International Journal of Pharmacy and Pharmaceutical Sciences
Vol 2, Issue 2, 2010

Research Article

BODY DISTRIBUTION AND STABILITY STUDIES ON MITOXANTRONE LOADED SOLID LIPID NANOPARTICLES CONJUGATED WITH CONCANAVALIN- A

ALOK MAHOR [1*], SHASHI ALOK [2], YASHWANT GUPTA [1], S. K. JAIN [1]

Department of Pharmaceutical Sciences, Dr. H. S. Gour University [1*], Sagar, M. P., India Institute of Pharmacy, Bundelkhand University[2], Jhansi, U. P., India. E mail: alokmpharma@gmail.com

Received: 16 Dec 2009, Revised and Accepted: 12 Jan 2010

ABSTRACT

The main objective of this work mainly strains on the distribution of the anticancer drug, mitoxantrone loaded into the SLNs. In vivo study using experimental animal model is the best way of validating and testifying the results in order to determine the efficacy, potency and medicinal nature of the drug. This can be achieved out by measuring the amount of drug or its metabolite concentration that have reached the serum or in urine, or by measuring the amount of drug that have reached to the tissues or organs. The significant part of this study was to evaluate in vivo performance of optimized formulations with respect to their capability to deliver the drug precisely to the desired site.

On the basis of in vitro characteristics optimized formulations $B_2B_{III}C_bS$ and SLN-C_3T_4 were taken for in vivo performance evaluation. A comparison between in vivo biodistribution of free drug administered and the drug plasma concentration profile and tissue distribution time profile of the uncoupled $B_2B_{III}C_bS$ and coupled SLN-C_3T_4 formulations was made.

INTRODUCTION

Cancer is fundamentally a disease of regulation of tissue growth. The difference between cancer cells and normal cells is profound, not only because of the way they look and the way they behave, but because of the radical difference in their lifespan. Placed in tissue culture, cancer cells can live forever. Normal cells, on the other hand, die after about 50 generations[1]. A single cancerous cell surrounded by healthy tissue will replicate at a rate higher than the other cells, placing a strain on the nutrient supply and elimination of metabolic waste products. Once a small tumor mass has formed, the healthy tissue will not be able to compete with the cancer cells for the inadequate supply of nutrients from the blood stream. Tumor cells will displace healthy cells until the tumor reaches a diffusion-limited maximal size[2].

Mitoxantrone, an antibiotic drug, antiviral, antibacterial and anticancer is probably due to inhibition of DNA and RNA synthesis through DNA intercalation[3] and possibly an electrostatic interaction with different cellular membranes[4].

In recent years, nanoparticles made from solid lipids are attracting major attention as a promising colloidal drug carrier [5]. They have been widely used for controlled drug delivery via intravenous, transdermal, ocular and oral administration routes. Solid lipid nanoparticles consist of toxicologically acceptable compounds, which are biodegradable. e.g. nutritional glycerides[6]. A full range of lipids and emulsifiers possessing the GRAS status (Generally Recognized as save) are available (Food Drug Cosmetic Law Reports, 1994). SLN can be used as carrier system for hydrophobic and hydrophilic drugs [7].

Conjugation of targeting ligands to drugs or drug carrier nanosystems is the most popular way of directing them to their target sites. To this end, various techniques have been devised, including covalent and non-covalent conjugation. The emphasis is that the ligand must be attached stably and accessibly to the drug carrier, so that the ligand is presented in its right orientation for binding to the target receptors. The coupling reactions must not affect the biological activity of ligand and should not adversely affect the structure of drug delivery nanosystems. Further, such coupling reactions must be optimized so that binding of ligands takes place in a homogeneous manner on the surface of the drug carrier nanosystems [8].

Site-specific drug delivery is feasible, because as part of the pathological phenotype of diseases, such as cancer, cells often express surface receptors that are not present under physiological conditions. Conjugating appropriate ligands for such a receptor should therefore result in a higher drug concentration at the tissue of interest and thus, increase the efficiency of the therapy. Different methods have been described for the conjugation of peptide ligands to SLNs surfaces. This maximizes the amount of drug that can reach the targeted tumor sites and minimizes systemic drug toxicity [9]. Lectins are one example, due to their binding specificity to carbohydrate structures, which are important in cellular processes, such as differentiation and recognition and immune response. For today, a more flexible interpretation would be accepted, e.g., "lectins are simply defined as proteins which specifically bind (or crosslink) carbohydrates" [10].

Among various lectins, Concanavalin A (Con A), a plant lectin with binding specificity for mannose and glucose, has been suggested as a model carbohydrate receptor recognizer and is one of the most extensively studied members of the lectin family for tumor vasculature targeting.. It has been shown that the carbohydrate-binding protein Concanavalin A (Con A) can agglutinate leukemic cells and cells transformed by polyoma virus, simian virus 40, chemical carcinogens, and X-irradiation. This protein did not agglutinate normal cells under the same conditions [11].

MATERIALS AND METHOD

Materials

Tristearin, cholesterol, steryl amine, ethyl alcohol and tween 80 (all purchased from Hi-media), mitoxantrone, soya lecithin and concanavalin- A purchased from Sigma- Aldrich, A-549 human cancer cell lines were purchased from NCCS, Pune, BALB/c mice (male, 8 weeks old, and weighing 20–25 g) were provided by the Dept. of Pharmaceutical Sciences, Dr. H. S. Gour University, Sagar, M. P., India with the approval of IAEC to perform experiments on animals.

Method

Preparation of solid lipid nanoparticles (Uncoupled)

Solvent injection method[12] is used in the present work for the preparation of solid lipid nanoparticles. This method offers clear advantages over the existing methods such as the use of pharmaceutically acceptable organic solvents, no need for high-pressure homogenization, easy handling and a fast production process without technically sophisticated equipment. It is based on lipid precipitation from a dissolved lipid in solution. For this purpose, a solution of the lipid in a water-miscible solvent or a

water-miscible solvent mixture is rapidly injected into an aqueous phase with or without surfactant.

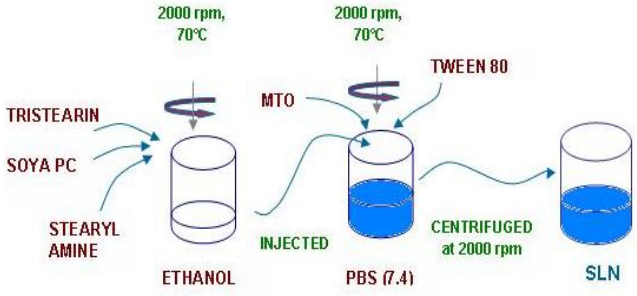

Fig. 1: preparation of uncoupled solid lipid nanoparticles

Briefly, tristearin, soya lecithin and stearyl amine were dissolved in a water-miscible solvent (ethanol) at 70˚C and was then rapidly injected through an injection needle into a stirred aqueous phase maintained at same temperature containing anticancer drug Mitoxantrone and a surfactant, Tween 80 which was previously dissolved in the aqueous phase. The resulting dispersion was then filtered with a paper filter in order to remove any excess lipid. The obtained formulation was subjeceted to optimization with the following parameters such as lipid con., drug conc., surfactant conc., processing variables and an optimized formulation was selected for preparation of coupled SLNs.

Preparation of solid lipid nanoparticles (coupled)

The ligand, Concanavalin A was coupled to Mitoxantrone loaded Solid Lipid Nanoparticles by conjugating amino group of stearylamine present on the surface of optimized SLN formulation with the carboxylic group of the lectin Concanavalin A with the aid of the linker, EDC. Amide linking is observed in the coupling reaction.

Fig. 2: Schematic diagram of coupling of Con-A with SLN

The optimized formulation $B_2B_{III}C_bS$ was selected for coupling. 10 mg of Con A was dissolved in PBS (pH 7.4) and to this; 20 mg of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was added. This whole solution was added to the optimized formulation and was solubilized with the aid of vortex mixer. This mixture was incubated for an optimized time period (4 hrs) at room temperature. Unbound EDC and Con A was removed by passing the formulation through Sephadex G-50 column. The coupled formulation was also subjected to optimization for the following variables such as ligand conc., conductance and the finally optimized coupled formulation SLN-C3T4 was selected for organ distribution and stability studies.

IN VIVO SERUM AND ORGAN DISTRIBUTION STUDIES

Tumor-bearing nude mice were prepared by injecting A 549 cancer cells (2 x 10^6 cells) subcutaneously into the right flank of the BALB/c mice (male, 8 weeks old, and weighing 20–25 g) and the tumor was allowed to grow until the mean tumor volume reaches 100±10 mm^3. The tumor bearing mice taken for present study were divided into four groups with five mice in each group. They were fasted overnight before administration of dose. To the first group, aqueous solution of Mitoxantrone was administered intravenously (via the tail vein). The dose given for mice was 5.0 mg/kg body weight.

To the second and third group of mice, mitoxantrone loaded SLN and Con A anchored mitoxantrone loaded SLN formulations were administered respectively, fourth group was kept as control.

One mouse from each group was sacrificed after 0.5 hr, 1 hr, 2 hr, 6hr and 24 hr and after administration of the formulations. Blood was collected by cardiac puncture. Different organs i.e. heart, liver, spleen, kidney and tumor were excised, isolated and dried with tissue paper and weighed. The amount of drug present in plasma and organs was determined by HPLC method.

Drug Assessment in Serum

Blood was collected from cardiac puncture in a centrifuge tube containing heparin sodium (anticoagulant) and centrifuged at 1600 rpm for 10 minutes. To prevent oxidative degradation of mitoxantrone (MTO), 20 µl of ascorbic acid solution was added. Supernatant was collected and 1 ml plasma sample or homogenized sample was mixed with 1 ml solution of hexane-sulphonic acid and ascorbic acid and then, 300 µl of 1 M NaOH was added and supernatant was collected. Ten µl of collected supernatant was filtered through 0.45-µm membrane filter and concentration was determined by HPLC method.

Table 1: Percent dose recovered in serum as free drug

Formulation	Percent dose recovered after (hr)				
	0.5	1	2	6	24
Free Drug (MTO)	9.45	8.21	6.14	5.16	3.78
MTO- SLN	7.45	9.03	5.78	4.22	3.08
Con A- SLN	5.98	5.25	4.16	3.48	2.95

Drug assessment in various organs

The isolated organs- tumor, lungs, liver, kidney, heart and spleen after drying using tissue paper were weighed and crushed into small pieces. One gram of each organ was homogenized with 2.0 ml of PBS (pH 7.4). To prevent oxidative degradation of mitoxantrone, 20 µl of ascorbic acid solution was added. The organs weighing less than one gram, whole organ was used. To the tissue homogenates, 1 ml solution of hexane-sulphonic acid and ascorbic acid and then, 300 µl of 1 M NaOH were added. The resultant suspension was centrifuged for 15 minutes at 3000 rpm and was filtered through 0.45 µm membrane filter. Tissue homogenate was analyzed for drug content as in serum by HPLC method.

Table 2: Distribution of mitoxantrone in various organs and in tumor after IV administration of free drug

Time (hrs)	Percent dose recovered					
	Tumor	Lungs	Liver	Kidney	Heart	Spleen
0.5	2.88	7.21	7.56	30.23	6.01	5.45
1	3.12	6.75	6.22	27.26	5.42	6.78
2	3.36	5.02	4.32	28.78	5.08	6.62
6	2.54	4.15	3.11	20.83	3.98	4.87
24	2.72	2.32	2.45	22.52	3.21	5.02

Table 3: Distribution of mitoxantrone in various organs and in tumor after IV administration of uncoupled formulation ($B_2B_{III}C_bS$)

Time (hrs)	Percent dose recovered					
	Tumor	Lungs	Liver	Kidney	Heart	Spleen
0.5	3.92	7.13	7.22	20.56	5.21	4.32
1	4.04	6.65	6.74	19.45	5.63	5.12
2	4.25	6.11	6.03	19.13	4.78	4.11
6	4.75	5.21	5.44	18.12	3.42	3.76
24	5.26	4.01	3.23	22.26	3.13	3.15

Table 4: Distribution of mitoxantrone in various organs and in tumor after IV administration of coupled formulation (SLN-C_3T_4)

Time (hrs)	Percent dose recovered					
	Tumor	Lungs	Liver	Kidney	Heart	Spleen
0.5	4.56	6.98	7.02	20.41	5.11	4.12
1	4.68	6.38	6.45	19.21	4.98	4.03
2	5.26	7.02	7.87	18.14	4.23	3.46
6	5.79	4.31	4.55	17.45	3.21	2.42
24	7.89	3.11	3.15	17.17	2.15	3.09

STABILITY TEST

The purpose of stability testing is to provide evidence on how the quality of a formulation varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light. Degradation is likely to occur under tropical conditions of higher ambient temperature and humidity. The present study is desired to test the stability of uncoupled and coupled SLN formulation.

$B_2B_{III}C_bS$ and SLN-C_3T_4 were the two optimized formulations, which are selected for stability testing under storage condition at 4°C and at room temperature. Both formulations were stored in screw capped, amber colored small glass bottles at 4±1°C and room temperature. Analysis of the samples was made on particle size and residual drug content after a period of 10, 20, 30, 45 and 60 days.

Effect of storage on Particle Size

Subsequent change in particle size was determined using a zetasizer (Malvern Instrument, UK) after a definite period of time of 10, 20, 30, 45 and 60 days when these formulations were stored at 4±1°C.

Table 5: Effect of storage on the particle size at 4±1°C

Formulation Code	Particle size (nm)					
	Initial	10 days	20 days	30 days	45 days	60 days
$B_2B_{III}C_bS$	275	278	282	285	291	299
SLN-C_3T_4	441	445	448	451	458	463

Table 6: Effect of storage on the particle size at 27±2 °C

Formulation Code	Particle size (nm)					
	Initial	10 days	20 days	30 days	45 days	60 days
$B_2B_{III}C_bS$	275	278	295	299	301	307
SLN-C_3T_4	441	454	461	472	480	485

Effect of Storage on Residual Drug Content

On storage for a specified period of time of 10, 20, 30, 45 and 60 days, the drug content of both the formulation was determined and % residual drug content was calculated. Free drug was separated by using Sephadex G-50 columnn and SLN were lysed with 0.1% triton, was filtered and drug content was determined using spectrophotometric techniques. 100 % initial drug content was taken for each formulation.

Table 6: Effect of storage on residual drug content at 4±1°C

Formulation Code	Percent residual drug content				
	10 days	20 days	30 days	45 days	60 days
$B_2B_{III}C_bS$	99.12	98.74	98.18	97.22	95.78
SLN-C_3T_4	98.65	98.18	97.71	96.45	95.33

Table 7: Effect of Storage on Residual Drug Content at 27±2°C

Formulation Code	Percent residual drug content				
	10 days	20 days	30 days	45 days	60 days
$B_2B_{III}C_bS$	97.12	96.74	94.18	91.22	89.78
SLN-C_3T_4	94.65	92.18	90.71	88.45	85.33

RESULTS AND DISCUSSION

In vivo studies were carried out to assess the therapeutic efficacy of the designed dosage form. The in vivo performance is the preliminary step for clinical evaluation of a drug or a formulation. Uncoupled $B_2B_{III}C_bS$ and coupled SLN-C_3T_4 formulations were administered intravenously with an equal amount of mitoxantrone to the Balb/C mice. In vivo biodistribution of free drug administered was compared with the drug plasma concentration profile and tissue distribution time profile of the uncoupled $B_2B_{III}C_bS$ and coupled SLN-C_3T_4 formulations. The free drug and prepared formulations were administered intravenously to Balb/C mice.

Free MTO (9.45%) was recovered in serum after a time interval of half hour but in case of uncoupled and coupled formulation, maximum concentration recovered was 9.03% and 5.25% respectively after one hour. From this data, it can be interpreted that plasma drug concentration is reduced when the drug is entrapped in SLN formulations. To this effect, coupled formulation showed even a lesser plasma drug concentration as compared to uncoupled SLN formulation. The reason behind this change is due to the entrapment of the drug with the SLN formulations.

Distribution of free drug in various body organs and in tumor was found to be maximum between half an hour to two hours. 3.36% of drug was recovered after half an hour in tumor, other body organs showed varied results at different time intervals; 30.23% in kidney (half hour), 7.21% in lungs (half hour), 7.56% in liver (half hour), 6.01% in heart (half hour) and 6.78% in spleen (one hour).

Accumulation seems to increase in uncoupled formulation in tumor as time duration in increased. The reason behind this may be an increase in permeability of tumor vasculature. Accumulation was found to decrease as time duration is increased in various body organs except in case of spleen where drug accumulation was

maximum, 4.52% (one hour). A minute increase was measured in the % dose recovered in heart, 5.63% and in spleen, 5.12% at one hour.

Coupled formulation SLN-C_3T_4 showed a marked increase in the drug accumulation in tumor. The % dose recovered increased as the time duration was increased in tumor i.e. from 4.56% (half hour) to 7.89% (24 hrs). This increase is due to the receptor mediated endocytosis of Con A coupled solid lipid nanoparticles. % dose recovered in the given time duration was maximum in kidney. Though a decrease was noticed in drug accumulation in different organs except in tumor, an increase or maximum accumulation was observed in kidney, lungs and spleen, 7.87%, 7.02% at (2 hrs) and 3.02% (24 hrs) respectively.

These results reveal that percentage of drug recovered from tumor was maximum when formulation SLN-C_3T_4 was given. It clearly shows that uptake of Con A conjugated SLNs was maximum in tumor as compared to plain and uncoupled drug loaded SLN formulations. This may be because of the uptake of Con A by the carbohydrate receptors, which are over expressed at the tumor vasculature.

Thus, it is inferred that SLN-C_3T_4 showed a maximum drug accumulation in tumor vasculature as the % dose recovered was maximum. The in vivo studies revealed that Con A conjugated solid lipid nanoparticles served the purpose of delivering maximum amount of drug at the target site.

Both uncoupled and coupled SLN formulation; $B_2B_{III}C_bS$ and SLN-C_3T_4 were subjected to stability studies. Both the formulations were stored at 4±1°C and room temperature. Variation in the particle size and residual drug content after time interval of 10, 20, 30, 45 and 60 days were analyzed.

The average particles sizes of the nanoparticles were found to increase on storage, which may be due to aggregation of particles. This effect was encountered lower in the case of formulation stored at 4oC, which signify that aggregation can be regulated by regulating temperature and hence ideal storage condition of SLNs are at 4oC than those stored at 27°C.

By keeping the initial drug content 100%, percentage of residual drug lasting in SLNs exposed that significant percent of drug was lost (3-4%) from the formulation within 60 days, which were stored at 4oC and only (11-12%) drug was lost from those stored at 27±2oC (Fig.7.3 and7.4) which could be due to more leaching of the drug from SLNs at room temperature. Data obtained from stability tests indicated that SLN formulations stored at 4oC were more stable than those stored at 27±2°C.

REFERENCES

1. Joseph Panno, Cancer, the role of genes, lifestyle and environment, Library of Congress Cataloging, 2005; 8-155
2. Peppas L. B., Blanchette J. O., Nanoparticle and targeted systems for cancer therapy, Advanced Drug Delivery Reviews, 2004; 56: 1649– 1659
3. Durr, F. E. Biologic and biochemical effects of mitoxantrone, Seminar on Oncology 1984; 11: 3–10.
4. Burns, C. P., Haugstad, B. N., Mossmann, C. J., North, J. A. And Ingraham, L. M., Membrane lipid alteration: Effect on cellular uptake of mitoxantrone, Lipids, 1988; 23: 393–397.
5. Vyas S.P. and Khar R.K., Targeted and Controlled Drug Delivery, CBS publishers and Distributors, New Delhi, 2002; First Edition :512-515
6. Muller R.H., Mehnert W., Lucks J.S., Schwarz C., Muhlen A. Z, Weyhers H., Freitas C., Ruhl D., Solid lipid Nanoparticles, An alternative colloidal carrier system for controlled drug delivery, European Journal of Pharmaceutics and Biopharmaceutics, 1995; 41: 62–69
7. H. Weyhers, S. Ehlers, H. Hahn, E. B. Souto, R. H. Muller, Solid Lipid Nanoparticles (SLN), Effects of lipid composition on in vitro degradation and in vivo toxicity, Pharmazie, June 2006; 61: Issue 6: 539-544
8. Vasir, J. K., Labhasetwar, V., Targeted Drug Delivery in Cancer, Technology in Cancer Research and Treatment, 2005; 4: 363-374
9. Wissing S.A., Kayserb O., Muller R.H., Solid lipid nanoparticles for parenteral drug delivery, Advanced Drug Delivery Reviews, 2004; 56: 1257– 1272
10. Inbar M., Sachs L., Interaction of the carbohydrate-binding protein Concanavalin A with normal and transformed cells, Weizmann institute of science, Rehovot, Israel, 1969; 1-8
11. Naeem, Aabgeena; Saleemuddin, M.; Hasan Khan, Rizwan, Glycoprotein Targeting and Other Applications of Lectins in Biotechnology, Current Protein and Peptide Science, June 2007; Volume 8: Number 3: 261-271
12. Schubert M.A., Muller C.C., Goymann, Solvent injection as a new approach for manufacturing lipid nanoparticles, evaluation of the method and process parameters, European Journal of Pharmaceutics and Biopharmaceutics, 2003; 55: 125–131