



# Development of Nanocochleates Containing Erlotinib HCl and Dexketoprofen Trometamol and Evaluation of *In Vitro* Characteristic Properties

## Erlotinib HCl ve Deksketoprofen Trometamol İçeren Nanokohleatların Geliştirilmesi ve *In Vitro* Karakteristik Özelliklerinin Değerlendirilmesi

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### ABSTRACT

**Objectives:** Erlotinib HCl is a tyrosine kinase receptor inhibitor and an anticancer agent that was first approved by the FDA in 2004 for treatment of non-small-cell lung cancer and pancreatic cancer. Dexketoprofen trometamol is a NSAID, but recent studies showed that dexketoprofen trometamol also had an effect in carcinoma due to its inhibitor effects on prostaglandins. The combination of dexketoprofen and anti-cancer agents reduces pain caused by cancer by diminishing the tumors pressure, which causes necrosis; it also lowers the poor prognosis of cancer. Combination therapy will make life easier for patients, considering drug administration and dosing. Nanocochleates are new drug delivery systems that have not been examined as much as liposomes, but they have more advantages than liposomes.

**Materials and Methods:** In this study, erlotinib HCl and dexketoprofen trometamol were loaded into nanocochleates with various formulations and particle sizes/distributions, polydispersity indexes, and zeta potential analyses were performed. Transmission electron microscopy imaging was performed with the obtained optimal formulation and drug-release studies using Franz diffusion cells were conducted.

**Results:** As a result, drug carrier systems with a particle size of 196.42-312.33 nm and zeta potential greater than 15 mV were produced. The highest encapsulation efficiency for the main active ingredient, erlotinib HCl, was obtained in the KOH-1B formulation with 86.22±1.45%.

**Conclusion:** This study showed that the drugs were successfully loaded into the nanocochleates and the nanocochleates actively released the drugs.

**Key words:** Nanocochleate, erlotinib HCl, Franz diffusion cell

### ÖZ

**Amaç:** Erlotinib HCl bir tirozin kinaz reseptör inhibitörü olup, FDA tarafından ilk kez 2004 yılında küçük hücreli olmayan akciğer kanseri ile pankreas kanserinin tedavisi için onaylanmış olan antikanserojen bir etkin maddedir. Deksketoprofen trometamol ise NSAİİ'dir. Ancak günümüzde prostaglandinler üzerindeki inhibitör etkisinden dolayı karsinomada da etkili olduğu yapılan çalışmalar ile gösterilmektedir. Bu nedenle günümüzde ayrı ayrı ürünler halinde kullanılan deksketoprofen ve antikanserojen madde kombine edildiğinde, hem hastaya alım kolaylığı sağlayarak sabit bir kan düzeyi sağlanabileceği hem de kansere bağlı ağrı ile tümörün basınç nekrozu yaratma ve kanser prognozunu kötü yönde artırma etkisinin azaltılması ve tümörün etkin bir şekilde tedavisinin mümkün olabileceği düşünülmüştür. Nanokohleatlar ise üzerinde lipozomlar kadar çok fazla çalışmanın yapılmadığı ancak lipozomlara kıyasla daha fazla avantaja sahip olan ilaç taşıyıcı sistemlerdir.

**Gereç ve Yöntemler:** Bu çalışmada, erlotinib HCl ve deksketoprofen trometamol çeşitli formülasyon çalışmaları ile nanokohleatlara yüklenmiş ve partikül büyüklüğü dağılımı, polidispersite indeksi ve zeta potansiyeli analizleri yapılmıştır. Elde edilen optimum formülasyon ile Franz difüzyon hücrelerinde salım çalışmaları ve geçirimli elektron mikroskobu görüntülemesi gerçekleştirilmiştir.

**Bulgular:** Yapılan çalışmalar sonucunda partikül büyüklüğü 196.42-312.33 nm aralığında ve zeta potansiyeli 15 mV'den büyük olan taşıyıcı sistemler üretilmiştir. Esas etkin madde olan erlotinib HCl için en yüksek yükleme verimliliği ise %86.22±1.45 ile KOH-1B formülasyonunda elde edilmiştir.

**Sonuç:** Çalışmalar sonucunda ilaçların nanokohleatlara başarılı bir şekilde yüklendiği ve yaklaşık %50 salım değerlerine ulaştığı tespit edilmiştir.

**Anahtar kelimeler:** Nanokohleat, erlotinib HCl, Franz difüzyon hücresi

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## INTRODUCTION

Erlotinib hydrochloride (ERLO) is an epidermal growth factor receptor inhibitor that was approved by the United States Food and Drug Administration (FDA) in 2004 for the treatment of non-small-cell lung cancer and its anticancer effects promised hope in various preclinical models.<sup>1</sup> Tablet forms containing ERLO are available in the market<sup>2</sup> but when the FDA-Orange Book for the USA market or the electronic Medicines Compendium for European Union 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 below pH 5.<sup>2</sup> ERLO has toxic effects such as diarrhea, skin rash, and fatigue, as well as toxic effects on pulmonary, hepatic and renal systems.<sup>3-6</sup> In order to overcome these toxic effects, it is aimed to load this drug into a nano drug carrier system. In a study on healthy rats in the literature, no toxic effect with ERLO was observed compared with its free form when it was encapsulated in polymeric nanoparticles.<sup>7</sup>

Dexketoprofen trometamol (DEX) is water-soluble but it has also some oil-solubility; DEX is a salt of the S-isomer of the racemic non-steroidal anti-inflammatory drug (NSAID) ketoprofen.<sup>8,9</sup> Although it inhibits cyclooxygenase (COX)-1 and COX-2 isoenzymes, it has partially selective activity for COX-1.<sup>10,11</sup> Recent studies with NSAIDs have shown that this drug has a protective effect against breast and colorectal cancers, which are frequently observed worldwide.<sup>12,13</sup> The underlying mechanism can be explained with angiogenesis by associated COX-derived prostaglandins.<sup>14</sup> 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<sup>2+</sup> and not containing water in the internal phase.<sup>15</sup> It is thought that as the mechanism of formation, fusion occurs through Ca<sup>2+</sup> 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.<sup>15,16</sup> Proteoliposome-derived cochleates are known to exhibit high 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.<sup>17</sup> 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 trapped molecules against harsh environmental conditions such as pH, lipase degradation, and temperature. They are also resistant to lyophilization. Cochleates include phosphatidyl serine (PS), dioleoylphosphatidylserine (DOPS), phosphatidic acid, phosphatidylinositol (PI), phosphatidylglycerol as soy-based phospholipids either alone or as mixtures.<sup>16</sup>

The aim of this study was 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,

adverse effects will be reduced, a low dose will provide more effective treatment, and combined drug administration will enhance treatment. Furthermore, the combination of an NSAID and an anticancer drug, which are currently used separately, will be more convenient for patients. Therefore, it was aimed to load ERLO, which is a hydrophobic drug, and DEX, a hydrophilic auxiliary drug, into nanocochleates and to characterize the system.

## MATERIALS AND METHODS

### Materials

DOPS and methoxy-poly(ethyleneglycol)2000-distearoylphosphatidylethanolamine (DSPE-PEG<sub>2000</sub>) were purchased from Avanti Polar Lipids, USA. Folic acid (FA), sodium acetate trihydrate, chloroform, and ethanol were from Sigma-Aldrich, Germany. ERLO was from Biotang, USA. DEX and calcium chloride dihydrate (CaCl<sub>2</sub>·2H<sub>2</sub>O) 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 12000 Da) was obtained from Sigma-Aldrich, USA.

### Analytical method and calibration

Ultra-performance liquid chromatography (UPLC), which is highly sensitive, was preferred to determine the drug-loading capacity and the cumulative drug-release studies. Initially, ERLO and DEX were scanned using an ultraviolet 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. One milligram of ERLO and 1 mg of DEX were weighed separately and transferred into a 100 mL volumetric flask. Twenty milliliters 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. In addition, 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, with a concentration of 10 µg/mL, was used as a stock solution. Solutions at concentrations ranging from 0.05 to 10 µg/mL were prepared by diluting the stock solution, samples were then analyzed using UPLC (6 replicates) and calibration curves were obtained. UPLC was found to be linear (r<sup>2</sup>=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 a rotary evaporator. Distilled water was added and vortexed for 15 min

followed by ultrasonification for 1 hour. Six millimolar  $\text{CaCl}_2$  was added dropwise to the liposome suspension with various ratios and vortexed for 30 min. Finally, the mixture was kept in a refrigerator at  $+4^\circ\text{C}$  overnight. The formulation contents and the amounts of these ingredients are shown in Table 1.

**Table 1. ERLO and DEX-loaded nanocochleate formulations**

Formulations code	KOH-1A	KOH-1B	KOH-1C	KOH-1D
DOPS	10 mg	10 mg	10 mg	10 mg
DSPE-PEG <sub>2000</sub>	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 $\text{CaCl}_2$	1:1	1:2	1:3	2:2

Determination of particle size distribution, polydispersity index and zeta potential of formulations, DOPS: Dioleoylphosphatidylserine, DSPE-PEG<sub>2000</sub>: Poly(ethyleneglycol)<sub>2000</sub>-distearoylphosphatidylethanolamine, FA: Folic acid, ERLO: Erlotinib hydrochloride, DEX: Dexketoprofen trometamol,  $\text{CaCl}_2$ : Calcium chloride

The particle sizes of the formulations were measured using laser light scattering. A Malvern Zeta-Nanosizer instrument was used to measure particle size distribution, the polydispersity index, and zeta potential. Three 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 18,000 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 drug in the formulation and the values are given as percentages. Three parallel measurements were made and mean and SD values were calculated.

#### Transmission electron microscopy (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 performed in the METU Central Laboratory. Prior to imaging, the samples were diluted 1:29 with distilled water.

#### Release studies using a Franz diffusion cell

Release studies were also performed using a 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 media.<sup>18,19</sup> When the release studies in the literature were considered, the most meaningful results were obtained in a pH 3 acetate medium; 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 the donor phase was 1.5 mL. The diffusional area of the Franz cells was measured as  $0.9\text{ cm}^2$ . During the studies, the temperature of the medium was kept constant at  $37\pm 0.2^\circ\text{C}$  and the stirring rate was maintained at 100 rpm. The experiment was conducted 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 using 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 are shown in Table 2.

### Results of encapsulation efficiency studies

Determined encapsulation efficiencies are presented in Table 3.

### TEM analysis image of the optimal formulation

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

### Release studies of the optimal formulation using a 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.

**Table 2. Results of the characterization studies**

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

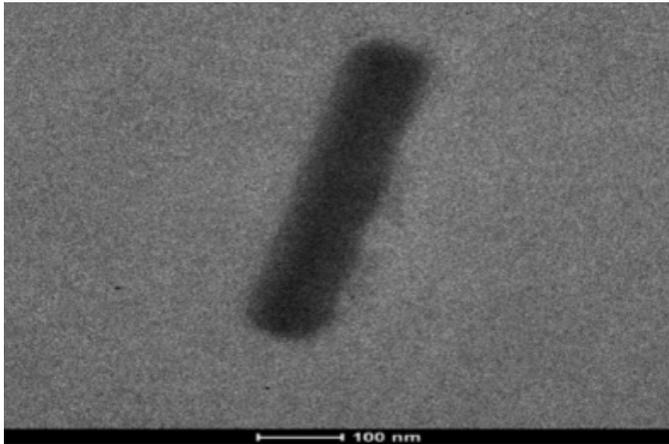
SD: Standard deviation

**Table 3. Encapsulation efficiencies**

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

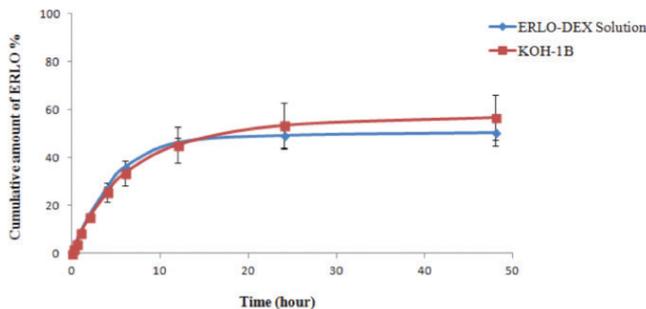
SD: Standard deviation, ERLO: Erlotinib hydrochloride, DEX: Dexketoprofen trometamol

When the kinetics of the release of drug solution and formulation were calculated, it was found that both formulations obeyed 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 kinetics results are shown in Table 4.



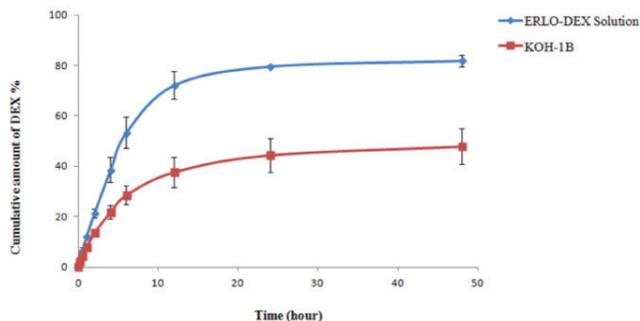
**Figure 1.** TEM image of the optimal formulation (x49,000)

TEM: Transmission electron microscopy



**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)

ERLO: Erlotinib hydrochloride, DEX: Dexamethasone trometamol



**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)

ERLO: Erlotinib hydrochloride, DEX: Dexamethasone trometamol

**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

ERLO: Erlotinib hydrochloride, DEX: Dexamethasone trometamol

## DISCUSSION

Erlotinib is an effective agent in the treatment of many types of cancer, but mainly non-small-cell lung and pancreatic cancers. 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 some adverse effects such as rash, fatigue, and loss of appetite with oral intake of drug.<sup>20</sup> In addition to providing more effective treatment with lower doses, nanocarrier systems would prevent these toxic effects because of targeting. For this purpose, the preparation of drug delivery systems that selectively target cancer cells should be considered. Finally, we decided to use nanocochleates, which were discovered by D. Papahadjopoulos in 1975 as a drug-delivery system and 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.<sup>17</sup> Cochleates has been chosen for 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.<sup>21</sup> The nano-sized particles must have a certain zeta potential in order not to be aggregated; this value is reported as  $\pm 30$  mV.<sup>22</sup> 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 purpose-made nanocochleate formulations. In considering the particle size results, it was found that the carrier systems had a size range of 196.42–312.33 nm. In addition to having small size, they also had suitable zeta potentials (more than 15 mV), which are a sign of stability. Although these results show a successful formulation design, the KOH-1B formulation had the highest encapsulation efficiency and was identified as the optimal formulation. KOH-1B formulation loaded with ERLO  $86.22 \pm 1.45\%$ , which has low water solubility and  $52.92 \pm 1.03\%$  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 were achieved.

When  $\text{CaCl}_2$  is used in a ratio of 1:1 or 2:2,  $\text{Ca}^{2+}$  ions are insufficient for a complete nanocochleate formation and a flabby spiral structure is formed. For this reason, only small amounts of DEX, which is a hydrophilic drug, can be loaded. When the  $\text{CaCl}_2$  ratio is increased, a negatively charged zeta potential of the nanoparticles is observed. This can create a problem for the stability of carrier systems over time.

The release of active substances is important and *in vitro* release profiles provide 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 a 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.<sup>23-27</sup> For this reason, Franz cell diffusion was preferred using a cellulose acetate membrane in our study. When the release profile of ERLO was considered, there was no significant difference between the nanocochleate and the drug solution, even though less drug was released from the drug solution. This is thought to be due to the dialysis membrane, which is hydrophilic, whereas ERLO is hydrophobic. This is because the ERLO has quickly reached saturation on surface of the membrane and the stagnant layer may be thick while in the solution phase and is held more strongly by the membrane. However, when it has been applied with a carrier system, ERLO has been 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, the situation is exactly the opposite; it quickly passes through the drug solution to the release environment because it has not reached any saturation on the surface of the hydrophilic membrane like itself. However, because it has been released from the carrier system, a lower release value is achieved compared with the drug solution.

When release kinetics were examined, it was determined that the drug solution and the formulation showed Hixson-Crowell release with the highest correlation coefficient. This model argues that drug release was achieved/controlled by diffusion. Drug release from cochleates cannot be achieved only through 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 was observed that ERLO and DEX active materials were successfully loaded into the carrier system in combination and nano-sized carrier systems were obtained using the simple thin film method. TEM analysis also supported this result. *In vitro* release studies showed that our systems released the drugs.

Tablet formulations containing only ERLO are currently available, but serious adverse effects are observed with the systemic circulation passage when the free drug goes to the

target site. With our drug-delivery system, this difficulty will be avoided.

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