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Formulation and *in vitro* bioactivity of mitoxantrone-loaded biodegradable microspheres on rat glioma (RG2) cells

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This study describes the preparation and evaluation of mitoxantrone (MTZ)-loaded chitosan, bovine serum albumin (BSA) and poly(D,L-lactide-co-glycolide) (PLGA) microsphere formulations in vitro *and investigation of the* in vitro *bioactivity of the released drug (for BSA microspheres) or that obtained by extraction from microspheres (microencapsulated drug) on the rat glioma (RG2) cells. The prepared microspheres were tested for the particle size, drug loading, surface morphology and release characteristics. Then, RG2 cells were used for evaluating the cytotoxicity of MTZ (the original drug or that microencapsulated or released from the microspheres) by methyl-thiazol-tetrazolium (MTT) assay. Chitosan and PLGA microsphere formulations exhibiting particle size from 7.7 to 59.8 µm, encapsulation efficiency from 8.2 to 55.5% were prepared. They were spherical in shape, had a smooth surface and homogenous distribution. For the release samples of MTZ-containing BSA microspheres, the cell death ratios were over 50% for all formulations. With regard to MTZ obtained by extraction from drug-loaded chitosan and PLGA microspheres the cell death ratios were between 57.1-68.5% and 74.3%, respectively. It was concluded that BSA, chitosan and PLGA microspheres-delivered MTZ has significant cytotoxic effect on RG2 glioma cells.*

Key words: Glioma – Mitoxantrone – Biodegradable microsphere – MTT test.

Malignant gliomas continue to be resistant to treatment. Despite advances in surgery, radiation therapy, and continued improvements in systemic chemotherapeutic agents, the median survival of patients remains approximately one year from the time of diagnosis [1]. A significant limiting factor in treating glioma is the inability to deliver therapeutic concentrations of chemotherapeutic drugs to the tumor without incurring unacceptable systemic side effects [2]. Numerous approaches over the past 10-20 years have focused on improving local exposure of brain tumors to chemotherapeutic drugs to enhance survival. One approach uses implantable, biodegradable polymers for local, sustained drug delivery directly to the tumor [1]. Polymers containing chemotherapeutic agents have been extensively evaluated in animal models of brain tumors [3-8] and used most recently in the treatment of human glioma [9-11].

Mitoxantrone(MTZ), an anti-tumor drug of the anti-tumor antibiotic classification, is a clinically well-established drug with high anti-tumor activity [12]. MTZ is used in the treatment of advanced breast cancer, non-Hodgkin lymphoma, acute non-lymphoblastic leukemia, and chronic myelogenous leukemia in blast crisis and has been approved for the treatment of hepatic and ovarian cancer. It has also been identified as one of the most potent drugs against malignant glioma cell lines *in vitro* [13, 14]. Despite its clinical effectiveness in the treatment of various malignancies and its *in vitro* activity against gliomas, data from clinical trials based on the systemic administration of MTZ for treatment of malignant brain tumors have failed to demonstrate efficiency. Systemic treatments have been hindered by the poor penetration of MTZ into the central nervous system (CNS) and dose-limiting myelosupression with resulting leukopenia [8]. DiMeco *et al*. [8] have incorporated MTZ into pCPP:SA (20:80) wafers and investigated the release kinetics, toxicity, distribution and efficacy of MTZ delivered from these wafers *in vivo*. The authors have reported that MTZ delivered from intracranially implanted biodegradable wafer was effective in the treatment of 9L gliosarcoma in Fisher 344 rats.

Microspheres have been proven to be efficient systems for delivery of a wide range of chemotherapeutic drugs to the brain [1, 15]. Furthermore, due to the their size, microspheres in suspension can be easily implanted by stereotaxy in discreet, precise and functional areas of the brain without causing damage to the surrounding tissue [16]. Therefore, our purpose is to prepare and to evaluate mitoxantrone (MTZ)-loaded chitosan, bovine serum albumin (BSA) and poly(D,L-lactide-co-glycolide) (PLGA) microsphere formulations *in vitro* and to investigate the *in vitro* cytotoxicity of MTZ (the original drug or that microencapsulated or released from the microspheres) on the rat glioma (RG2) cells using the methyl-thiazol-tetrazolium (MTT) test.

I. EXPERIMENTAL

1. Materials

Mitoxantrone dihydrochloride, dimethyl formamide (DMF) and sodium dodecyl sulphate (SDS) were purchased from Sigma (USA) and 50:50 poly(D,L-lactide-co-glycolide)(Resomer RG503) from Boehringer Ingelheim (Germany). Chitosan (Protosan CL 213) was supplied by FMC Biopolymer (Norway). Polyvinyl alcohol (M_w 72,000) and aqueous glutaraldehyde solution (25% w/w) were provided by Merck (Germany). Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine and sodium pyruvate were supplied by Biological Industries (Israel). Fetal bovine serum (FBS) and penicillin G sodium-streptomycin sulfate solution were provided by Biochrom (Germany). RG2 cell lines purchased from American Type Culture Collection (ATCC, USA). All other chemicals were obtained commercially as high pressure liquid chromatography (HPLC) or analytical grade reagents.

2. HPLC assay of MTZ

Determination of MTZ was performed by HPLC according to the modified method of Hu *et al*. [17]. The modular HPLC consisted of Waters Assoc. Model 510 constant flow pump, a Waters Assoc. 717 autosampler and IBM Model (pc/2, 80/386) chromatography workstation. Samples were analyzed on a Waters Assoc. μBondapak C_{18} column (10 μm particle size, 25 cm x 4 mm i.d.) and MTZ and haloperidol as an internal standard were quantitated in a Waters Assoc. Model 490-e UV detector set at 242 nm. The mobile phase consisted of methanol:10 mM KH_2PO_4 buffer (pH 3.0)(55:45, v/v), containing 0.09% 1-pentanesulphonic acid sodium salt and was run through the HPLC system at a rate of 1.5 ml/min at room temperature. The mean

peak area ratio used throughout this study was calculated by dividing the MTZ peak area by the haloperidol peak area. The chromatographic method was validated by linearity, sensitivity, precision, accuracy and specificity. The lower limit of the detection is 0.1 μg/ml.

3. Preparation of microspheres

3.1. Preparation of chitosan microspheres

Preparation of MTZ-loaded chitosan microspheres was performed according to the modified method of Jameela *et al*. [18]. Twenty four milligrams of the drug was mixed with 6 g of 4% (w/v) solution of chitosan in 5% (v/v) acetic acid containing 2% (w/v) NaCl and dispersed in a mixture of 35 ml liquid paraffin and 25 ml petroleum ether containing 0.85% (w/v) sorbitan sesquioleate as stabilizer in a beaker using a homogenizator (Ultra Turrax T25 Basic, Ika Labortechnik, France) for three min at 11,000 rpm. The mixture was then transferred into a 100-ml round-bottomed flask and stirred using a paddle stirrer at 2,000 rpm for 5 min. Then, glutaraldehyde-saturated toluene (GST) prepared according to the method of Longo *et al*. [19] was added (*Table I*). After stirring for 2 h, the hardened spheres were centrifuged, washed several times with petroleum ether, twice with 5% (w/v) sodium bisulphite, followed by water and finally with acetone and dried.

Table I - Characteristics of MTZ-loaded chitosan (C) and PLGA (P) microspheres[a, b].

Formulation code	Amount of cross-linking agent	Encapsulation efficiency	Average particle size (μm)
C1c	1 ml	8.2 ± 0.1	21.9 ± 1.1
C2c	2 ml	24.2 ± 1.3	23.4 ± 2.0
C4c	4 ml	15.8 ± 1.0	20.5 ± 1.9
P1	-	55.5 ± 1.5	59.8 ± 0.4
P4	-	47.7 ± 2.4	35.4 ± 3.2
P6	-	29.7 ± 2.3	7.7 ± 0.1

[a]Data represents the mean ± standard deviation (n = 3).
[b]Theoretical drug loading of microspheres with MTZ was targeted as 10%.
[c]Glutaraldehyde-saturated toluene (GST) was used as a cross-linking agent.

3.2. Preparation of BSA microspheres

MTZ-loaded and unloaded albumin microspheres were manufactured and characterized as described previously [20]. MTZ was dissolved in an aqueous solution of BSA (17.5% w/v). The aqueous phase was dispersed in 10 ml dichloromethane containing poloxamer 188 by vortexing. The formed droplets were hardened by addition of a 10% (w/v) solution of previously purified glutaraldehyde in dichloromethane (PGD). Twenty five, 50, 100 or 500 μl of PGD solution were used for formulation A25, A50, A100 or A500, respectively. After initial cross-linking for 30 s, 1-butanol was added and mixed for 15 min. Finally, the microspheres were washed with acetone and cold water then lyophilized. To determine whether washing procedure affected the release characteristics of the microspheres, a batch of BSA microspheres (formulation A100) were washed with sodium bisulphide solution instead of water (formulation AS100).

3.3. Preparation of PLGA microspheres

MTZ-loaded PLGA (Resomer RG 503, 50:50) microspheres were prepared using the water-in-oil-in-water (w/o/w) technique [21]. PLGA was dissolved in 1.8 ml methylene chloride (6% or 10% PLGA-methylene chloride, w/w) and 0.4 ml of distilled water containing MTZ was added and mixed by vortexing for 2 min at room temperature. The primary w/o dispersion was injected with a syringe into aqueous 1, 4 or 6% PVA (w/v) for formulation P1, P4 or P6, respectively, while being mixed with a Silverson Laboratory Mixer-L4R (Silverson Machines, UK) at 300 rpm for formulation P1 and P4 or 2,500 rpm for formulation P6. The solvent was extracted by transferring the resulting w/o/w emulsion into 150 ml of 0.35% PVA solution and stirring for 1 h at room temperature. The solidified microspheres were recovered by centrifugation and dried under vacuum at room temperature.

4. Characterization of microspheres

4.1. Particle size distribution

Particle size distribution of microspheres was determined by laser diffractometry using a Malvern Mastersizer (Malvern Instruments Ltd., UK). Samples were prepared by suspending microspheres in distilled water containing 0.01% Tween 80 for 1 min in an ultrasonic bath and analyzed. The average particle size was expressed as the volume mean diameter.

4.2. Surface morphology

The surface morphology of the microspheres was examined by scanning electron microscopy (SEM) (Jeol-SEM 1200 EX, Japan). Microspheres were mounted on metal stubs with conductive silver paint and then sputtered with a 150 Å thick layer of gold using a Bio-Rad apparatus.

4.3. Determination of MTZ loading

The amount of MTZ incorporated in the chitosan microspheres was determined by extracting the drug containing microspheres using methanol. The microspheres (5 mg) were added to 15 ml of methanol and stirred magnetically for 24 h. The supernatant solution was assayed with the HPLC.

To determine the drug content of PLGA microspheres, 10 mg of MTZ-loaded microspheres was washed three times by 1 ml of 10 mM KH_2PO_4 buffer (pH 3.0) in order to determine the active substance existing on the surface of the microspheres. Then, it was centrifuged at 10,000 rpm for 4 min and the supernatant was assayed with the HPLC. Subsequently, remaining microspheres were dried and dissolved in 1 ml of methylene chloride. 0.5 ml volume of 10 mM KH_2PO_4 buffer (pH 3.0) was added to extract the entrapped MTZ, and following centrifugation at 10,000 rpm for 4 min, the aqueous layer containing the MTZ was transferred into a fresh tube and assayed with the HPLC.

4.4. *In vitro* release study

The *in vitro* MTZ release from microspheres was determined in phosphate buffered saline (PBS, pH 7.4). Twenty milligrams of chitosan or 10 mg of PLGA microspheres were suspended in 1 ml of PBS in polypropylene tubes placed in a shaker bath (37ºC) at 50 cpm. At predetermined time intervals, samples were centrifuged at 2,000 rpm for 5 min and supernatant removed for HPLC assay. Fresh replacement media was added to re-suspend the microspheres.

5. Cell culture

RG2 cells were maintained in DMEM supplemented with 10% FBS, L-glutamine (0.584 g/l), sodium pyruvate (0.11 g/l), penicillin G sodium (50 units/ml) and streptomycin sulfate (50 μg/ml) at 37°C in a humidified incubator containing 5% CO_2. Cells were grown in 75 cm^2 culture flasks. Confluent cell monolayers were trypsinized and cells in exponentially growing phase were used in cytotoxicity experiments.

6. Evaluation of bioactivity *in vitro*

The cytotoxicity of MTZ (the original drug or released from BSA microspheres or obtained by extraction from chitosan, BSA and PLGA microspheres) against RG2 cells was assessed using MTT assay. MTT assay measures the ability of viable cells to reduce a water-soluble, yellow tetrazolium salt (MTT) into a purple, insoluble formazan product. The color reaction is used as a measure of cell viability and proliferation [22]. RG2 cells in 80 μl of culture medium were seeded