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RESEARCH ARTICLE

Nanoparticle and Liposome Formulation of Doxycycline and Investigation of Transport Properties Through Caco-2 Cell Lines

Çiğdem Yücel was awarded the Young Scientist prize of FABAD for her article.

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Summary

Controlled release systems are nowadays successfully used for treating diseases. Nanoparticles and microparticles are used to improve the quality of life, to decrease drug dosages, to prolong dosage intervals and are used for protection from harmful side effects. Glioblastoma multiforme is the most common, disruptive and fast growing malign tumor in the central nervous system. Doxycycline is a type of tetracycline antibiotic that plays a key role in the growth and spread of the tumor as it inhibits the MMP-2 enzyme and produces an antitumor effect. In this study, doxycycline liposome and nanoparticle formulations were prepared. In order to determine the various concentrations of the different substances to be used in different formulations, MTT tests were performed, penetration specifications of the cell cultures of doxycycline Caco-2 were investigated, by comparing the release kinetics of the formulations. Doxycycline encapsulation efficiencies were calculated as 67.4% for the liposomes, whereas this figure for the prepared nanoparticles was calculated as 53.4%. As the result of the MMP-2 test, inhibition percentage of the doxycycline liposomes was found to be 96%, which is thought to help to stop the dissemination of the glioblastoma cells.

Key Words: *Doxycycline, Nanoparticle, Liposome, Glioblastoma multiforme.*

Doksisiklinin Nanopartikül ve Lipozom Formülasyonu ve Caco-2 Hücrelerinden Geçiş Özelliklerinin İncelenmesi

Özet

Kontrollü salım sistemleri, günümüzde birçok hastalığın tedavisinde başarı ile uygulanmaktadır. Nanopartiküler ve mikropartiküler ilaç taşıyıcı sistemler yaşam kalitesini artırmak, ilaç dozunu azaltmak, dozlama aralığını uzatmak ve yan ve zararlı etkilerden korumak amaçlı olarak kullanılmaktadır. Glioblastoma multiforme merkezi sinir sisteminin en yaygın, tahrip edici, hızla yayılan malign tümörüdür. Doksisiklin, tümörün yayılması ve metastazında önemli rol oynayan bir enzim olan MMP-2'nin inhibisyonuyla antitümör etki yapan tetrasiklin türevi bir antibiyotiktir. Bu çalışmada, doksisiklinin lipozom ve nanopartikül formülasyonu hazırlanmıştır. Formülasyonlarda kullanılacak maddelerin farklı derişimleri için MTT testleri yapılmış, doksisiklinin Caco-2 hücre kültürlerinden geçiş özellikleri, formülasyonlardan salım kinetikleri kıyaslanarak incelenmiştir. Hazırlanan lipozomlarda doksisiklinin enkapsülasyon verimi, lipozomlar için %67.4, hazırlanan nanopartiküllerde ise doksisiklinin enkapsülasyon verimi %53.4 olarak hesaplanmıştır. MMP-2 testi sonucunda, yüzde inhibisyon yüzdesi doksisiklin içeren lipozomlarda %96 olarak bulunmuş ve bu inhibisyon yüzdesinin glioblastoma hücrelerinin yayılmasını önlemede katkıda bulunacağı düşünülmüştür.

Anahtar Kelimeler: *Doksisiklin, Nanopartikül, Lipozom, Glioblastoma multiforme*

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INTRODUCTION

Doxycycline is a semisynthetic antibiotic with a broad spectrum of activity against a wide range of gram negative and positive pathogens (1). The present data in the literature indicate that doxycycline as a cytotoxic agent and matrix metalloproteinases inhibitor, can be a reasonable candidate for the treatment of glioblastoma multiforme (2). Liposomes can act as biocompatible, biodegradable, non-immunogenic drug carriers, hence the advantages of liposomal-encapsulated drugs are their prolonged duration of exposure (3). Nanoparticles are submicron-sized polymeric colloidal particles with a therapeutic agent of interest encapsulated within their polymeric matrix, or adsorbed or conjugated onto the surface (4). Caco-2 cells are human colon cancer derived cells, which are increasingly used in in-vitro blood-brain barrier (BBB) models, in passive permeability and membrane transport studies (5,6). In this study, Caco-2 cells were used as a model. The cytotoxic effects of doxycycline on Caco-2 cells, the penetration of doxycycline nanoparticle and liposome formulations through Caco-2 cells were investigated. MTT test was performed to test doxycycline effect on the viability of Caco-2 cells.

MATERIAL and METHODS

Preparation of nanoparticles

Doxycycline nanoparticles were prepared using emulsion polymerisation. Eudragit-RS-100 was used as a polymer. The polymer (2 g) was dissolved in methanol at room temperature, then was injected slowly (0.5 mL.min^{-1}) into aqueous phases containing Doxycycline and 0.4% PVA (polyvinilalcohol). The mixture was stirred at 8000 rpm by Ultraturrax® for 5 minutes. After evaporation, nanoparticle suspensions were ultracentrifuged at 15000 rpm at 25°C for 35 min. Supernatants and nanoparticles were separated.

Preparation of liposomes

Doxycycline liposomes were prepared using dry film hydration method. Cholesterol, phospholipid (dipalmytoilphosphatidiylcholine-DPPC) and doxycycline were added to the flask. After it was dissolved in chloroform, was then evaporated under rotavapor at ~44°C. The dry film was hydrated by phosphate buffer (pH 7.4) and vortexed. Liposome suspensions were ultracentrifuged at 15000 rpm at 25°C for 60 min. Supernatants and liposomes were separated.

Preperation of cell culture

Caco-2 cells were seeded on polycarbonate filter inserts for 21 days (1.2 cm diameter, 0.4 µm pore size) with 80000 cells/cm^2 density (7).

MTT test

MTT test was performed to study the doxycycline effect on viability of the Caco-2 cells. Cell viability was also investigated using DMEM for 24 hours periods. Various doxycycline concentrations (20, 15, 10, 5, 2.5 µg/mL), and liposome formulations (75, 50, 25 µl volumes) were studied in DMEM.

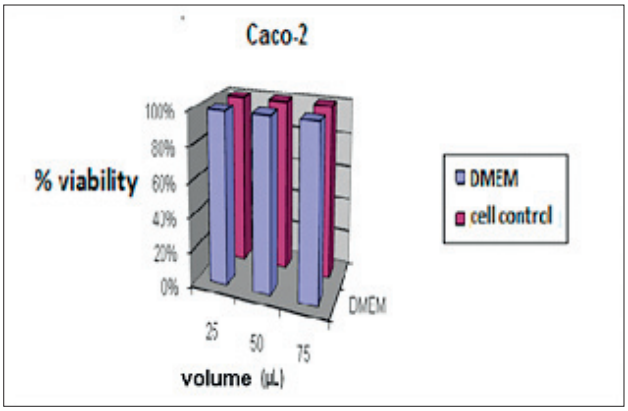
Penetration Experiments

According to MTT test results, the cell viability seemed to be higher with DMEM and it was used for penetration studies. Doxycycline concentration did not exceed to 10 µg/mL for 24 hours of penetration period. (Figure 1). Caco-2 monolayer containing membrane was placed between donor and receptor compartments of vertical diffusion cells. 95% O_2 and 5% CO_2 were delivered to the system within the experiment period at 37ºC to maintain cell viability. Penetration experiments were performed from apical to basolateral compartment. Dialysis membrane penetration studies were also performed at 37ºC using pH 7.4 phosphate buffer. Samples were taken out at specified time periods. Removed sample volumes were always replenished with fresh DMEM. The doxycycline content of the samples were analyzed by a UV spectrophotometer.

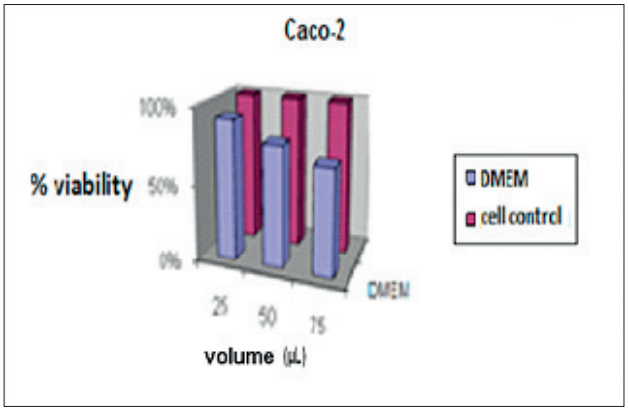
Apparent permeability coefficients

Apparent permeability coefficient (Papp = k) of doxycycline was calculated according to the following equation:

$$P\text{app} = \frac{dQ}{dt}\,\frac{1}{AC_0}$$



(1)



(2)

Figure 1. Effects of doxycycline liposome (1) and nanoparticle (2) formulation on the Caco-2 cell viability.

dQ/dt refers to permeability rate, A (cm^2) refers to membrane diffusion area, C_0 (µg/mL) refers to the initial concentration of doxycycline in the donor compartment.

MMP-2 determination

Approximately $5x10^5$ Caco-2 cells were seeded to 35 mm wells. They were incubated for two days and washed with DMEM. Studied groups were incubated with 1 mL DMEM for 18 h. After the cells were centrifugated, MMP-2 was determined from the upper clear part of the kit.

RESULTS

The cumulative amount of doxycycline nanoparticle and liposome formulations transported through apical to basolateral side of Caco-2 cells was found to be 53.4% and 67.4%(n = 3) for doxycycline nanoparticles and liposomes, respectively (Figure 2).

Table 1. Apparent permeability coefficients (k) of doxycycline liposome and nanoparticle.

Samples	Apparent permeability coefficients (k)
Doxycycline solution	0.881 ±0.030
Doxycycline nanoparticle	1.21 ±0.17
Doxycycline liposome	2.82 ±0.15

Apparent permeability coefficients (k) were calculated as 1.21 ±0.17, 2.82 ±0.15 (n = 3) for doxycycline nanoparticles and liposomes, respectively (Table 1). Based on the secretion of MMP-2 from cancer cells, various formulations containing MMP inhibitor, doxycycline, have been tested and it was found that the growth of the cancer cells was inhibited by developed doxycycline formulations. The best inhibition percentage of 96.6 ±0.003%(n = 4) was seen significantly with doxycycline liposomes ($p < 0.001$) (Table 2).

DISCUSSION

In the present study MLV liposomes were prepared by the dry film hydration method using DPPC and

Table 2. MMP-2 determination of the percentage of results.

Formulations	concentration%
Control grup	100 ±0.00 (n = 7)
Doxycycline liposome	96.6 ±0.003 (n = 4)
Doxycycline nanoparticle	98.9 ±0.002 (n = 4)

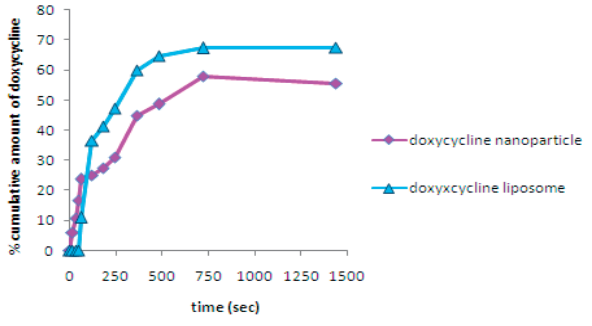


Figure 2. Cumulative amount of doxycycline nanoparticle and doxycycline liposome.

cholesterol. The amount of doxycycline captured in doxycycline liposomes were 37.6%. It is possible to achieve an encapsulation efficiency above 70% theoretically, but this cannot be reached practically. The encapsulation efficiency of 50% has been reported as quite high for liposomes.

The amount of doxycycline captured in doxycycline nanoparticles were 50.8%. Encapsulation efficiencies obtained from the literature were used to compare with varying formulation parameters up to 70% encapsulation efficiencies obtained, and reported to be good enough (7).

The penetration across Caco-2 cells studies were performed for solution, liposome and nanoparticle formulations. In literature, Caco-2 cells are used for blood-brain mimicking (6) and Caco-2 cells are also used to estimate blood-brain barrier (BBB) permeability (6,8). Permeability coefficients were also calculated from solution, nanoparticle, liposome formulations and the lowest value was 0.881 cm/h for doxycycline solution, the penetration value found for the nanoparticle was 1.21 cm/h for doxycycline and from the liposome penetration data, highest value was found to be 2.82 cm/h for doxycycline liposome. These results indicate that the membrane structure of liposome, due to the similarities in the cell membrane structures, liposomes are able to penetrate more into the cells and get endositozed easier by the cells. Consequently, doxycycline transport is found higher in liposome, in comparison to doxycycline nanoparticle. Finally, MMP2 was found to be inhibited more with Doxycycline when liposome formulation was used.

CONCLUSION

It was concluded that doxycycline permeability coefficient and% cumulative amount of doxycycline were found to be higher in liposome formulation compared to nanoparticles. Inhibition percentage of liposome formulations showed that this was the best inhibition of MMP-2. This suggests that liposome formulations can be more effective and can be used for treating glioblastoma multiforme.

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