-Aldrich, USA.

Analytical method and calibration

The UPLC method, which is a highly sensitive, was preferred to determine the drug loading capacity and the cumulative drug release studies. Initially, ERLO and DEX were scanned by UV spectrophotometer to determine their maximum absorbance wavelengths in distilled water containing ethanol (20%) and pH 3 acetate buffer and they were found as 244 and 260 nm for ERLO and DEX respectively. 1 mg of ERLO and 1 mg of DEX were weighed separately and transferred into a 100 mL volumetric flask. 20 mL of ethanol was added and the flask was sonicated to dissolve all the contents for 10 min, and then diluted up to 100 mL with distilled water. On the other hand, 1 mg of ERLO and 1 mg of DEX were weighed separately and transferred into a 100 mL volumetric flask. A portion of pH 3 acetate buffer was added and the flask was sonicated to dissolve all the contents for 10 min, and then diluted up to 100 mL with pH 3 acetate buffer. Finally ERLO and DEX together having a concentration of 10 µg /mL was used as stock solutions. Solutions at concentrations ranging from 0.05 to 10 µg /mL were prepared by diluting the stock solutions, samples were then analyzed by UPLC (6 replicates) and calibration curves were obtained. UPLC method was found to be linear (r^2=0.999) and reproducible for both mediums.

Development of ERLO and DEX loaded nanocochleate formulations

The Bangham method was preferred because it was an easy method for preparing liposomes. In this context, DOPS, PEG-DSPE, FA, ERLO, and DEX were placed in a round bottom flask, and chloroform was added to dissolve all the materials. The organic phase was evaporated at 42°C using rotary evaporator. Distilled water was added and vortexed for 15 minutes followed by ultrasonication for 1 hour. 6 mM $CaCl_2$ was added dropwise to the liposome suspension with various ratios and vortexed for 30 minutes. Finally the mixture was kept in the refrigerator at +4°C for an overnight period. Formulation contents and the amounts of these ingredients are shown in Table 1.

Table (1) ERLO and DEX loaded nanocochleate formulations

Formülasyon Kodu	**KOH-1A**	**KOH-1B**	**KOH-1C**	**KOH-1D**
DOPS	10 mg	10 mg	10 mg	10 mg
DSPE-PEG_{2000}	10 mg	10 mg	10 mg	10 mg
FA	20 mg	20 mg	20 mg	20 mg
ERLO	6 mg	6 mg	6 mg	6 mg
DEX	3 mg	3 mg	3 mg	3 mg
Chloroform	5 mL	5 mL	5 mL	5 mL
6 mM $CaCl_2$	1:1	1:2	1:3	2:2

Determination of particle size distribution, polydispersity index and zeta potential of formulations

The particle sizes of the formulations were measured by the laser light scattering method. The Malvern Zeta-Nanosizer instrument was used to measure particle size distribution, polydispersity index, and zeta potential. 3 parallel measurements were made and mean and standard deviation (SD) values were calculated.

Determination of encapsulation efficiency of formulations

In order to determine the encapsulation efficiency of the formulations, the formulations were first centrifuged at 18000 rpm for 40 min and the supernatant fractions were analyzed to determine the amount of free drug. The amount of drug loaded into the formulation was determined by subtracting this value from the total amount of the drug in the formulation and the values were given as percentages. 3 parallel measurements were made and mean and standard deviation (SD) values were calculated.

TEM analysis of the optimal formulation

TEM imaging was performed for the most appropriate formulation in terms of drug encapsulation efficiency, particle size distribution, polydispersity index, and zeta potential. These analyses were carried out in METU Central Laboratory. Prior to imaging, the samples were diluted 1:29 with distilled water.

Release studies using Franz diffusion cell

Release studies have also been performed using the Franz diffusion cell for the optimal formulation. In the release studies of the nanoparticulate systems containing ERLO, pH 3 acetate, pH 5.2 acetate and pH 7.4 phosphate buffers were used as release medium (18,19). When the release studies in the literature were considered, the most meaningful results were obtained in the pH 3 acetate medium and therefore the pH 3 acetate buffer release medium was selected. The volume of the receptor medium was 2.5 mL, and the sample volume added to donor phase was 1.5 mL. The diffusional area of Franz cells was measured as 0.9 cm^2. During the studies, medium temperature was kept constant at 37 ± 0.2° C and the stirring rate was maintained at 100 rpm. The experiment was carried out taking the entire sample from the receptor medium and replenished with fresh medium. When no release was observed the experiment was terminated. All samples were analyzed by UPLC.

RESULTS

Results of particle size distribution, polydispersity index and zeta potential studies

In vitro characterization studies, particle size distribution, polydispersity index, and zeta potential were investigated and the results were found as shown in Table 2.

Table (2) Results of the characterization studies

Formulation	PSD±SD (nm)	PI±SD	Zeta potential±SD (mV)
KOH-1A	312.33±31.93	0.349±0.076	-17.05±2.26
KOH-1B	218.90±13.14	0.285±0.07	-21.10±0.93
KOH-1C	211.43±13.21	0.300±0.131	-19.52±2.02
KOH-1D	196.42±9.71	0.196±0.021	-22.93±0.41

Results of encapsulation efficiency studies

Determined encapsulation efficiencies are presented in Table 3.

Table (3) Encapsulation efficiencies

Formulation	Encapsulation efficiency±SD(%)	
	DEX	ERLO
KOH-1A	48.54±1.47	84.38±0.79
KOH-1B	52.92±1.03	86.22±1.45
KOH-1C	47.43±0.98	85.55±1.17
KOH-1D	40.45±1.63	81.89±2.17

TEM analysis image of the optimal formulation

As a result of the characterization studies, KOH-1B formulation, which has the highest encapsulation efficiency for the drugs and the most suitable values in terms of PSD, PI and zeta potential, has been determined as the optimal formulation. TEM imaging of the optimal formulation confirmed that a successful formulation was performed. The visual image of the TEM analysis is shown in Figure 1.

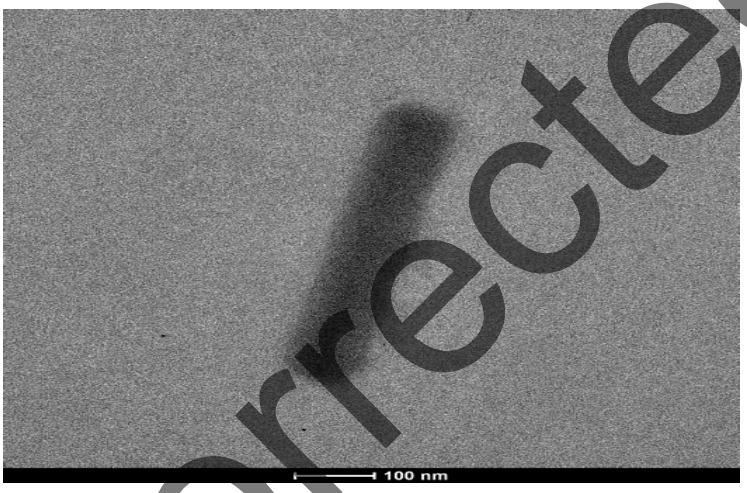

Figure (1) TEM image of the optimal formulation (x49000)

Release studies of the optimal formulation using Franz diffusion cell

The release studies of the optimal formulation and the drug solution in the pH 3 acetate buffer for 48 hours resulted in 56.73% and 50.50% for ERLO and 47.83% and 81.89% for DEX, respectively. The results of the Franz-cell diffusion studies are shown in Figure 2 and Figure 3.

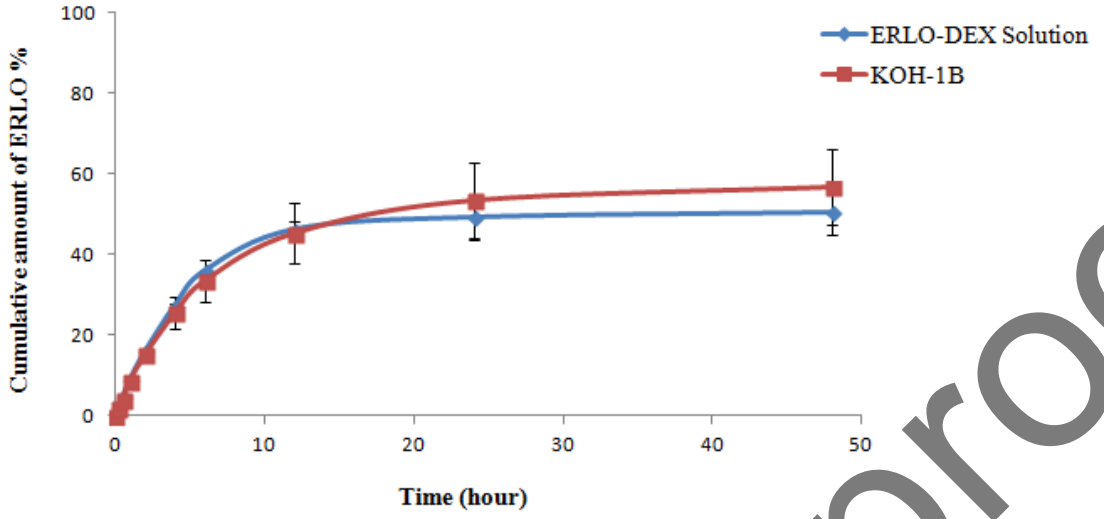

Figure (2) Franz-cell diffusion release profiles of optimal formulation (◊) and drug solution (□) for ERLO at pH 3 acetate buffer (error bars represent standard deviations, n=3)

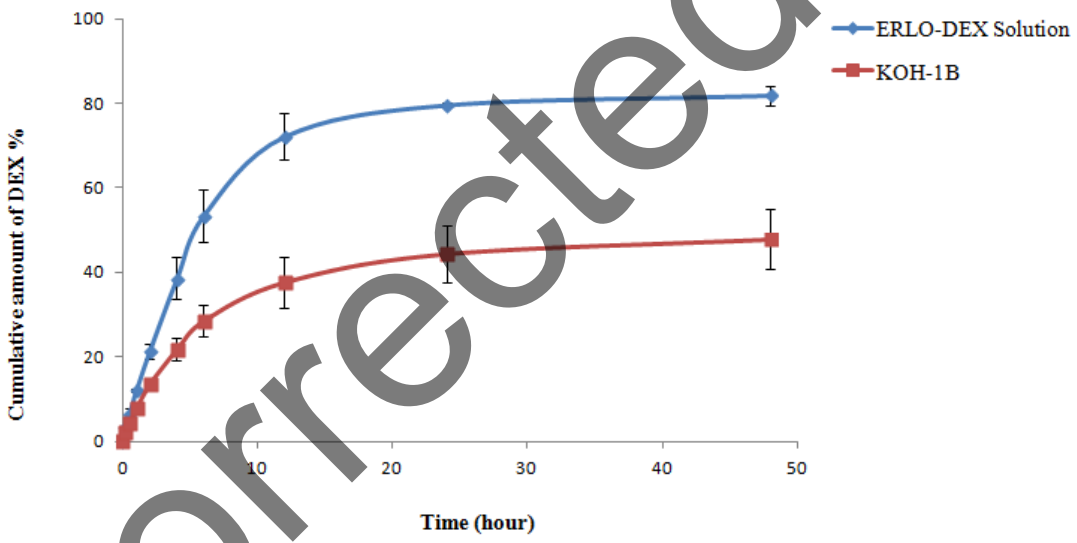

Figure (3) Franz-cell diffusion release profiles of optimal formulation (◊) and drug solution (□) for DEX at pH 3 acetate buffer (error bars represent standard deviations, n=3)

When the kinetics of the release of drug solution and formulation were calculated, it was found that both formulations were obeyed with Hixson-Crowell kinetics for ERLO and DEX, and the correlation coefficients were 0.9984 and 0.9961 for ERLO and 0.9996 and 0.9993 for DEX, respectively. The evaluated results of kinetic are shown in Table 4.

Table (4) Correlation coefficients of various release kinetics

	Drug Solution (ERLO-DEX Solution)		Formulation (KOH-1B)	
	ERLO	**DEX**	**ERLO**	**DEX**
Zero Order	0.9975	0.9986	0.9947	0.9990
First Order	0.9096	0.9121	0.8889	0.9160
Higuchi	0.9783	0.9804	0.9801	0.9766
Hixson Crowell	0.9984	0.9996	0.9961	0.9993
Korsmeyer Peppas	0.9921	0.9941	0.9944	0.9921

DISCUSSION

Erlotinib is an effective agent for the treatment of mainly non-small cell lung and pancreatic cancers and many other types of cancer. The studies show that erlotinib binds to human serum albumin while circulating in the bloodstream before going to the target site. This interaction can lead to observe some side effects such as rash, fatigue, and loss of appetite in the oral intake of drug (20). In addition to providing more effective treatment with lower doses, when nanocarrier system was used it will also prevent these toxic effects because of targeting. For this purpose, the preparation of drug delivery systems, which go selectively to the target site, should be considered. Finally it was decided to use nanocochleates that were discovered by D. Papahadjoupoulos in 1975 as a drug-delivery system and which began to be used in vaccine therapy in the 80's and 90's. Because, cochleate technology is known to be effective in the oral administration of hydrophobic drugs such as ERLO (17). Cochletates has been chosen to use as delivery system specifically for ERLO and DEX.

It is estimated that the pore size of blood vessels of tumor tissues is in the range of 400-600 nm. For this reason, the particle size of the carrier system should be 200 nm or less to reach the tumor tissue and to exploit the EPR effect (21). The nano-sized particles must have a certain zeta potentials in order to be not aggregated and it is stated that this value is ±30 mV (22). In this context, the developed carrier system should be evaluated in terms of particle size and zeta potential as well as encapsulation activities. Encapsulation efficiency, particle size distribution, polydispersity index, and zeta potential analyses were performed with four different nanocochleate formulations prepared for the purpose. In considering the particle size results, it was obtained that the carrier systems prepared have a range of 196.42-312.33 nm. Additionally, although they have small size and suitable zeta potentials (more than 15 mV) which are a sign of stability. While these results show us a successful formulation design, the KOH-1B formulation with the highest encapsulation efficiency was identified as optimal formulation. KOH-1B formulation loaded with ERLO 86.22±1.45%, which as has low water solubility and 52.92±1.03% with DEX which is water soluble. This is possibly due to the lack of an aqueous phase in the structure of the nanocochleates and thus higher encapsulation efficiencies for hydrophobic drugs have been achieved.

When $CaCl_2$ is used in a ratio of 1: 1 or 2: 2, the Ca^{+2} ions have not been enough for a complete nanocochleate formation and a flabby spiral structure has been formed. For this reason, especially DEX, which is a hydrophilic drug, can not be loaded too much. When the $CaCl_2$ ratio has been increased, a negatively charged zeta potentials of the nanoparticles observed. This can create a problem for stability of carrier systems over the time.

The release of active substances are important and the in vitro release profiles provide an information about the structure and behavior of the formulation, the possible interactions between the drug and the carrier system, and their effects on the rate and mechanism of drug release. Franz cell release studies are useful method for determining in vitro release of the drug

from micro- and nano-particles. This method is used to determine the release kinetics of various formulations including liposomes and nanoparticles (23-27). For this reason Franz cell diffusion method was preferred by using cellulose acetate membrane in our study. When the release profile of ERLO was considered, there is no significant difference between the nanocochleate and the drug solution even though less drug has been released from the drug solution. This is thought to be due to the dialysis membrane which is hydrophilic and where ERLO is hydrophobic. This is because the ERLO has quickly reached the saturation on surface of the membrane and the stagnant layer may be thick while it is in solution phase and has been hold more strongly by the membrane. However, when it has been applied with a carrier system, ERLO has slowly released to the receptor environment without reaching saturation on the membrane surface, and therefore the amount of released drug was still increasing with respect to the solution. For DEX, which has high water solubility, it is exactly the opposite. Since it has not reach any saturation on the surface of the hydrophilic membrane like itself, it has quickly passed through the drug solution to the release environment. However, since it has been released from the carrier system, a lower release value has been achieved compared to the drug solution.

When release kinetics were examined, it was determined that the drug solution and the formulation showed the Hixson-Crowell release with the highest correlation coefficient. This model argues that drug release is achieved/controlled by diffusion. Drug release from cochleate cannot be achieved by only diffusion; the dissolution of the drug particles from the surface and opening of the cochleates may also enhance the dissolution and its rate.

CONCLUSION

When all the results are considered, it is observed that ERLO and DEX active materials are successfully loaded into the carrier system in combination and nano-sized carrier systems are obtained by using a simple method such as the thin film method. TEM analysis also supports this result. In vitro release studies have shown that our systems released the drugs.

Nowadays, tablet formulations containing only ERLO are available, but serious side effects are observed with the systemic circulation passage when the free drug goes to the target site. With the drug delivery system we have designed, this difficulty will be avoided.

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