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RESEARCH ARTICLE

Development of Liposome Formulations of Tamoxifen and Assessment of Caco-2 Cell Transportation Properties

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SUMMARY

Breast cancer is the most common type of cancer and known to be the second cause of death among the women worldwide. Nano-sized drug delivery systems such as liposomes and nanoparticles have been widely used to enhance permeability and bioavailability of the cancer agents with low solubility. In addition, these systems provide enhanced efficacy and/or reduced toxicity and less side effects. Tamoxifen is the first representive of the SERM (Selective Estrogen Receptor Modulators) group drugs, which is approved by FDA for the treatment of estrogen positive breast tumors and the most common hormonal treatment for all stages of breast cancer. In this study, new oral liposome formulations of tamoxifen were developed using dimethyl-β-cyclodextrin (DM-β-CD) and sodium taurocholate (NaTC) as absorption enhancers. Caco-2 (Human colorectal carcinoma cell line) model was used to investigate oral absorption properties of developed liposome formulations. The liposome formulations' cytotoxic properties, Caco-2 transportation properties were investigated comparatively with solutions, transepithelial electrical resistance (TEER) values were measured and apparent permeability coefficients (Papp) were calculated. According to the results, DM-β-CD successfully enhanced tamoxifen transportation. While the initial TEER value of Caco-2 cell monolayer was 285 Ω, 226 Ω was measured for tamoxifen+DM-β-CD liposomes at the end of 24 hours. This result indicated that DM-β-CD in liposome formulation, increased tamoxifen transport by opening tight junctions. Liposome formulation is a promising approach for oral tamoxifen treatment.

Key Words: *Breast cancer, Selective Estrogen Receptor Modulators (SERM), absorption enhancement, tamoxifen, liposomes, dimethyl-β-cyclodextrin (DM-β-CD)*

Tamoksifenin Lipozom Formülasyonlarının Geliştirilmesi ve Caco-2 Hücre Geçiş Çalışmalarının Değerlendirilmesi

ÖZET

Meme kanseri tüm Dünya'da kadınlar arasında en sık rastlanılan kanser türü olup, kanser kaynaklı ölümlerde ikinci sıradadır. Lipozomlar, nanopartiküller gibi nanometre boyutlarında ilaç taşıyıcı sistemler düşük çözünürlüğe sahip antikanser ajanların membranlardan geçişini ve biyoyararlanımını artırmak için yaygın olarak kullanılmaktadır. Ayrıca, bu sistemler, farmakolojik etkinliğin artmasını, ilaç kaynaklı toksisitenin ve yan etkilerin azaltılmasını sağlamaktadır. SERM (Selektif Östrojen Reseptör Modülatörleri) grubu ilaçların ilk temsilcisi olan tamoksifen FDA tarafından östrojen pozitif meme tümörlerinin tedavisinde kullanılmak için onaylanmış olup, meme kanserlerinin her döneminde en yaygın olarak kullanılan hormonal terapidir. Bu çalışmada dimetil- β-siklodekstrin (DM-β-CD) ve absorpsiyon artırıcı olarak sodyum taurokolat (NaTC) kullanılarak tamoksifenin oral yolla kullanılacak lipozom formülasyonları geliştirilmiştir. Caco-2 (İnsan Kolorektal Karsinoma hücre hattı) model olarak kullanılarak geliştirilen formülasyonların oral absorpsiyon özellikleri incelenmistir. Formülasyonların sitotoksik etkileri ve Caco-2 transport özellikleri çözeltilerle karşılaştırılmalı olarak incelenmiştir, transepitelyal elektrik rezistansı (TEER) değerleri ölçülmüş, geçiş katsayıları (Papp) hesaplanmıştır. Sonuçlara göre, DM-β-CD'nin tamoksifen geçişini artırdığı görülmüştür. 24 saatlik deney süresi sonucunda, tamoksifen+ DM-β-CD lipozomları ile, başlangıç Caco-2 hücre tek tabakası TEER değerinin 285 Ω'dan, 226 Ω'a düştüğü gözlenmiştir. Bu sonuç, DM-β-CD'nin lipozom formülasyonunda hücrelerdeki sıkı bağlantı bölgelerini açarak tamoksifen geçişini artırdığını işaret etmiştir. Lipozom formülasyonunun oral tamoksifen tedavisi için gelecek vadeden bir formülasyon olduğuna karar verilmiştir.

Anahtar Kelimeler: *Meme kanseri, Selektif Östrojen Reseptör Modülatörleri (SERM), Tamoksifen, absorpsiyon artırma, lipozomlar, dimetil- β-siklodekstrin (DM-β-CD)*

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INTRODUCTION

Breast cancer is known to be the most common type of cancer as well as the second common cause of death from cancer in women all over the world (Cardoso et al.,2013; Acevedo et al., 2014). The common reason for breast cancer related mortality is the metastatic breast cancer development (Grobmyer et al., 2012). The treatment of breast cancer is often a combination of several different methods. Firstly, surgical procedures come to mind as the most effective method (Alison, 2001) followed by chemotherapy and/or radiotherapy which usually leads unselective damages on healthy tissues (Hull et al., 2014). Another treatment approach has been hormonal therapy for estrogen receptor (ER) and progesterone receptor-positive breast cancers. Since %75 of breast cancers are ER positive (ER +), hormonal therapy is promising as an effective and quite well-tolerated treatment (Barse, 2000). There are two main drug groups for hormonal therapy which are aromatase inhibitors and estrogen receptor modulators (Riggs et al., 2003; Smith et al., 2003). Aromatase inhibitors, suppress circulating estrogen up to 90% by inhibiting peripheral aromatase (Minckwitz et al., 1998). The other group, Selective Estrogen Receptor Modulators (SERM) is a specific chemical group, structurally different from estrogen, configured as nonsteroidal estrogen (Jordan, 2007). They bind to estrogen receptors and constitute agonist or antagonist estrogen effects by interacting with the receptor in many different target tissues thereby inhibit the binding of estrogen to mimic or block its effects (Goldstein et al., 2000; McDonnell 2005). The first representative of SERM group is tamoxifen (O'Regan et al., 2002). It has been approved by FDA for breast cancer prevention and adjuvant therapy.

Liposomes, colloidal lipid vesicules have been used successfully in drug delivery for over 40 years with taking advantage of their unique structure similar to cell membrane structure; biocompatible and biodegradable properties coming through the lipid content (Lasic 1998; Allen 1997; Çoban and Değim 2013). Nanometer sized drug delivery systems such as liposomes and nanoparticles are taken up by cells more efficiently than larger micromolecules. In this concept, they are advantageous, and effective for drug transport and delivery.

Oral drug delivery is the most common and acceptable route of drug administration for many years. However, the formulation development of hydrophobic drugs is a challenge. Nanotechnology concept has been applied to the oral drug delivery with the aim of enhancing bioavailability after oral administration. There are numerous forms of reported drug delivery systems such as lipid based systems (liposomes, microemulsions, self-nanoemulsifying systems, solid lipid nanoparticles), polymer based systems, nanosuspensions (Sim et al., 2016; Kalepu et al., 2015). In oral drug development studies, the Caco-2 (Human Colon Carcinoma cell line) model is widely employed to predict the gastrointestinal permeability of drugs and formulations. This cell line expresses basic characteristics of the human small intestine like cytochrome P450 enzymes, transporters, microvilli and enterocytes (Awortwe et al., 2014).

In light of the foregoing data, the primary aim of this study was to develop liposome formulations of a low soluble BCS (Biopharmaceutics Classification System) Class II drug, tamoxifen and enhance its' transportation properties through Caco-2 monolayers. For this aim, a cyclodextrin derivative dimethyl-β-cyclodextrin (DM-β-CD) and a bile salt sodium taurocholate (NaTC) was utilized in formulation development as absorption enhancers. Caco-2 transportation studies were carried out comparatively with solution formulations, permeability coefficients were calculated and TEER values were measured.

MATERIALS AND METHODS

Materials

L-α-Dipalmitoil Fosfatidil Choline (DPPC) was purchased from Avanti Polar Lipids Inc, (USA). Tamoxifen citrate, sodium taurocholate, dimethyl-β-cyclodextrin, chitosan and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. LLC (USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Bichrom (Germany) and all cell culture equipments were provided from Grenier GmbH (Germany). All other chemicals and solvents used in experiments and analysis were in high pressure liquid chromatography (HPLC) grade and purchased from Merck (Germany).

Preparation of Tamoxifen Liposomes

The liposomes were prepared by reverse phase evaporation method with some modifications which was described previously (Mertins et al., 2009). 3 mg of tamoxifen was dissolved in 0.5 mL of methanol, 30 mg of DPPC was dissolved in 4.5 mL of ethyl acetate and mixed in a round bottom glass flask. The absorption enhancers sodium taurocholate (NaTC) or dimethyl-β-cyclodextrine (DM-β-CD) were added to the flask to obtain liposomes with absorption enhancers. 1mg/mL chitosan solution was prepared in (0.02 M acetate buffer/0.1 M NaCl) (pH 4.5) solution and 100 µL of this solution was added to the flask dropwisely to the flask in ultrasonic sonication bath.

The organic phase was removed by using a rotatary evaporator at 30-35 °C. Then an gel-like lipid film was obtained and hydrated with 5 mL of physiological saline (0.9 % sodium chloride) (PS) under sonication. The final liposome suspensions were centrifuged at 20000 g for 15 minutes at 25°C and the liposomes and supernatants were separated, the liposomes were collected at the bottom of the centrifuge tubes.

Characterization of Liposomes

The particle size and zeta potential of liposomes were determined using Zetasizer-Nano ZS-Malvern (Germany) by dispersing liposomes in PS. The liposomes were imaged by Transmission Electron Microscope (TEM).

The tamoxifen contents of liposomes were determined using HPLC. Mobile phase for tamoxifen analysis was adopted from literature with modifications of the mobile phase, column type and flow rate. The mobile phase 0.025 M dipotassium hydrogen phosphate buffer (pH was arranged by acetic acid to 8.7): methanol (10:90 %). ODS 2, 4.6x150 mm column was used at 25 °C at the wavelength of 238 nm with the flow rate of 1 mL/ minute (Fontana et.al., 2005) (Table 1).

Table 1. Validated HPLC assay conditions of tamoxifen

Column	ODS 2, 4.6x150 mm, 120 A (pore size)
Mobile phase	0.025 M dipotassium hydrogen phosphate buffer (pH was arranged by acetic acid to 8.7) : methanol (10:90 %)
Column temperature	25 °C
Flow rate	1 mL/min
Wavelength	238 nm
Retention time	6–8 min

The encapsulation efficiencies were calculated as it was reported in the literature (Mutlu et al.,2011; Mutlu Ağardan et al., 2016) by the following equation:

$$\text{EE \%} = \frac{\text{TD} - \text{UED}}{\text{TD}} x\,100$$

where EE % is percentage of encapsulation efficiency, TD is the total drug concentration, and UED is the concentration of unencapsulated drug.

Caco-2 Transportation Studies

Before Caco-2 cell transport studies MTT tests were carried out with tamoxifen (1200, 600, 300, 120, 60, 30 µg/mL), NaTC (7.5, 3.75, 1.875, 0.005 mM), DM-β-CD (5, 1.5, 0.375, 0.15 %), drug free liposomes (75, 50, 25 %), and PG/DMEM (Propylene glycol/ Dulbecco's Modified Eagle's Medium) (60:40%) with 24 hours time period to observe the effects on cell viability.

Caco-2 cells were seeded on semipermeable polycarbonate filter inserts for 21 days (1.2 cm diameter, 0.4 µm pore size) with 80,000 cells/mL density (Stevenson et al., 1999; Ottman et al., 2007). The tamoxifen solutions were prepared in PG/DMEM (60:40%) and the liposomes also were dispersed in PG/DMEM (60:40%). The transport studies were performed from apical to basolateral side of the diffusion cells at 37°C. The samples were withdrawn at predetermined time periods (15 min, 30 min, 45 min, 1h, 2h, 4h, 6h, 8h, 12h, 24h) and replaced with fresh DMEM. Tamoxifen that passed through the apical to basolateral side was analyzed with HPLC and Papp values were calculated according to the following equation (Raiman et al, 2003; Mutlu et al, 2011):

$$Papp = \frac{dQ}{dt}\frac{1}{A.C0}$$

According to this equation, dQ/dt refers permeability rate, A (cm^2) refers membrane diffusion area and C0 (µg/mL) refers the initial concentration in the donor compartment. Transport studies were evaluated with all types of liposomes and solutions for comparison.

RESULTS AND DISCUSSION

The liposomes were prepared as given in the Methods section. Following preparation liposomes were characterized by particle size, zeta potential, polidispersity index measurements and encapsulation efficiency calculations. The characterization data were given in Table 2. In this table PS, PDI, ZP, EE and SD refers to particle size, polidispersity index, zeta potential, encapsulation efficiency and standard deviation respectively.

Table 2. Formulation characteristics of liposome formulations (n=3)

Formulation Type	PS (nm±SD)	PDI±SD	ZP (mV±SD)	EE (%±SD)
Tamoxifen Liposomes	208.0 ± 4.0	0.374±0.07	11.7 ± 2.1	81.8 ± 4.6
Tamoxifen - DM-β CD Liposomes	244.7 ± 8.1	0.332±0.08	-14.8 ± 0.3	45.1 ± 6.1
Tamoxifen – NaTC-Liposomes	243.3 ± 5.0	0.214±0.17	29.3 ± 7.9	70.3 ± 6.3

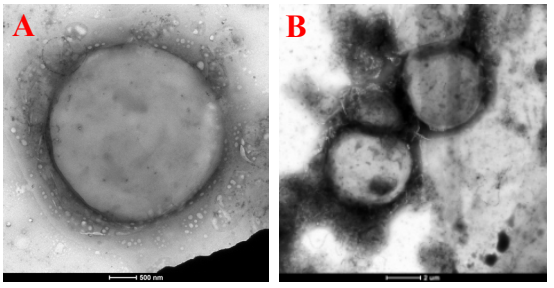

Figure 1. TEM images of (A) drug free liposomes (B) tamoxifen and DM-β-CD liposomes

The liposomes were viewed by TEM microscope and images of (A) drug free liposomes (B) tamoxifen and DM-β-CD liposomes were given in Figure 1.

Before Caco-2 cell transport studies MTT tests were carried out with tamoxifen (1200, 600, 300, 120, 60, 30 µg/mL), NaTC (7.5, 3.75, 1.875, 0.005 mM), DM-β-CD (5, 1.5, 0.375, 0.15%), drug free liposomes (75, 50, 25 %), and PG/DMEM (60:40%) with 24 hours time period to observe the effects on cell viability. The cytotoxicity data also utilized in the formulation development to choose the amount of tamoxifen, NaTC, DM-β-CD, lipid contents. Thus we made certain that our formulations could be used safely during cell transportation and in vivo studies. PG/DMEM (60:40%) had been also tested in MTT studies because it was the medium used in Caco-2 transportation studies. The aim of using PG in addition to DMEM was to solubilize tamoxifen and provide sink conditions (Yener, et.al, 2003). According to MTT test results, it was decided to use tamoxifen in 120 µg/mL, DM-β-CD in %0.15, NaTC in 0.005 mM concentrations with the cell viability results of 78.4%, 66,7, 68.4%, respectively. As a result of natural lipidic contents of liposomes, there were no significant cytotoxicity observed. Lastly, 77.4% was provided with PG/DMEM (60:40%) (Figure 2).

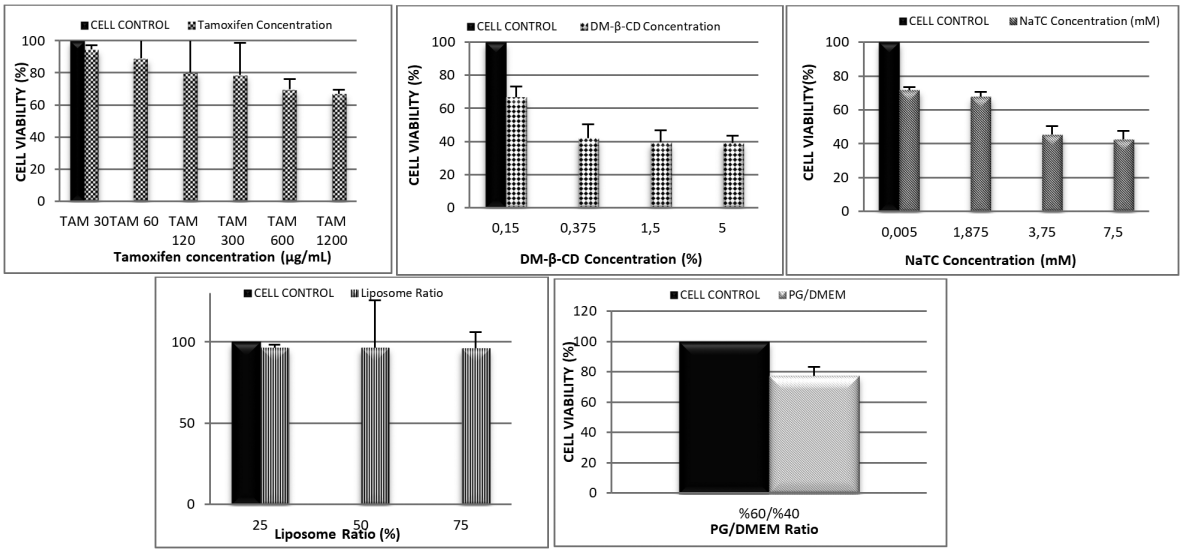

Figure 2. Cytotoxicity test (MTT) results (n=3). The error bars represent standard deviation.

The liposomes and solutions were prepared concerning MTT test results for transportation studies. The experiments were carried on at the end of 21 days cultivation period of Caco-2 cells. The samples were withdrawn at predetermined time periods and samples were analyzed with HPLC for tamoxifen. TEER values of Caco-2 monolayers were measured at the beginning and end of 24 hours experiment period. The TEER value of Caco-2 monolayer was measured as 285 Ω, initially. Then, Papp values were calculated for either solutions or liposomes. The Papp and TEER data were given in Table 3. According to

Papp and TEER data, DM-β-CD was clearly enhanced tamoxifen transportation. Lipophilic tamoxifen was encapsulated in lipidic bilayers of liposomes as a result, only tamoxifen loaded liposomes could not release tamoxifen adequately. The mechanism of DM-β-CD increasing transportation of tamoxifen was considered as increasing the solubility. In addition, DM-β-CD could also enhanced opening of tight junctions. Although NaTC was not as successful as DM-β-CD at liposome formulations, it was still effective but its effect was not significant.

Table 3. Papp and TEER data of formulations

Formulation Type	Papp (cm/hour) (n=3)	TEER Values (Ω)
Tamoxifen solution	1.58 ± 0.01	244
Tamoxifen + DM-β-CD solution	1.98 ± 0.01	226
Tamoxifen+ NaTC solution	1.68 ± 0.03	252
Tamoxifen liposomes	0.611 ± 0.020	255
Tamoxifen + DM-β-CD liposomes	1.22 ± 0.06	226
Tamoxifen + NaTC liposomes	1.20 ± 0.02	253

With DM-β-CD including tamoxifen liposomes, a significant TEER value decrease was measured. The initial TEER value 285 Ω, dropped to 226 Ω. Among the Papp values, the highest permeability coefficient (1.98 ± 0.01 cm/hour) with a TEER value 226 Ω, was belong to DM-β-CD including tamoxifen solution. Likewise, DM-β-CD including tamoxifen liposomes has the highest Papp value, 1.22 ± 0.06 cm/hour. This results confirmed the potential DM-β-CD at enhancing tamoxifen absorption.

CONCLUSIONS

In conclusion, liposome formulations of tamoxifen were developed using absorption enhancers. The effects of DM-β-CD and NaTC were tested by the means of cytotoxicity and Caco-2 cell transportation studies. DM-β-CD was considered as a suitable absorption enhancer for tamoxifen especially in liposome formulations. Caco-2 model was a very convenient method to figure out early absorption studies. Lastly, in vivo studies were planned to confirm efficacy of developed formulations.

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REFERENCES

Acevedo, C., Amaya,C. , López-Guerra, JL., 2014. Rare breast tumors: Review of the literature. Reports of practical oncology and radiotherapy 19(4), 267-274.

Alison, M.R. (2001). Encyclopedia of life sciences: Cancer. Nature Publishing Group.

Allen, T.M., 1997. Liposomes: Opportunities in drug delivery. Drugs 4, 8-14.

Awortwe, C., Fasinu, P.S., Rosenkranz, B., 2014. Application of Caco-2 cell line in herb-drug interaction studies: current approaches and challenges. Journal of Pharmaceutical Sciences 17(1), 1-19.

Barse, P.M., 2000. Issues in the treatment of metastatic breast cancer Seminars in Oncology Nursing 16(3), 197-205.

Cardoso, F., Bischoff, J., Brain E., Zotano, Á., Lück, H.J., Tjan-Heijnen, V.C., Tanner, M., Aapro, M., 2013. A review of the treatment of endocrine responsive metastatic breast cancer in postmenopausal women. Cancer Treatment Reviews 39, 457–465.

Çoban, Ö., Değim, Z., 2013. Liposomes containing imatinib mesylate and dexketoprofen trometamol: Development and characterization. FABAD Journal of Pharmaceutical Sciences 38(3), 121-126.

Fontana, G., Maniscalco, L., Schillaci, D., Cavallaro, G., Giammona, G., 2005. Solid lipid nanoparticles containing tamoxifen characterization and in vitro antitumoral activity. Drug Delivery 12, 385-392.

Goldstein, S.R., Siddhanti, S., Ciaccia, A.V., Plouffe, L., 2000. A pharmacological review of selective ostrogen reseptor modulators. Human Reproduction Update 6(3), 212-224.

Grobmyer, S.R., Zhou, G., Gutwein, L.G., Iwakuma, N., Sharma, P. ,Hochwald, S.N., 2012. Nanoparticle delivery for metastatic breast cancer. Maturitas 73, 19–26.

Hull, L.C., Farrell, D., Grodzinski, P., 2014. Highlights of recent developments and trends in cancer nanotechnology research-view from NCI alliance for nanotechnology in cancer. Biotechnology Advances 32(4), 666-678.

Jordan, V.C., 2007. Tamoxifen or raloxifene for breast cancer chemoprevention: a tale of two choices-point. Cancer Epidemiology Biomarkers&Preventation 16(11), 2207-2209.

Kalepu, S., Nekkanti, V., 2015. Insoluble drug delivery strategies: review of recent advances and business prospects. Acta Pharmaceutica Sinica B 5(5), 442-453.

Lasic, D.D., 1998. Novel applications of liposomes. Trends in biotechnology 16(7), 307- 321.

McDonnell, D.P., 2005. The molecular pharmacology of estrogen receptor modulators: implications for the treatment of breast cancer, Clinical Cancer Research (Suppl.) 11(2), 871s-877s.

Mertins, O., Lionzo, M.I.Z., Micheletto, Y.M.S., Pohlmann, A.R., Silveira, N.P., 2009. Chitosan effect on the mesophase behavior of phosphatidylcholine supramolecular systems. Materials Science and Engineering C 29(2), 463–469.

Minckwitz, G., Kaufmann, M., 1998. New endocrine approaches in the treatment of breast cancer. Biomedicine& Pharmacotherapy 52(3), 122-132.

Mutlu Ağardan, N.B., Değim, Z., Yılmaz, Ş., Altıntaş, L., Topal, T., 2016. The effectiveness of raloxifene-roaded liposomes and cochleates in breast cancer therapy. AAPS PharmSciTech. 17(4), 968-977.

Mutlu, N.B., Değim, Z., Yılmaz, Ş., Eşsiz, D., Nacar, A., 2011. New perspective for the treatment of Alzheimer diseases: Liposomal rivastigmine formulations. Drug Development and Industrial Pharmacy 37(7), 775–789.

O'Regan, R.M., Jordan, V.C., 2002. The evolution of tamoxifen therapy in breast cancer: selective estrogen-receptor modulators and downregulators, The Lancet Oncology 3(4), 207-214.

Raiman, J., Törmälehto, S., Yritys, K., Junginger, H.E., Mönkkönen, J., 2003. Effects of various absorption enhancers on transport of clodronate through Caco-2 cells. Int J Pharm 261, 129–136.

Riggs, B.L., Hartmann, L.C., 2003. Selective estrogen-receptor modulators- mechanisms of action and application to clinical practice. New England Journal of Medicine 348 (7), 618-629.

Sim, T., Lim, C., Hoang, N.H., Joo, H., Lee, J.W., Kim, D., Lee, E.S., Youn, Y.S., Kim, J.O., Oh, K.T., 2016. Nanomedicines for oral administration based on diverse nanoplatform. Journal of Pharmaceutical Investigation 46(4), 351-362.

Smith, I.A., Dowsett, M., 2003. Aromatase inhibitors in breast cancer. New England Journal of Medicine 348 (24), 2431-2442.

Yener, G., Gönüllü, Ü., Üner, M., Değim, T., Araman, A., 2003. Effect of vehicles and penetration enhancers on the in vitro percutaneous absorption of celecoxib through human skin. Pharmazie 58(5), 330-333.