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TARGETING OF ANTICANCER DRUG IMPROVES THERAPEUTIC RESPONSE OF ASCITES DALTON'S LYMPHOMA TUMOR IN ANIMAL MODEL

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ABSTRACT
Poly (lactide-co- glycolide) has gained attention for preparation of variety of drug delivery systems. In the present work PLGA were employed for the preparation of microparticles as controlled drug delivery and drug targeting, focused to explore the preparation of PLGA microparticles containing carboplatin as model drugs. Carboplatin microparticles were prepared by single and double evaporation method, solvent diffusion and the physicochemical properties such as particle size, drug entrapment efficiency, drug loading capacity and yield content were analyzed. In-vitro drug released were measured verses time factor. The incorporation efficiency of carboplatin was higher with the single emulsion evaporation method and the loading efficiency of drug in PLGA microparticles was determined. Drug was administration in to the tumor induced experimental animals as per stated in the methods. The efficiency of drug release form prepared formulae was studied using *in vivo* technique with Ascites Dalton's lymphoma tumor induced animal model. Glutathione–S-transferase (GST) activities are playing significant role in development of resistance in the presences of tumor under various newly developed platinum derivative factors. Western blot analysis showed induction in GST expression in microsphere loaded drug treated samples confirmed carboplatin's anticancer activity leading to tumor regression and the ascites was completely diminished.

KEYWORDS: Animal model, Carboplatin, Dalton's lymphoma tumor, drug loaded, PLGA.

INTRODUCTION
Medical nanotechnology offers the possibility of designing novel drugs with greater cell specificity and new drug-release systems that act selectively on specific targets and protect the drug from degradation. Advances in nanotechnology have led to the development of new nanomaterials whose physiochemical properties differ from those of their larger counterparts due to their higher surface-to-volume ratio. These novel properties make them excellent candidates for biomedical applications, given the range of biological processes that occur at nanometer scale.[1] The impact of nanotechnology in medicine can mainly be seen in diagnostic methods, drug-release techniques and regenerative medicine.[2] This procedure offers a range of new solutions for diagnoses and smart treatments by stimulating the body's own repair mechanisms.

Microspheres are one of the multi particulate drug delivery systems and are prepared to obtain prolonged (or) controlled drug delivery, to improve bioavailability or stability and to target drug to specific sites. Microspheres can be defined as solid, approximately spherical particles ranging from 1 to 1000μm, containing dispersed drug in either solution (or) microcrystalline form. The microspheres can be produced by several methods utilizing emulsion system (o/w, w/o, o/w/o and w/o/w). The common emulsion system used oil-in-water (o/w), with microspheres being produced by the emulsion solvent evaporation method. This relatively simple method enables the entrapment of a wide range of hydrophobic drugs.[3] Polylactic acid and its copolymers with glycolic acid (PLGA) are widely employed for the preparation of sustained release preparations and PLGA microparticle have successfully employed as anti cancer drug carrier with injectable drug release has been observed in mice.[4] The formulation was subsequently explored as an anticancer drug carrier in order to avoid the discomfort associated with subcutaneous injection.[5] However, oral PLGA microparticle suffered from several drawbacks such as low drug encapsulation, high polymer composition, sustained drug release for 3-4 days and a partial therapeutic benefit.[6] The administration routes of PLGA nano/micro particle may vary from parenteral, oral, dermatological, pulmonary and nasal to ocular. Recently, diclofenac sodium-loaded PLGA nanoparticles were developed for ocular use and found good biocompatibility with eye.[7] The use of microsphere-based therapy allows drug release to be carefully tailored to the specific treatment site through the choice and formulation of various drug–polymer combinations and

has been used to produce dry powders, granules, or agglomerates from drug suspensions.[8]

Biological drugs and targeted therapies are aimed at a specific cellular target, such as small molecules that inhibit a specific protein molecule that is a key player in signal transduction, in apoptosis[9] in the cell cycle or in other important cellular pathways. Carboplatin, cis-diammine (1,1-cyclobutanedicarboxylato) platinum (II), has a cyclobutanedicarboxylato leaving group that facilitates a slower reaction with glutathione and metallotheonines compared to cisplatin.[10] The objective of the present study was to prepare the carboplatin drug-loaded microparticle and investigate drug to be released in Ascites Dalton's lymphoma tumor induced experimental animals were evaluated to confirm the low dose of novel anticancer drug potential therapeutic response in animal models reliably predict human clinical outcomes.

MATERIALS AND METHODS

Materials

Carboplatin was obtained from M/s LI TAKA Drugs Ltd. Pune, India, as a gift sample. Poly (lactide-coglycolide) (50:50) was obtained from Boehringer Ingelheim Pharma, Germany as a gift sample. All other ingredients used were of analytical grade. Cell Line: was Dalton's lymphoma Ascites (DLA) cell line was obtained from Amla Cancer Institute, Thrissur, Kerala.

Carboplatin – loaded microspheres were prepared through single emulsion evaporation method. 160mg of PLGA and 40mg of carboplatin were dissolved in 2ml of dichloromethane. This solution was added drop wise to an aqueous 20ml PVA (3%, w/v) solution under sonication for 20 min (in pulsed manner, 40% intensity) using a probe sonicator (Lapsonic®) over an ice bath. The solvent was evaporated at room temperature (28˚C) for 12h, under magnetic stirring. Carboplatin loaded microparticles were isolated by 30 min centrifugation at 35,000 rpm. After centrifugation, the supernatant was recovered and assayed for unentrapped drug and sediment was washed using the same amount of distilled water as of the supernatant and again centrifuged at 35,000 rpm for 20 min. The washing process was repeated 3 times and all the washed solutions were collected and assayed for unentrapped drug. The loading efficiency of drug in PLGA microparticles were separated from the aqueous medium by ultracentrifugation at 35,000 rpm for 30 min. The amount of drug present in the microparticles was determined as the difference between the total amount of drug used to prepare the microparticles and the amount of drug present in the aqueous medium.

$$\text{Drug content (\% w/w)} = \frac{(\text{Mass of the total drug} - \text{Mass of free drug}) \times 100}{\text{Mass of nanoparticles}}$$

The morphology of carboplatin loaded PLGA microparticles were analyzed using a scanning electron microscope (Hitachi High Technology). Samples were prepared from dilutions in distilled water of particle suspensions and dropped onto stubs. After air drying, particle were coated with a thin layer of gold and then examined by scanning electron microscopy.

Tumor maintenance

Inbred Swiss albino mice were maintained the laboratory with free access to commercially available food pellets and water. For each experimental determination, 4-5 mice aged 10-12 weeks were used. Ascites Dalton's lymphoma tumor was maintained in vivo by intraperitoneal (ip) transplantation of $1X10^7$ tumor cells per animal (0.25 vol in phosphate - buffered saline, PBS). PBS was prepared by adding 0.15 M NaCl to 0.01 M sodium phosphate buffer, pH 7.4. Since tumor-transplanted animals usually survived for 18-20 days and were allowed to grow with tumor (tumor growth started on 7^{th} day after inoculation and treatment was started on 12^{th} day for the experimental design it was considered as the initial stage of tumor growth.

Drug delivery in experimental animals: scheduled given below

The drugs are administered intravenously, leading to non-specific systemic distribution and experimental scheduled given below: Experimental animals were divided into three treatment groups involving 60 mice of 4 batches containing 15 mice each and injected as follows.

Batch 1: animals received normal saline and were used as control (saline 10ml /kg of body wt intraperitoneal (i.p) injection (n=5).

Batch 2: animals were induced cancer with Dalton's lymphoma ascites transplantation of $1X10^7$ tumor cells per animals were allowed to grow with tumors and the batch 2 served as normal.

Batch 3: animals received a single dose of drug carboplatin was administrated to tumor bearing mice on the12^{th} day of tumor growth (maintained as experimental group 1) Carboplatin Dose (10 mg /ml was dissolved in 5% dextrose in water and use immediately).

Batch 4: animals received a single dose of drug carboplatin loaded microsphere was administrated to tumor bearing mice on the 12^{th} day of tumor growth (maintained as experimental group 2).

Treatment was started 12^{th} days after the tumor inoculation and continued observation for another for 5 days, body weight of animals were noted daily in all groups during treatment period. On the 17th day animal in all the groups mice were subjected for experimental studies, DLA bearing animals were sacrificed by anesthesia and organs tissue were isolated (liver, blood,

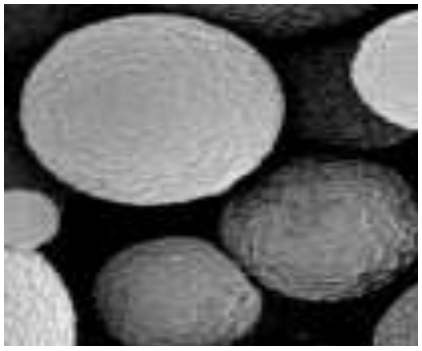
ascites tumor) frozen in liquid nitrogen and stored at -80°C until biochemical analysis could be completed. If any death, of the animals in different groups were recorded daily and the survival pattern of the animals were determined for different group and drug loaded and in- vitro drug release studies were carried out using scanning electron microscopy.

Western blotting

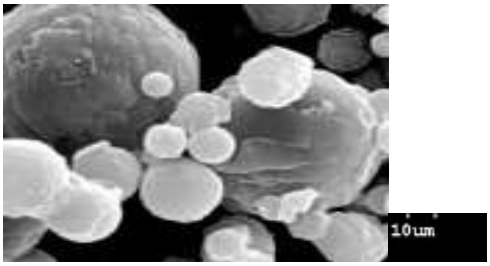
Liver tissues crude extract were used for estimation of GST activity according to the method of.[11] Extracted proteins (40µgprotein per lane) were analyzed by 15% SDS-PAGE.[12] Proteins were transferred electrophoretically to nitrocellulose filters (for 3 h at1A) using an immunoblot transfer apparatus. After transfer, the nitrocellulose was incubated for 1 h at room temperature in 3% (w/v) BSA in Tris-buffered saline (TBS; 500mM NaCl and 20mM Tris-HCl pH 7.5) to block non specific binding. The blot was incubated overnight at 4°C with 3% (w/v) BSA in TBS containing antiserum at a dilution of 1:500. After three 15 min washes with TBS containing 0.1% BSA and 0.2% Nonidet P40, the blot was incubated for 1 h at room temperature with peroxidase- conjugated goat anti (mouse immunoglobulin) diluted at 1:1000 in 3% BSA in TBS. The blot was again washed three times with TBS containing 0.1% BSA and 0.2% Nonidet P40. Antibodies were visualized using a chem.-illuminescence detection system.

RESULTS AND DISCUSSION

The SEM pictures of microspheres (control) and drug-loaded microsphere formulations are presented in Fig1. It can be seen that microspheres (control) are spherical with wrinkles on their surface (Fig. 1A). In case of drug-loaded microspheres, the formulation parameters such as drug loading, and concentration and molecular weight induced remarkable change in the surface morphology of the microspheres [Fig.1B]. Average particle size and polydispersity index of microspheres were measured by Laser Particle size analyzer after suitable dilution and Data was discussed (Data not given). Particle size density was also measured by Zetasizer (Malvern, UK).



B



A

Figure1: Scanning electron microscope image of microspheres.

Legend

Scanning electron microscope pictures of drug-free microspheres (A) and drug-loaded microsphere formulations (B).

The in-vitro release profile was biphasic with an initial burst release (7.266%) in 1 days attributed to surface associated drug, followed by a slower release phase as the entrapped drug slowly diffused out into the release medium and 65.49% drug was released after 30 days. A low burst release of the drug was observed due to washing of prepared PLGA particles. There was a sustained release of drug at a constant rate. The concentration of drug was above MIC (invitro) even after 30 days. The adsorbed molecules on the surface of particles are rapidly desorbed when in contact with the dissolution medium. The diffusion of the drug, the erosion and swelling of polymer matrix and the degradation of polymer were the main mechanism for the drug release (Fig 2). Since degradation of PLGA is slow, the release of carboplatin from particles would mainly depend on the drug diffusion, in the time of experimental studies. Samples of tumor were taken and immediately frozen for sectioning and imaging.

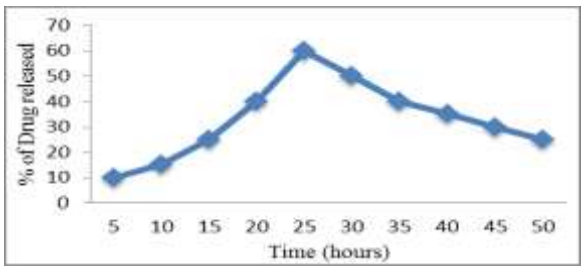


Figure 2: Response of Drug Release from Microspheres.

In Vitro drug release study microspheres did not maintain the form of spheres in an acidic medium. The microspheres swelled quickly and dissolved. Similar observations have been reported with non-cross-linked and cross-linked (glutaraldehyde and formaldehyde) chitosan microspheres.[13] Cytotoxic drugs cannot discriminate between healthy or cancerous tissue, targeting rapidly dividing cells, therefore systemically administered chemotherapy kills both the tumour and healthy tissue. Further, many drugs are insoluble making it impossible to deliver therapeutic doses to the site of

action (tumour). These drugs may be "solubilized" with the use of nano-sized colloidal carriers, ie by the creation of crystalline nanosuspensions that were stabilized by surfactants, or by combining them with organic or lipid nanoparticles. This has the effect of maintaining the drugs in the circulation for greater periods of time, enabling a therapeutic dose to be delivered to the tumour.[14]

Table 1: Characterization of Parameters of Carboplatin drug on Microspheres.

S. No.	Parameters (% w/w)	Mean ± SD n=5
1	Drug Content	26.9
2	Drug Entrapment	15.8
3	Drug Loading	18.95
4	Process Yield	56.8

The properties of microspheres, encapsulation efficiency loaded with the drug has shown in [Table1]. However, the yield was high in case of formulations prepared using low molecular weight than those prepared using higher (medium and high). The encapsulation efficiencies of microspheres were affected by the concentration and molecular weight of loaded material and volume of 1% wt/vol solution added. This may be explained on the basis that an increase in viscosity of the solution with increase in concentration prevents drug crystals from leaving the droplet. Microspheres containing drug (carboplatin) were prepared. With increase in drug loading, encapsulation efficiency of the microspheres increased when increasing volume of 1% wt/vol solution was used for cross-linking reaction. There was no significant increase in particle size and percent drug entrapment as polymer concentration was increased. The results indicate the optimal drug/polymer ratio to be 1:4 w/w for maximal micro particles formulation. Sonication time was also optimized in order to achieve stable formulation with minimum average particle size and maximum per cent drug entrapment.

Excessive amount of ascitic fluid was formed in mice of tumor developed group without treatment at the end point of the experiment (3 ml ascites/mouse in average). Drug loaded treatment alone significantly decreased the volume of ascitic fluid to an average of 1.1 ml/mouse at 20 mg/,these effects were comparable to carboplatin drug alone given and carboplatin loaded in microsphere released showed formation of ascetic fluid was completely eradicated. Non-blood cells in the ascitic fluid were counted as an estimation of tumor cells in the ascites. The results were shown as cell numbers/ml of ascites. Whereas carboplatin at the used does did not significantly reduce the number of cells in ascites, Microsphere loaded drug showed a strong effect in decreasing cell numbers/ml of ascites by 64% and 79% at the indicated doses. As there was no ascitic fluid in the drug released treatment groups. All mice did not show observable toxicity associated with the treatments and found no tissue damages were detected in the treatment groups and there was not significant differences between control group and treated groups.

Proteins were isolated from tumor samples of the treated and control mice. Western blot analysis showed GST protein expression also increased activity in microsphere loaded drug treated samples. These results confirmed the in vitro data that drug induced apoptosis in tumor cells. The elevated drug uptake by the tumor cell under the condition of increased glutathione concentration and GST activity after treatment could be an important contributory factor to carboplatin's anticancer activity leading to tumor regression [Figure 3]. GST represents an integral part of the detoxification system and protects cells against oxidative and chemical –induced toxicity and stress by catalyzing the S-conjugation between the thiol group of GSH and the electrophilic moiety of toxic substrates including carboplatin. The increase in GST activity identified in mice model, there was a specific induction in GST expression which paralleled the increase in total GST activity. Firstly, a reduction of intracellular CBDCA accumulation occurs in the majority of resistant cells. Secondly, increases in intracellular glutathione (GSH) concentration and glutathione S-transferase (GST) activity have been reported. GSH and GST are believed to play important roles in drug detoxification. The in vitro results were consistent with in vivo inhibition of tumor growth and ascites formation in cancer-bearing mice. Remarkably, with carboplatin microparticle treatment almost complete tumor inhibition (97%) was achieved and the ascites was completely diminished. This effect was not achieved by carboplatin microsphere treatment alone.

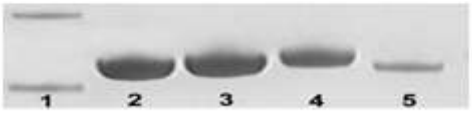


Figure 6: Western blot analysis of GST expression in mice model.

Legends
Lane1.GST molecular weight markers, Lane [36.5KDa; 54.5KDa]; Lane 2. GST expression in tumor treated with carboplain loaded microsphere. Lane 3. GST expression in tumor treated with carboplain. 4. GST in normal mice, Lane 5. GST in tumor induced mice.

Therefore, it was of interest to determine GSH levels in various tissues during ascites Dalloton's lymphoma growth *in vivo* and carboplatin treatment. Platinum uptake and glutathione-S-transferase (GST; EC 2.5.1.18) activity were also measured in Dalton's lymphoma cells collected from mice under different experimental conditions in an attempt to identify the mechanism of GSH changes and its significance in carboplatin mediated cancer chemotherapy. Alkylating agents stunt tumor growth by cross-linking guanine nucleobases resulting in abnormal base pairing or DNA strand breaks.

Tumor DNA is unable to uncoil and separate which prevents the cell from dividing. Typically, alkylating agents act nonspecifically requiring conversion into active substances *in vivo*.[15]

Carboplatin is one of the most widely used antineoplastic alkylating agents for the treatment of certain cancers such as testicular, ovarian carcinomas and carcinomas of the head and neck.[16] Therapeutic efficiency of nanoparticles to carry their contents deep within the tumor would be of huge benefit to enhancing the efficacy of chemotherapeutics. Therapeutic efficacy was determined utilizing a human breast carcinoma MCF-7 bearing murine model and significant MCF-7 tumor regression due to apoptosis was seen after intravenous injections of the liposome encapsulated cisplatin.[17] Additionally, studies have begun to focus on developing an understanding of how nanoparticle characteristics such as size, shape and surface properties affect their ability to penetrate into tumors. Targeting carboplatin increased its antitumor activity and markedly decreased its toxicity, allowing therapeutic responses not possible with a free drug.

CONCLUSION

Present study confirmed that the microspheres may be a suitable device for administration of carboplatin. The development of a system whereby drugs could be administered in a single dose, that maintained active levels of the drug for a prolonged period would be the ideal system. Tumor responses to treatment have conventionally been assessed from imaging measurements of tumor and its shrinkage may take several weeks to manifest. Exciting new methods are being developed to formulate nanoparticles out of a seemingly endless number of compositions expressing an even greater number of functions in the fight against cancer. Their inherently small size and modifiability are allowing for innovative controlled and targeted techniques resulting in a drastic reduction in anticancer treatment side effects and increased antitumor efficacy. Anticancer nanoparticulate technology is being developed with the goal to minimize side effects for nanoparticles treatments that are perfectly engineered to attack cancer in a decisive manner with healthy tissues suffering no undesirable consequences at the initial stages of cancer cell development. Thus, future anticancer therapies using nanomedicine can be envisioned to specifically kill all cancer cells within the tumor while leaving normal tissue in the body virtually untouched.

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