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IN-VITRO EVALUATION OF ZIDOVUDINE NIOSOMES FOR ANTI-HIV ACTIVITY

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ABSTRACT

The present study investigates the drug targeting of anti-retroviral drugs, such as zidovudine, by incorporation into niosomes and its anti-HIV activity was compared with zidovudine solution using the TZM- bl assay. Niosomal formulations containing tween 60, tween 80 were prepared and characterized for mean vesicle size, entrapment efficiency and *in-vitro* release. Zidovudine niosomes with tween 80 has the greater entrapment efficiency of 80.32 % than with tween 60 having 76.24 %. The niosomal formulation with tween 80 was chosen as the best formulation as it has greater entrapment efficiency and sustains the release of drug for longer time. Zidovudine niosomes has shown slightly greater % inhibition against the HIV-1 viruses (Ada5, and VB 59) when compared with the drug in solution.

KEYWORDS: Zidovudine, Niosomes, TZM-bl assay.

INTRODUCTION

Nonionic surfactant vesicles (niosomes) formed from self-assembly of hydrated synthetic nonionic surfactant monomers are capable of entrapping a variety of drugs and have been evaluated as an alternative to liposomes [1]. Nonionic surfactants form unilamellar and multilamellar vesicles that have similar physical properties to liposomes and are a relatively inexpensive drug delivery system. In niosomes, soluble drug molecules are present in the aqueous compartments between the bilayer whereas insoluble ones are entrapped within the bilayer matrix. The use of niosomes for drug delivery can alter the biodistribution to provide a greater degree of targeting of the drug to selected tissues, sustained release and altered pharmacokinetics [2-4].

Zidovudine (AZT) the first anti-HIV compound approved for clinical use is still widely used alone or in combination with other antiviral agents for treatment of AIDS and AIDS-related complex. The main limitations on the therapeutic effectiveness of AZT are its dose-dependent hematological toxicity, high first-pass metabolism, poor bioavailability and very short biological half-life [5]. After oral administration it is rapidly absorbed from the gastrointestinal tract with a peak plasma concentration of 1.2µg/ml, at 0.8 hr. It is also rapidly metabolized to the inactive glucuronide with a mean elimination half-life ($t_{1/2}$) of 1 hr. This necessitates frequent administration of large doses (200 mg every 4 hrs) since it is crucial to maintain the systemic drug concentration within the therapeutic level throughout the treatment course.

HIV primarily infects helper T cells, macrophages and dendritic cells, which are vital to the human immune system. Vesicles or particles from 150 nm to 2 µm exhibit maximal phagocytosis by macrophages [6], while vesicles in the size range of 70 to 150 nm prolong plasma drug concentrations.

The purpose of this study was to perform the *in-vitro* evaluation of the zidovudine niosomes for the anti-HIV activity using the TZM-bl assay in which TZM-bl is a HeLa cell clone that was engineered to express CD4 and CCR5 and contains integrated reporter genes for firefly luciferase and *E. coli* β-galactosidase under control of an HIV-1 long terminal repeat sequence.

MATERIALS AND METHODS

Materials

AZT was a gift from Aurobindo Ltd (India). Cholesterol (CHOL), tween 60, tween 80, was from Sigma (USA). All materials used in the study were of analytical grade.

Methods

Formulation of niosomes

Multilamellar niosomes were prepared by the thin-film hydration method. Accurately weighed quantity of drug (75 mg), surfactant (Tween 60 or Tween 80), and cholesterol (ratio of cholesterol: non-ionic surfactant is 1:4.5) were dissolved in chloroform in a round-bottom flask. The chloroform was evaporated at 60°C under reduced pressure using a rotary flask evaporator (Superfit, India). After chloroform evaporation the flask was kept under vacuum overnight in a nitrogen atmosphere to remove residual solvent. The thin films were hydrated [7] with 6 ml of phosphate buffered saline (PBS), pH 7.4, and the flask was kept rotating at 60°C. Formulations were sonicated 3 times at 50 Hz in a bath-sonicator (Ralsonics model RP 120, Mumbai, India) for 15 min with 5 min interval between successive times. Vesicle suspensions were also sonicated for 5 min.

Microscopy

The size, shape and lamellar nature of vesicles in unsonicated formulations were observed by optical microscopy [8] using a calibrated eyepiece micrometer, and photographs were taken at 400x magnification with a digital camera (Olympus, 8.1 megapixel, Japan).

Characterization of sonicated vesicles by transmission electorn microscopy

A drop of the sample was placed onto a carbon-coated grid and allowed to dry to a thin film. Before drying of this film on the grid, it was negatively stained with 1 % phosphotungstic acid (PTA). For this, a drop of staining solution was pipeted onto the film and the excess drained off with filter paper. The grid was allowed to air dry thoroughly and then examined using a transmission electron microscope [9] with an accelerating voltage of 80 kV.

Determination of vesicle diameter

The z-average diameter of vesicles was determined by dynamic light scattering [10] using a Zetasizer, Nano ZS 90, (Malvern instruments). For the measurement, 100 µl of the formulation was diluted with an appropriate volume of PBS, pH 7.4, and the vesicle diameter, polydispersity index was determined.

Determination of drug entrapment in vesicles

Zidovudine niosomal formulations were centrifuged at 15,700 x g for 90 min at 4°C using a refrigerated centrifuge (Eppendorf, 5415 R, Germany) to separate niosomes from non-entrapped drug. Concentration of the free drug in the supernatant was determined by measuring absorbance at 267 nm with a UV spectrophotometer (Shimadzu, model UV 1650 PC, Kyoto, Japan). The percentage of drug entrapment in niosomes was calculated [11]. This process was repeated thrice to ensure that free drug was completely removed.

% Drug entrapment =
[Total drug – Drug in supernatant/Total drug] X 100

In-vitro release studies

In-vitro release was studied using a dialysis bag (Himedia dialysis membrane, 12,000 -14,000 molecular weight cut-off) as a 'donor compartment'. Niosomes containing entrapped zidovudine obtained after centrifugation of 2 ml of the formulation were re-suspended in 1 ml of PBS, pH 7.4, and used for the release study. The dialysis membrane was

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soaked in warm water for 10 min, one end was sealed with a clip, the niosome preparation or free zidovudine solution was pipetted into the bag and the bag was sealed with another closure clip to prevent leakage. The dialysis bag was placed in 100 ml of PBS, pH 7.4, at 37 ± 2°C. The medium, which acted as the receptor compartment, was stirred at 100 rpm. Samples of medium (5 ml) were withdrawn hourly and replaced with fresh buffer and zidovudine absorbance at 267 nm was measured using PBS as blank [12]. Results were the mean values of three runs.

Table 1. Determination of vesicle size and % Entrapment efficiency for Zidovudine niosomal formulations containing Tween 60 and Tween 80

S. No.	Formulation cholesterol: surfactant ratio (1: 4.5)	Average vesicle size of unsonicated formulation(µm)	% Entrapment Efficiency
1	Tween 60 + Chol	2.48 ± 0.288	76.24 ± 0.38
2	Tween 80 + Chol	2.66 ± 0.190	80.32 ± 0.84

Table 2. TZM-bl assay of Zidovudine solution

Concentration (µg/ml)	Cytotoxicity results(% Viability with product)	Anti-HIV testing(% Inhibition)			
		IIIB	Ada5	UG070	VB59
390.6	54	98	100	98	98
195.3	61	99	99	97	97
97.65	70	99	99	97	97
48.825	87	99	100	98	97
24.4125	88	99	100	NC	NC
12.20625	96	98	100	NC	NC
6.103125	99	97	100	NC	NC
3.051563	100	91	99	NC	NC

*NC denotes not carried out

Table 3. TZM-bl assay of Zidovudine niosomes (containing Tween 80)

Concentration (µg/ml)	Cytotoxicity results(% Viability with product)	Anti-HIV testing(% Inhibition)			
		IIIB	Ada5	UG070	VB59
780	68	100	100	98	99
390	77	98	100	98	99
195	94	98	100	98	98
97.5	95	98	100	98	97
48.75	96	99	100	NC	NC
24.375	96	99	100	NC	NC
12.1875	96	99	100	NC	NC
6.09375	100	96	100	NC	NC

*NC denotes Not Carried Out

***In-vitro* anti – HIV testing**

The best formulation of Zidovudine niosomes and Zidovudine solution were tested for determining anti-HIV activity against cell associated HIV using TZM-bl assay. TZM-bl is an adherent cell line that is maintained in T-75 culture flasks. Complete growth medium (GM) consists of DMEM (Dulbecco's Modified Eagles Medium) supplemented with 10% fetal bovine serum (FBS, heat-inactivated), 25 mM HEPES and 50 µg/ml gentamicin. Using the 96 well flat bottom culture plates this assay was performed separately for zidovudine niosomes and solution.. 150 µl of GM was taken in all wells of column 1 (cell control) and 100 µl in all wells of columns 2-10 (column 2 was the virus control).

An additional 40 µl was taken in all wells of columns 3-10. 11 µl of test samples Zidovudine niosomes and Zidovudine solution to columns 3-10 were taken. The required number of vials of pseudovirus was thawed by placing in an ambient temperature water bath. When completely thawed, the virus in GM was diluted to achieve a TCID (Tissue Culture Infectious Dose) of approximately 150,000 RLU (Relative Luminescence Units) equivalents (+/- 15,000 RLU). 50 µl of cell-free virus was dispensed to all wells in columns 2-10, covered with plates and incubated for 1 hour. A suspension of TZM-bl cells (trypsinize aproximately 10-15 minutes prior to use) at a density of 1 x 10^5 cells/ml in GM containing DEAE-Dextran (25 µg/ml) was prepared. 100 µl of cell suspension (10,000 cells per well) was dispensed to each well in columns 1-10, covered with plates and incubated for 48-72 hours. The final concentration of DEAE-Dextran is 10 µg/ml. 150 µl of culture medium was removed from each well, leaving approximately 100 µl. 100 µl of Britelite plus Reagent was added to each well, incubated at room temperature for 2 minutes to allowed for complete cell lysis. Mixed by pipet action (at least two strokes) and 150 µl was transferred to corresponding 96-well plates of zidovudine niosomes and solution. The two plates were read immediately in a luminometer. The IIIB, Ada5, UG070 and VB59 are the four HIV-1 strains used for anti-HIV testing here.

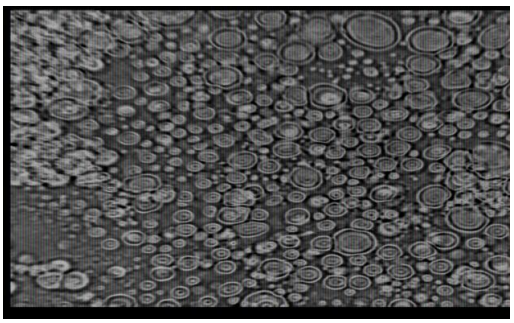

Figure 1. Optical microscopic image of unsonicated niosomal formulation

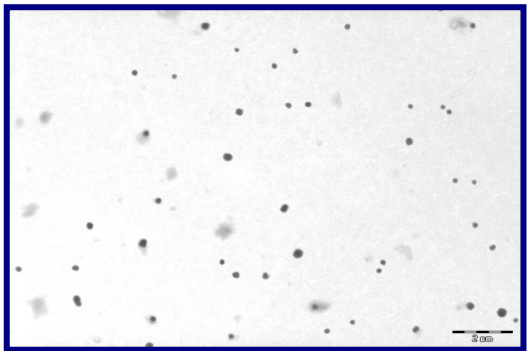

Figure 2. TEM image of sonicated niosomes

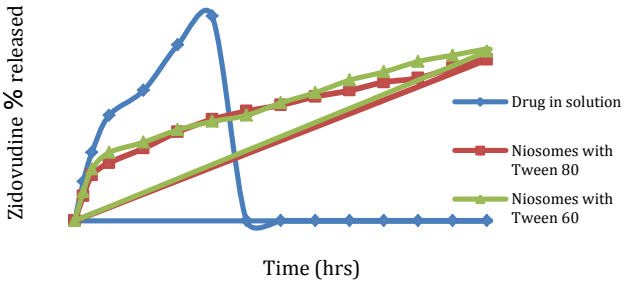

Figure 3. Comparative *in-vitro* release study of Zidovudine formulations

RESULTS AND DISCUSSION

Preformulation studies

Preformulation studies were carried out with different µ molar ratios of cholesterol and nonionic surfactant Tween 80 (1:1.5, 1:2.5, 1:3.0, 1:3.5, 1:4.5, 1:6.0). The niosomal formulation with the cholesterol: non-ionic surfactant ratio of 1:4.5 has shown the greater entrapment efficiency and sustained the release of drug for longer time. So this ratio of cholesterol: non-ionic surfactant was chosen for the preparation of niosomes with different surfactants.

Vesicle size and shape

Photomicrographs revealed that niosomes were spherical and multilamellar (Figure 1). Non-sonicated vesicles with Tween 60 showed average vesicle size of 2.48 ± 0.288 µm and with Tween 80 of 2.66 ± 0.190 µm (Table 1). Addition of cholesterol to the formulation led to spherical vesicles that were stable on sonication under the study conditions. Cholesterol abolished the phase transition in a manner that may be analogous to that observed for liposomes [13].

Niosomal vesicles from non-sonicated Tween 80 formulations (2.66 ± 0.190 µm) were larger than those with Tween 60 (2.48 ± 0.288 µm). This suggests that when the hydrophilicity of the surfactant increases, the vesicle size increases. Similar results were observed by Agarwal R *et al*. The sonicated vesicles of Tween 80 formulation showed the average vesicle size of 0.282 ± 0.13 nm and the polydispersity index was found to be 0.49 ± 0.05.

Characterization of sonicated vesicles by transmission electorn microscopy

TEM images of niosomes (Figure 2) show that multilamellar niosomes prepared by thin film hydration were spherical and that the sonicated Tween 80 vesicles were nanosize. Vesicle size in niosomes depends upon the properties of the molecules in the bilayers and also on the interaction between bilayers.

Entrapment efficacy

AZT entrapment was influenced by the affinity of the drug for the niosome material, the thickness of the niosome bilayers, the drug solubility in water and the compatibility between the drug and niosome material[6]. The niosomal formulation with Tween 80 showed the greater entrapment efficiency of 80.32% when compared with the tween 60 having 76.24 % (Table 1).

In-vitro release

The *in-vitro* release of zidovudine from the drug in solution, niosomes containing Tween 60 and Tween 80 were determined. Niosomal zidovudine formulations with Tween 60 and 80 show significant reduction in *in-vitro* drug release ($p<0.001$) in 4 hrs compared with drug in solution (Figure 3). The niosomal formulation with Tween 80 released 78.12% and with Tween 60 released 82.86% in 12 hrs which indicates that greater sustained release of drug was achieved with Tween 80.

***In-vitro* anti-HIV testing**

The Zidovudine niosomes (containing tween 80) and Zidovudine solution were tested for determining anti-HIV activity against cell associated HIV using TZM-bl assay (Table 2 and 3). From the results of the TZM-bl assay it was found that Zidovudine solution showed greater cytotoxicity of 46 % when compared to the Zidovudine niosomes of 23 % at the concentration of 390µg/ml. It can be due to the immediate action of the Zidovudine solution when compared to the niosomes which release the drug slowly. Study results confirm that even the low concentrations of both zidovudine niosome and solution are effective in inhibiting the attack of the HIV-1 viruses (IIIB, Ada5, UG070 and VB59) in the TZM-bl cells.

CONCLUSION

Zidovudine niosomes formulated with Tween 80 entrapped high amounts of drug and sustained the drug release for a longer time. Zidovudine niosomes has shown slightly greater % inhibition against the HIV-1 viruses (Ada5, and VB 59) when compared with the drug in solution.

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