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ORIGINAL ARTICLE

Complexation behavior of antiestrogen drug tamoxifen citrate with natural and modified β-cyclodextrins

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Abstract Inclusion complexes of the poorly-soluble antiestrogen drug tamoxifen citrate (TMX) were prepared with β-cyclodextrin (β-CD) and 2,3-di-*O*-hexanoyl-β-cyclodextrin (β-CDC6) being natural and amphiphilic cyclodextrins, respectively using the co-lyophilization technique. Complexation occurred in aqueous medium for natural cyclodextrin β-CD and a medium of water:ethanol mixture for the amphiphilic cyclodextrin β-CDC6. The complexes were characterized using analytical techniques including Differential Scanning Calorimetry (DSC), Fourier Transform Infrared spectroscopy (FTIR) and proton Nuclear Magnetic Resonance Spectrometry (^{1}H NMR). Anticancer efficacies of the complexes were determined against MCF-7 human breast carcinoma cell line with MTT assay. It was found that tamoxifen citrate can be incorporated in the cavity for β-CD and both in the cavity and the aliphatic chains for β-CDC6. The latter having two hydrophobic sites for inclusion of water-insoluble drug exhibited significantly higher anticancer efficacy accordingly.

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Introduction

Antiestrogen drug Tamoxifen has been the clinical choice for the antiestrogen treatment of advanced or metastatic breast cancer for more than 20 years [1–3]. However, while tamoxifen is antiestrogenic to the breast, it also acts as an estrogen to the uterus. One of the most severe side effects of tamoxifen administration is reported to be its proliferative effect on the endometrium [4, 5]. Other side effects include liver cancer, increased blood clotting and ocular side-effects such as retinopathy and corneal and opacities. These effects were reported to be dose-dependent suggesting the use of lower doses with colloidal delivery systems to be the key approach for the formulation of tamoxifen for long-term chemoprevention of breast cancer [6]. This approach was based on achieving required amount of drug at tumor site for a certain period of time and minimizing side effects on other organs of the body.

Cyclodextrins have been used to solubilized or stabilize drugs by forming inclusion complexes resulting in the masking of physicochemical properties of the free drug [7, 8]. Amphiphilic cyclodextrins are chemically obtained derivatives of natural cyclodextrins (α-, β- and γ-cyclodextrin) modified on the primary and/or secondary face with aliphatic chains of varying length (C2–C18) and structure (linear or branched) linked with different chemical bonds including ester, ether, thiol or amide bonds [9, 10].

In this study an approach based on the hypothesis of localization of Tamoxifen citrate (TMX) in the target tissue with lower dose administration was used. To achieve this goal, TMX was complexed to natural cyclodextrin, β-cyclodextrin (β-CD) and modified, amphiphilic β-cyclodextrin, β-CDC6, to increase its limited aqueous solubility and to achieve similar anticancer efficacy with a lower dose of TMX incorporated in an inclusion complex with natural and/or amphiphilic β-cyclodextrins.

Experimental

Materials

Tamoxifen citrate (TMX) was a kind gift from TEVA Pharmaceuticals (Plantex Netanya), Israel. Amphiphilic β-cyclodextrin, β-CDC6, modified on the secondary face with 6C aliphatic ester has been synthesized, purified and characterized as reported previously [11]. β-cyclodextrin (Kleptose®) was purchased from Roquette Freres, France. All organic solvents were of HPLC grade and were used without further purification.

Preparation of inclusion complexes

Inclusion complexes, TMX:β-CD and TMX:β-CDC6 were prepared with 1:1 molar ratio using the modified co-lyophilization techniques to fit the physicochemical properties of β-CD and β-CDC6 [12].

In this technique, briefly, TMX and β-CD were dispersed in deionized water and stirred to equilibrium for 5 days. Resulting suspension was filtered off and the filtrate was lyophilized to achieve the TMX:β-CD in powder form. On the other hand, TMX and β-CDC6 were both dissolved in absolute ethanol. Deionized water twice the volume of ethanol was added to form a dispersion which was stirred for equilibrium for 5 days followed by the evaporation of ethanol and lyophilization of aqueous disperson.

Characterization of inclusion complexes

Resulting inclusion complexes were characterized by techniques such as Fourier Transform Infrared (FTIR) spectrometry (Nicolet 520 FTIR spectrophotometer (Thermo Electron Corp, Waltham, MA)), Differential Scanning Calorimetry (DSC) (DuPont 910 differential scanning calorimeter (Wilmington, DE)) Proton NMR spectrometry (^{1}H NMR) (Bruker DPX 400 Digital FT-NMR Spectrophotometer, Germany) and Scanning Electron Microscopy (SEM) to demonstrate the formation and nature of inclusion complexes.

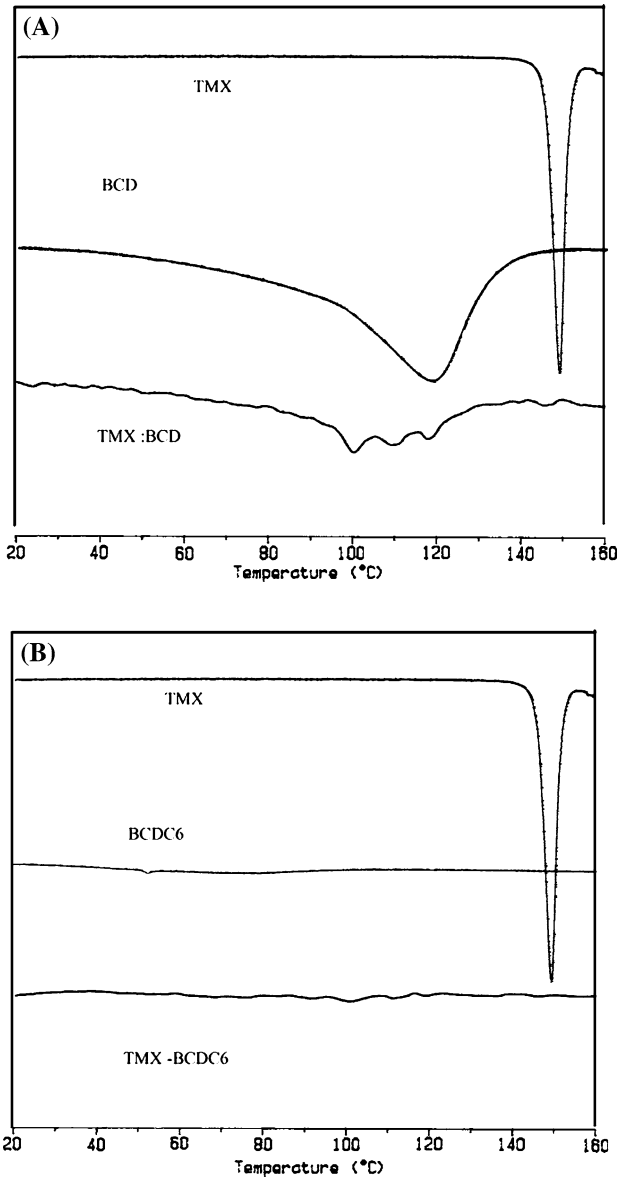

Fig. 1 (**A**) DSC thermograms of TMX, β-CD and TMX:β-CD co-lyophilizate, (**B**) DSC thermograms of TMX, β-CDC6 and TMX:β-CDC6 co-lyophilizate

Determination of anticancer efficacy of complexes

Finally, the anticancer efficacy of TMX:β-CD and TMX:β-CDC6 complexes of 0.1 mg/mL each were compared using MCF-7 human breast cancer cell line (1,000 cells/well) with MTT assay to determine cell viability. MCF-7 cells were maintained in DMEM supplemented with 10% Fetal Calf Serum at 37 °C in a humidified incubator containing 5% CO_2. Confluent cell monolayers were trypsinized and cells in

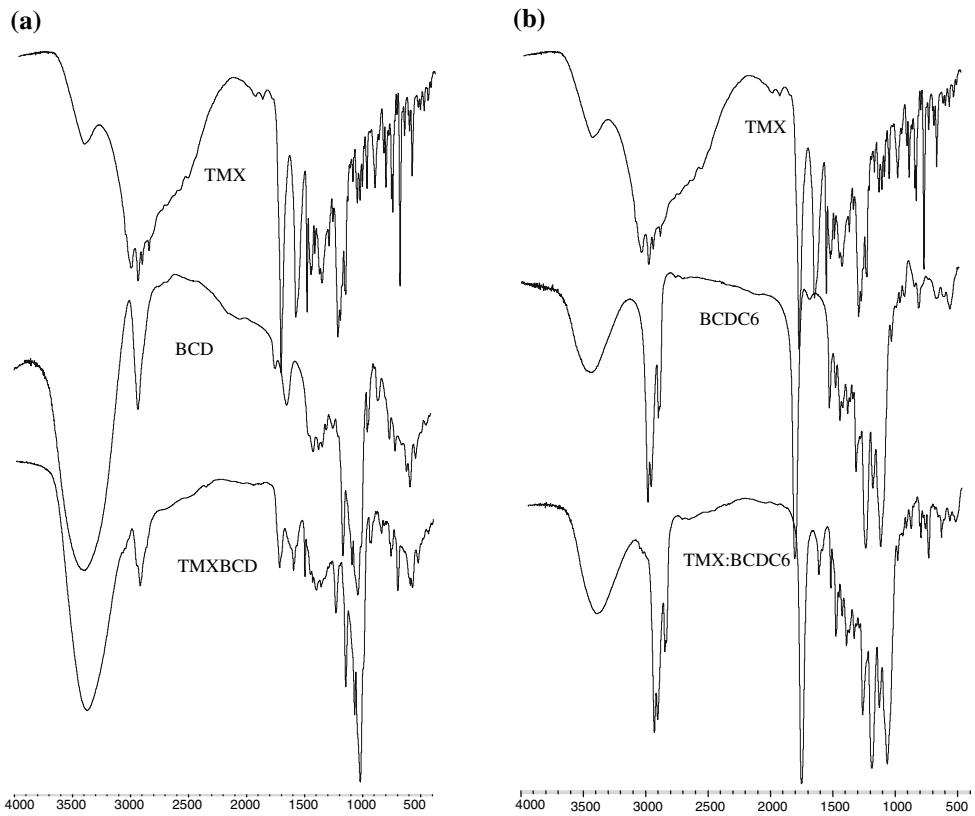

Fig. 2 (a) FTIR spectra of TMX, β-CD and TMX:β-CD co-lyophilizate, (b) FTIR spectra of TMX, β-CDC6 and TMX:β-CDC6 co-lyophilizate

Table 1 ^{1}H NMR chemical shifts, δ (ppm), of C–H protons of β-CD and tamoxifen citrate:β-cyclodextrin complex taken in DMSO

	β-CD	Complex
H-1	4.82	4.81
H-2	3.30	3.30
H-3*	3.70	3.65
H-4	3.20	3.20
H-5*	3.65	3.55
H-6a,b	3.75	3.75

* Internal protons of cyclodextrin molecule that are expected to be affected by a potential inclusion complex formation

Table 2 ^{1}H NMR chemical shifts, δ (ppm), of C–H protons of β-CDC6 and tamoxifen citrate:β-CDC6 complex taken in DMSO

	β-CDC6	Complex
H-1	5.07	5.05
H-2	4.75	4.75
H-3*	5.30	5.05
H-4	3.70	3.70
H-5*	4.10	4.05
H-6a,b	3.90	3.90

* Internal protons of cyclodextrin molecule that are expected to be affected by a potential inclusion complex formation

exponentially growing phase were used in cytotoxicity experiments. The cytotoxicity of tamoxifen citrate complexes with β-CD and β-CDC6 against MCF-7 cells was assessed using MTT assay [13]. After 48 h incubation, 20 μL MTT solution (5 mg/mL) was added to each well and the plates were incubated for further 2 h. The solution in each well containing media, unbound MTT and dead cells were removed by suction and 100 µl of DMSO was added to each well. The plates were then shaken and the optical density (OD) was read on Σ960 ELISA reader at test wavelength of 570 nm. Cells incubated in culture medium alone served as a control for cell viability (untreated wells).

Results and discussion

Various techniques were used in order to elucidate the structure and the formation of 1:1 inclusion complexes of tamoxifen citrate with natural cyclodextrin β-CD and amphiphilic cyclodextrin β-CDC6. Each technique confirms or completes the previous technique and the combined data obtained from these techniques add up to decide whether a total or partial inclusion complex is formed [14–16].

In this context, thermal behavior of the drug in free form and in complex were determined by DSC analysis. Figure 1a, b displays the DSC thermograms of lyophilized

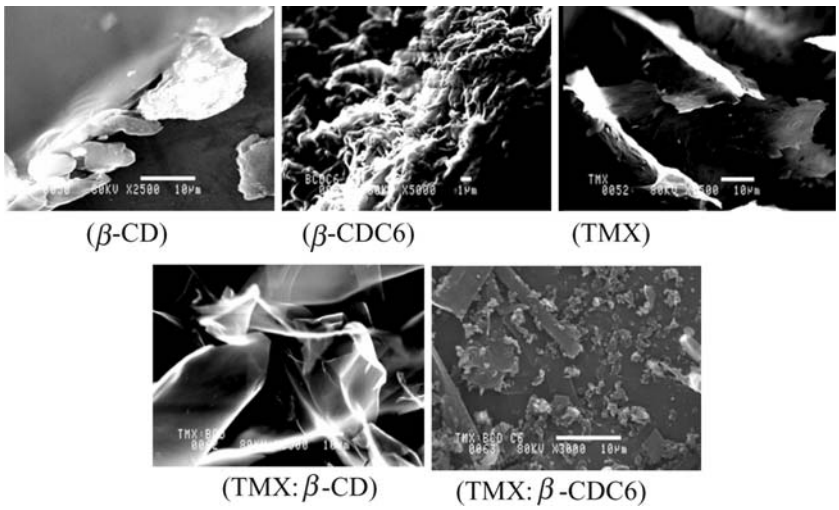

Fig. 3 SEM photomicrographs of β-CD, β-CDC6, TMX and the co-lyophilizates

TMX, cyclodextrin and the co-lyophilizate for TMX:β-CD and TMX:β-CDC6, respectively. Thermograms indicate the absence of free crystalline TMX due to the disappearance of typical TMX melting endotherm at 149 °C. DSC thermogram of TMX was taken after lyophilization of the drug in order to avoid alterations in the crystalline structure to amorphous state during the lyophilization process. FTIR spectra of the co-lyophilizate also indicated the disappearance of typical bands of tamoxifen citrate such as tertiary amine bands at 900–1100 cm^{-1}, C=C stretching peaks at 1600–1700 cm^{-1} and –COOH band at 3000 cm^{-1}. This indicates that the drug's chemical properties are masked due to inclusion in CD cavity for both natural and amphiphilic cyclodextrins. Corresponding FTIR spectra are seen in Fig. 2a, b. H NMR spectra taken at 400 MHz indicate signal shifts in the internal protons of the cavity H-3 and H-5 with shifts for H-3 being more significant for β-CDC6 compared to β-CD. Chemical shifts in proton signals for β-CD complex and β-CDC6 complex of tamoxifen citrate, respectively are given in Tables 1 and 2. Finally, SEM photomicrographs given in Fig. 3a–e indicate that the crystalline structures of the co-lyophilizates are significantly different than the free drug and the cyclodextrins. This could be due to the co-lyophilization techniques that is used to prepare the complexes which is very similar to the spherical crystallization techniques used in the manufacturing of nanoparticles. Crystalline structures of the free drug and the cyclodextrins are changed after the co-lyophilization process with a completely new crystalline or amorphous structure for the complex [17].

Anticancer efficacy of TMX:β-CDC6 was found to be significantly higher than the TMX:β-CD as shown in Fig. 4. This was attributed to the fact that β-CDC6 possesses two hydrophobic zones that the drug may be

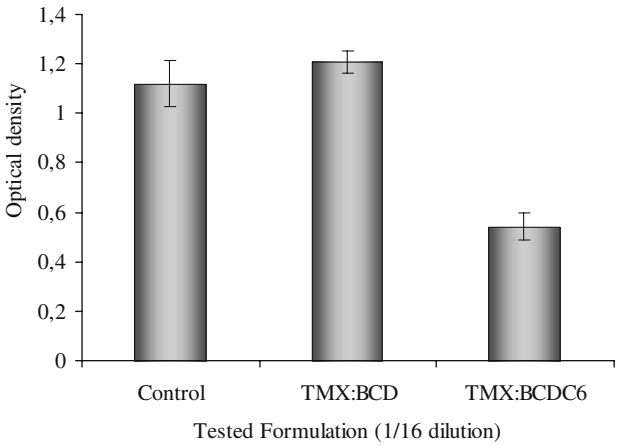

Fig. 4 Anticancer efficacy expressed by cell viability (OD) of MCF-7 cells after administration of TMX:β-CD, TMX:β-CDC6 and control (treated with PBS pH 7.4)

incorporated within; the CD cavity and the long aliphatic chains [17]. β-CDC6 is aligned with 14 aliphatic chains of 6C length which provides an advantage for interaction with lipophilic molecules. Thus, it is believed that higher amount of TMX was believed to be entrapped in amphiphilic cyclodextrin; β-CDC6 when compared to natural cyclodextrin, β-CD leading to significantly higher anticancer efficacy against human breast carcinoma cell line MCF-7.

Conclusion

Poorly-soluble antiestrogen drug tamoxifen used in the treatment of metastatic breast cancer can be complexed to both natural and amphiphilic β-cyclodextrins. Complexation to amphiphilic β-cyclodextrin seems to result in high anticancer efficacy for the drug. CD

complexation may be an alternative in the formulation development of tamoxifen to reduce severe side effects associated with dose.

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References

1. O'Reagan, R.M., Jordan, V.C.: The evolution of tamoxifen therapy in breast cancer: selective oestrogen-receptor modulators and downregulators. Lancet Oncol. **3**, 207–214(2002)
2. Pappas, S.G., Jordan, V.C.: Chemoprevention of breast cancer: current and future prospects. Cancer Metastasis Rev. **21**, 311–321 (2002)
3. Powles, T.J.: Anti-oestrogenic chemoprevention of breast cancer- the need to progress. Eur. J. Cancer **39**, 572–579 (2003)
4. Peters-Engl, C., Frank, W., Danmayr, E., Friedl, H.P., Leodolter, S., Medl, M.: Association between endometrial cancer and tamoxifen treatment of breast cancer. Breast Cancer Res. Treat. **54**, 255–260 (1999)
5. Cohen, I.: Endometrial pathologies associated with postmenopausal tamoxifen treatment. Gynecol. Oncol. **94**, 256–266 (2004)
6. de Lima, G.R., Facina, G., Shida, J.Y., Chein, M.B.C., Tanaka, P., Dardes, R.C., Jordan, V.C., Gebrim, L.H.: Effects of low dose tamoxifen on normal breast tissue from premenopausal women. Eur. J. Cancer **39**, 891–898 (2003)
7. Albers, E., Müller, B.W.: Cyclodextrin derivatives in pharmaceuticals. CRC Crit. Rev. Ther. Drug Carrier Syst. **12**(4), 311–337 (1995)
8. Loftsson, T., Masson, M., Brewster, M.E.: Self-association of cyclodextrins and cyclodextrin complexes. J. Pharm. Sci. **93**(5), 1091–1099 (2004)
9. Duchene, D., Ponchel, G., Wouessidjewe, D.: Cyclodextrins in targeting. Applications to nanoparticles. Adv. Drug Deliv. Rev. **36**, 29–40 (1999)
10. Memisoglu, E., Bochot, A., Şen, M., Özalp, M., Duchene, D., Hıncal, A.A.: Direct formation of nanospheres from amphiphilic β-cyclodextrin inclusion complexes. J. Pharm. Res. **20**(1), 117–125 (2003)
11. Memisoglu, E., Bochot, A., Şen, M., Charon, D., Duchene, D., Hıncal, A.A.: Amphiphilic β -cyclodextrins modified on the primary face: synthesis, characterization and evaluation of their potential as novel excipients in the preparation of nanocapsules. J. Pharm. Sci. **91**(5), 1214–1224 (2002).
12. Hedges, A.R.: Industrial applications of cyclodextrins. Chem. Rev. **98**, 2035–2044 (1998).
13. Yang, X.H., Sladek, T.L., Liu, X., Butler, B.R., Froelich, C.J., Thor, A.D.: Reconstitutionof caspase 3 sensitizes MCF-7 breast cancer cells to doxorubicin- and etoposide-inducedapoptosis. Cancer Res. **61**, 348–354 (2001)
14. Hedges, A.R., Shieh, W.J.: Complexation in modified cyclodextrins compared with unmodified cyclodextrins. In: Duchene, D. (ed.) New Trends in Cyclodextrins and Derivatives. Editions de Santé, Paris (1991)
15. Schneider, H.J., Hackle, F., Rudiger, V.: NMR studies of cyclodextrins and cyclodextrin complexes. Chem. Rev. **98**, 1755–1785 (1998).
16. Thompson, D.O.: Cyclodextrins as enabling excipients: their present and future use in pharmaceuticals. CRC Crit. Rev. Ther. Drug Carrier Syst. **14**(1), 1–104 (1997).
17. Memisoglu-Bilensoy, E., Bochot, A., Trichard, L., Duchene, D., Hıncal, A.A.: Amphiphilic cyclodextrins and microencapsulation. In: Benita, S. (ed.) Microencapsulation-2nd and Revised Edition, pp. 269–295, Taylor & Francis Ltd, New York, (2003)