INTRODUCTION

The treatment of brain cancers remains tremendous challenge due to restrained delivery of therapeutic agents to the tumors. Glioblastoma represents 54% of all the gliomas and 18% of all brain tumors. Glioblastoma is the most aggressive, invasive and malignant type of glioma: A tumor which arises from the glial cells in the brain. Treatment of glioblastoma involves surgery, radiation followed by chemotherapy. Temozolomide (TMZ) has been shown to be one of the most effective antineoplastic agents for treating high-grade glioma. Despite, treatment of glioblastoma with a combination of surgical resection, radiotherapy and chemotherapy, the median survival span of patient (1 year) has not been improved significantly for last 30 years [1]. The reason for this therapeutic inadequacy is due to existence of an insurmountable obstacle like the blood-brain-barrier (BBB) [2,3]. In addition, due to its short half-life of approximately 1.8 hrs in plasma, TMZ has to be administered with a high systemic doses to reach therapeutic brain levels, which simultaneously brings about a series of side effects including bone marrow depression, oral ulcerations, nausea, vomiting, fatigue, and headache [4]. Therefore, to get better efficacy for treating brain tumors and to reduce dose-related toxicity and side effects during chemotherapy, it is necessary to identify the alternative way to achieve drug targeting at specific tumor site.

In the past few years, a number of different approaches have been developed to overcome obstacle due to BBB. The methods include direct injection into the brain [5], osmotic opening of the tight junctions [6], drug structural modifications [7], chemical drug delivery systems, and nanoparticulate carriers such as liposomes [8,9], solid lipid nanoparticles, or polymeric nanoparticles [10,11]. Among these several strategies, liposomes seem to be one of the most promising and alternative techniques for targeted delivery to brain tumor microenvironment. Liposomal formulations of various anti-cancer drugs have been reported previously and have shown encouraging efficacy in preclinical studies [12]. Liposomes are increasingly demonstrating their advantage of effective transporting of various drugs across the BBB on the basis of their small size and lipid nature of the surface. Through encapsulation of drugs in a macromolecular carrier like liposome, may help in significant reduction in volume of distribution as well as significant increase in the concentration of drug in the tumor [13]. This results in a decrease in the amount and types of nonspecific toxicities and an increase in the amount of drug that can be effectively delivered to the tumor.

In this investigation, hydrogenated soya phosphatidylcholine (HSPC) was used as a lipid to prepare liposomes. HSPC liposomes are found to be more stable than phosphatidylcholine liposomes [14,15]. We report here efficacy of TMZ loaded HSPC liposomes and compare it with the efficacy of plain TMZ through cell culture technique.

MATERIALS AND METHODS

Materials

TMZ was received as a gift from Naproad Life Science, Mumbai, India. Steryl amine (SA) was from Sigma-Aldrich, India. Cholesterol (CHL) was obtained as a gift sample from VAV Life Science, Mumbai, Maharashtra, India. HSPC was a gift from Lipoid, Germany. Human glioblastoma cell line (U87MG) was procured from National Centre for Cell Science, Pune, Maharashtra, India. All other chemicals used were of analytical reagent grade.
Methods

Preparation of TMZ loaded HSPC liposomes
Liposomes were prepared by a modified ethanol/solvent injection method [16,17]. TMZ (100 mg) and lipid (HSPC-CHL-SA: 150 mg) were dissolved in ethanol (5 ml). An ethanolic solution of the drug and lipid was injected in aqueous phase (20 ml, phosphate buffer saline, pH 7.4). A syringe fitted with a 26 gauge needle was used to inject ethanolic solution. During addition of ethanolic phase, the aqueous phase was magnetically stirred at 700 rpm and was maintained at 55°C. On addition of ethanolic solution to aqueous phase, the formation of liposomes occurred spontaneously. Liposomes dispersion was stirred overnight at room temperature using a magnetic stirrer to allow ethanol to evaporate. Then after, liposomal dispersion was centrifuged (Centrifuge 3K30, Sigma) at 15,000 rpm for 30 minutes. The supernatant was collected and analyzed for TMZ content. The pellet was transferred to a vial and stored in a refrigerator. Four batches were prepared to optimize the composition of liposomes.

Estimation of TMZ
Supernatant was diluted with 0.1 N HCl. Diluted sample (20 µl) was injected in HPLC column. The condition of HPLC analysis was as indicated in Table 1.

Characterization of TMZ loaded HSPC liposomes

Particle size analysis
The particle size of formulation was measured by dynamic light scattering technique (Malvern Nano S90; Malvern Instruments, U.K). Appropriate dilution was done with double distilled water and light scattering was measured at 25°C at 90° angle. Particle size distribution is reported as polydispersity index (PDI).

Entrapment efficiency
The % entrapment efficiency was calculated using the following equation.

\[ \text{Entrapment efficiency (\%)} = \frac{T_a - T_b}{T_a} \times 100 \]

Where, \( T_a \) = Amount of TMZ taken for preparation of liposomes (100 mg)

\( T_b \) = Amount of TMZ found in supernatant through HPLC method of estimation.

Scanning electron microscopy (SEM)
Double-sided carbon tape was affixed on aluminum stubs. A drop of liposomal dispersion was put on slide. Slide was allowed to dry and the resultant film was placed on the aluminum stubs. These aluminum stubs were then placed in the vacuum chamber of microscope. The gaseous secondary electron detector with working pressure of 0.8 Tor, and acceleration voltage of 30 KV was used to observe the morphological characteristics of the TMZ loaded HSPC liposomes.

In vitro drug release study
In vitro drug release from liposomal dispersion was performed by dialysis bag diffusion technique. TMZ loaded HSPC liposomes were filled in dialysis bag (MWCO 12-14 k Da, pore size 2.4 nm) that was immersed in a receptor compartment containing 150 ml of phosphate buffer pH 7.4. The system was stirred at 50 rpm and maintained at a temperature 37°C±0.5. At predetermined time intervals, samples (500 µl) were withdrawn from receptor compartment. TMZ was estimated in the samples by HPLC method described above.

Cell cytotoxicity assay
3 (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is colorimetric determination of cell viability [18]. In MTT assay, cells are exposed to drug or therapeutic system. MTT enters the cells and in mitochondria by succinic dehydrogenase and reduced to insoluble dark purple formazan product. The formazan concentration is directly proportional to the cell viability [20,21,23]. MTT reduction is possible only in metabolically active cells so the level of activity is the measure of viability of cell. U87MG cells (1×10⁶ cells/ml per well) were seeded in two culture plates containing 96-wells for 24 hrs at 37°C and cell were kept in CO₂ incubator. The non-adherents cells were removed by washing twice with 500 µl warm PBS, using gentle swirling action. The cells were treated with 500 µl each of solutions of pure drug and TMZ loaded liposomal formulation at different concentrations (1, 10, 20, 40, 80, and 100 µg/ml) and cells were incubated at 37°C for 24 hrs in CO₂ incubator. After incubation, 10 µl of MTT was solubilized in PBS pH 7.4 solution (5 mg/ml) and added to each well. The plates were kept for incubation for 2-3 hrs in CO₂ incubator at 37°C. After incubation the supernatant was removed, and 200 µl of dimethyl sulfoxide was added to dissolve the formazan crystals [18,19]. The absorbance at 570 nm was taken with microplate reader (Elx800-MS, Biotek, VT, USA).

The cell viability was calculated from the following equation:

\[ \text{Cell viability (\%)} = \left( \frac{\text{Abs of treated cells}}{\text{Abs of control untreated cells}} \right) \times 100 \]

Cell uptake study of TMZ loaded HSPC liposomes
Cell uptake study was performed to confirm the interaction of TMZ loaded HSPC liposomes with the glioblastoma tumor cell. The synthesis of fluorescein isothiocyanate (FITC) labeled liposomes was based on the reaction between the isothiocynate group of FITC and the amino group of SA [20]. Briefly, 10 mg of FITC was added in the PBS pH 7.4 during the preparation of liposomes using ethanol injection method. U87MG human glioblastoma cells were plated at density of 1.5×10⁴ cells/cm² in 24 well plates and were used for uptake studies after culturing when they formed confluent monolayers. The uptake of FITC labeled Liposomes was visualized using fluorescence microscope after 4 hrs of incubation.

![Scanning electron microscope image of temozolomide loaded hydrogenated soya phosphatidylcholine liposome](image)

Table 1: Conditions for HPLC method of analysis

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>0.1% aq acetic acid-acetonitrile (90:10 V/V)</th>
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<tbody>
<tr>
<td>Pump mode</td>
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<td>Column</td>
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<td>Column temp</td>
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</tr>
<tr>
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</tr>
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<td>Injection volume</td>
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<tr>
<td>Flow rate</td>
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<tr>
<td>Run time</td>
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</table>

Fig. 1: Scanning electron microscope image of temozolomide loaded hydrogenated soya phosphatidylcholine liposome
RESULT AND DISCUSSION

Characterization of TMZ loaded HSPC liposome

Vesicle size and PDI

Table 2 indicates composition, vesicle size, vesicle size distribution, and entrapment efficiency of TMZ loaded liposomes. Vesicle size and entrapment efficiency were selected to calculate overall desirability function as per following equation [22].

\[
\text{OD} = \left( \frac{1}{n} \sum_{i=1}^{n} \left( \frac{Y_i - \mu}{\sigma} \right)^2 \right)^{-1/2}
\]

Vesicle size plays an important role with respect to permeation of liposomes through BBB as well as its permeation and retention in tumor microenvironment [23]. Particles/vesicles having size <200 nm may effectively cross BBB as well as permeate in tumor microenvironment through fenestration in capillaries supplying blood to tumors [23].

In this investigation, average vesicle size of TMZ loaded liposomes varied from 105.7 nm to 204.2 nm. It indicates that intravenous administration of TMZ loaded liposomes may effectively cross BBB and may get retained in tumor microenvironment.

In a liquid dispersion of neutral liposomes, increase in vesicle size may occur due to aggregation [10]. Charged liposomes offer greater physical stability as compared to neutral liposomes. SA was used to prepare positively charged liposomes.

Vesicle size distribution is indicated by PDI. The range of PDI is 0 to 1. The value close to zero indicates narrow vesicle size distribution and the value greater than 0.5 indicate heterogeneous size distribution [22]. The statistically significant difference (*p<0.05) was found between PDI values of Batch B1 and Batch B4. This may be attributed to higher amount of SA present in Batch B4, resulting in greater charge density leading to disaggregation of liposomes in the system. Higher vesicle size of Batch B1 of liposomes was observed when compared with vesicle size of Batch B4 may be due to partial aggregation.

% Drug entrapment efficiency

The % drug encapsulation efficiency for respective batches are shown in Table 2. The % EE was found to be in the range of 60.30-83.77%. The % entrapment efficiency of optimized batch was found to be 78.25%.

SEM

Photomicrograph of TMZ loaded HSPC liposomes (Batch B2) is shown in Fig. 1. The image indicates that liposomes of spherical shape were formed by the method employed to prepare them. Spherical and rod-shaped particles effectively adhere to cells [1]. Hence, there is a greater probability for TMZ loaded liposomes to adhere to cells. Photomicrograph also indicates that there is no aggregation of liposomes. Low PDI value (0.217) may be an outcome of no or less aggregation.

In vitro drug release study

Fig. 2 shows in vitro drug release profile. It indicates a biphasic curve. Drug release rate is faster during the first phase up to 8 hrs as compared to during the second phase of 8-14 hrs during the first phase 60% of entrapped drug gets released where during the second phase approximately 15% of entrapped drug gets released. The release characteristic could be attributed to the fact that TMZ was trapped by the lipid, and therefore, TMZ might get released gradually from the lipid vesicles. The release of TMZ from liposomes may take place by dissolution and diffusion process.

In vitro drug release of temozolomide loaded hydrogenated soya phosphatidylcholine liposomes in phosphate buffer pH 7.4

Cell uptake study of fluorescein isothiocyanate labeled temozolomide loaded hydrogenated soya phosphatidylcholine liposomes

% Drug Release: Temozolomide loaded HSPC Liposomes

Fig. 3: Cell uptake study of fluorescein isothiocyanate labeled temozolomide loaded hydrogenated soya phosphatidylcholine liposomes

Table 2: Composition and characteristics of TMZ loaded HSPC liposomes

<table>
<thead>
<tr>
<th>Batch code</th>
<th>Composition*</th>
<th>Characteristics</th>
<th>Overall desirability</th>
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<tr>
<td></td>
<td>Lipid (mg)</td>
<td>VS (nm)</td>
<td>PDI</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>B4</td>
<td>90</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

*Initial amount used to prepare liposomes. Every batch was prepared using 100 mg TMZ. TMZ: Temozolomide, HSPC: Hydrogenated soya phosphatidylcholine, PDI: Polydispersity index, SA: Steryl amine
Cell uptake study

Cell uptake study was conducted to demonstrate the infiltration of liposomes into human glioblastoma cells (U87 MG). The internalization of FITC labeled liposomes was visualized using fluorescence microscope after 4 hrs of incubation (Fig. 3). The results indicate that these HSPC loaded temozolomide liposomes interact with the glioblastoma tumor cell.

Cell cytotoxicity assay (MTT Assay)

The anticancer activity of the TMZ loaded HSPC Liposomes at concentrations of (1, 10, 20, 40, 80, and 100 µg/ml) were compared with free drug at same concentration against glioblastoma cell lines by MTT assay. Fig. 4 indicates % cell inhibition of TMZ and drug loaded HSPC liposomes at various concentration. The cytotoxicity induced by TMZ loaded HSPC liposomes was substantially higher (*p<0.05) compared with that induced by plain drug. It was found that about 54% cells were killed at the concentration of 40 µg/ml of HSPC liposomes. However, at same concentration % cell inhibition was comparatively lower about 46% in case of pure drug. In a nutshell, the IC50 value of the TMZ loaded HSPC liposomes was less than that of pure TMZ. The higher cell inhibition through HSPC liposomes may be due to lower particle size and better infiltration of liposomes into human glioblastoma tumor cells.

CONCLUSIONS

The present results demonstrate the feasibility of encapsulating the alkylating agent, TMZ, into desired size Liposomes by ethanol injection method. TMZ loaded HSPC liposomes exhibited biphasic release in vitro. Cell uptake study revealed that the drug loaded HSPC liposomes are efficiently taken up by glioblastoma cell. Furthermore, based on the cell culture study it has been confirmed that the drug loaded HSPC liposomes are efficient in killing glioblastoma tumor cell where compared with drug as IC50 value of drug loaded liposomes was found to be lower as compared to pure drug which may be more useful for treating brain tumors. Hence, the prepared formulation may improve the availability of drug at the target site and also reduce the dose-related toxicity of chemotherapy. However, to serve as a drug delivery system in the treatment of malignant brain tumors, further investigations are required at pharmacokinetic level.

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REFERENCES