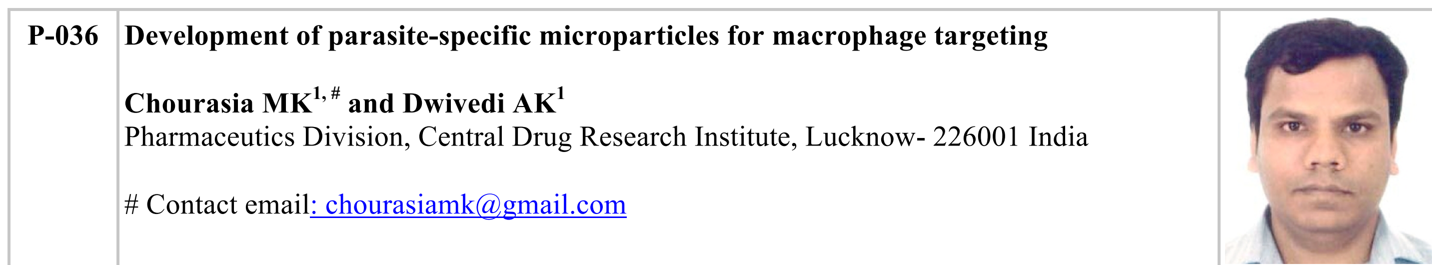

P-036

Development of parasite-specific microparticles for macrophage targeting

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INTRODUCTION AND OBJECTIVES

Visceral leishmaniasis (VL) is a disease caused by an intracellular protozoan most common in rural areas of developing countries, affecting around 10 million people in the world. Drug toxicity after administration of drugs through conventional dosage form is the major limitation associated with drug therapy against VL. Amphotericin has been considered as drug of choice for the treatment of VL. Doxorubicin (DOX) always ranked superior among other chemotherapeutics for solid tumors. Counter intuitively, DOX was reported to be equally effective anti leishmanial agent based on studies conducted by Sett et al in 1992 and Mukherji et al., 2004. Although highly potent, activity of DOX was limited by its fatal toxicity and cost, a point of concern when the affected population is rural strata of society especially in developing countries. We have developed a site specific drug delivery system with a view towards vectoring DOX to reticuloendothelial system.

MATERIALS AND METHODS

DOX was received as gift sample from SPARC, India. Span 85, tween 20, chitosan, dextran sulphate (DS), GI-1640 medium, eagles medium and locke's solution were obtained from Sigma Chemicals, USA. Glutaraldehyde, and triethylamine were purchased from Qualigens Fine Chemicals, India.

Optimized procedure for the formulation of chitosan microparticles Chitosan solution (1%w/v) was prepared by dissolving chitosan in 3% GAA (glacial acetic acid) and stirred overnight. DOX incubated with DS (1:1) in GAA (3%v/v) for 20-30 min was added to chitosan solution, resulting in Chitosan-DOX-DS complex. Light and heavy liquid paraffin oil (1:1) was taken and a mixture of Span 85, Tween 20 (7-8% v/v) in a ratio of 10:1 was added to this, which was then homogenized (16,000 rpm) for few min to ideally disperse surfactants in oil base. During homogenization, chitosan-DOX-DS solution was slowly injected into oil phase to form o/w emulsion. Homogenization carried out at 16,000 rpm for about 30 min resulted in formation of primary emulsion. Cross-linking agent toluene-saturated-glutaraldehyde (1:8) was added to it stage by stage (every hour for a period of 3h). Preparation was then transferred to magnetic stirrer and left for 4 to 5 hrs for stabilization and cross-linking. Formulation was centrifuged at 8000 g and sediment recovered, washed with n-hexane and acetone thrice.

Characterization Particle size was determined by dynamic light scattering (Malvern instrument). DSC was performed to check interaction between drug and formulation excipients. Intrinsic auto fluorescent property of DOX was exploited for image analysis. For determination of entrapment efficiency, weighed amount of MPs were dispersed in 3% (v/v) GAA solution and vortexed for 3 to 4 hrs. Digested chitosan was centrifuged, filtered and analyzed for drug content. In vitro phagocytic uptake study was conducted against intracellular amastigotes in macrophages. For in vivo studies, laboratory-bred male golden hamsters (Mesocricetus auratus) were used as experimental host. All animal care and experimental use of animals conformed to CPCSEA guidelines for laboratory animal facility and was approved by institutional animal ethics committee of CDRI. Animals were infected intracardially with 1x107 amastigotes in 0.1 ml PBS. Animals carrying 25–30 days old infection were employed for drug screening. A dose of 500 µg/Kg body weight/day was administered to hamsters (n=4) for 4 consecutive days. Three animals were sacrificed on day 7 post treatment and the dab smears were prepared from spleen, liver and bone marrow.

RESULTS AND DISCUSSION

Formulations of MPs were carried out using emulsion cross-linking method (Ya et al., 1996). Preliminary stage of this procedure includes formulation of emulsion followed by chemical cross-linking. Careful optimization of process parameters were carried out to obtain an ideal dosage form for *in vivo* administration. Incorporation of positively charged DOX into cationic polymer (chitosan) was a real challenge solved using polyanion, namely DS. Cationic DOX was masked by pairing amino groups with sulphate group of DS. In other words, protonable groups in DOX molecule were expected to interact with deprotonable groups of DS. Toluene saturated glutaraldehyde were used instead of glutaraldehyde to ensure effective miscibility of glutaraldehyde in oil base. Particle size was measured for different formulations (0.1, 0.5, 1, 2 and 3% v/v chitosan solution) using DLS. A linear relationship between particle size and concentration was observed. Surface morphology studies deserve attention especially when mode of administration of drug is systemic. Spherical MPs are more favorable for systemic drug administration as it can easily traverse blood vessels to reach its stipulated target of action. DOX loaded chitosan MPs exhibited higher degree of morphology with spherical structure as proven from fluorescence microscopic im-

ages. Smooth surfaces of these chitosan MPs were a result of toluene- saturated glutaraldehyde used for surface cross linking (Fig. 1).

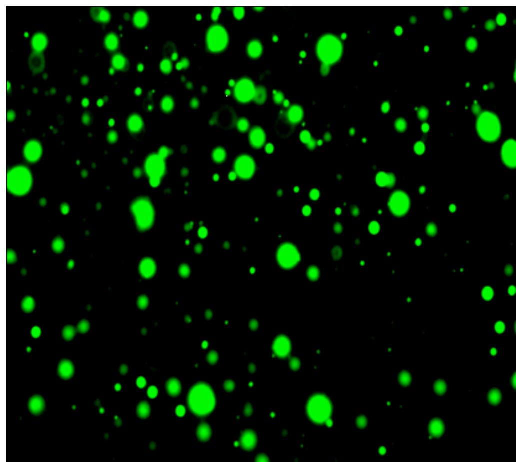

Figure 1 : Fluorescent imaging of doxorubicin loaded microparticles

Qualitative analysis of in vitro phagocytic uptake of DOX MPs was studied using macrophage J774.1 cells in light microscopy (Fig. 2). DOX MPs were non-specifically taken up by murine macrophage cell lines and maximum uptake observed at 60 min. Uptake of MPs by macrophages is a saturable process and MPs containing traces of surfactants can cause decrement in phagocytic uptake (Ahsan F et al., 2002) and so, it was carefully cleansed during formulation process as mentioned before. pH is another important factor that needs consideration while targeting macrophages for VL. Subsequent to uptake, phagocytized MPs fused with lysosomes (pH 3-6) resulting in degradation of polymer and release of drug therein. Chitosan, because of its acid resistive property buffers lysosomal pH by using $-NH_2$ moiety and thereby ensures sustained release of drug without bulk disruption for a short duration. This rationalizes our choice for chitosan as an intracellular drug delivery agent against VL.

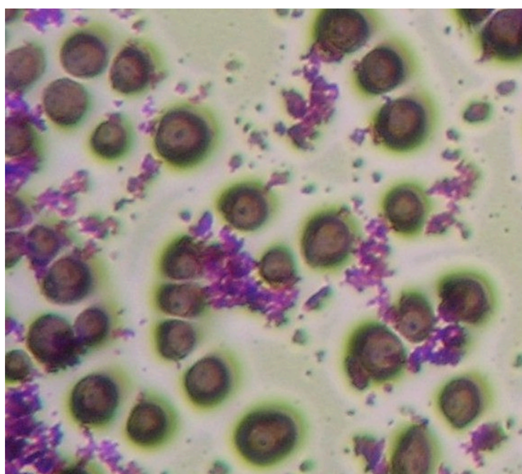

Figure 2 : *In vitro* phagocytic uptake studies of doxorubicin microparticles

From *in vivo* experiments, it was clearly evident that almost 78.2±10.4% inhibition of amastigotes was observed (Fig. 3). Free drug parasite inhibition was found to be 33.3±2.4%, which clearly depicts that DOX was distributed elsewhere in the body and its bioavailability towards spleen was very minimal. Ultimately, it was explicitly clarified that an optimal quantity of DOX required is reaching spleen and liver via macrophage transport. Hamsters infected with *L. donovani* seem to be an ideal model for study of progressive VL, where disease ultimately proves fatal thus simulating human *Kala azar*. *In vivo* studies carried out in hamsters ensured a safe, efficient, and targeted delivery of DOX. As seen from the graph, maximum suppression of parasite was governed in chitosan MP loaded DOX formulation. A 78.2±10.4% parasitic suppression indicates that our proposed delivery system can be a promising tool for anti leishmanial therapy (Fig. 3).

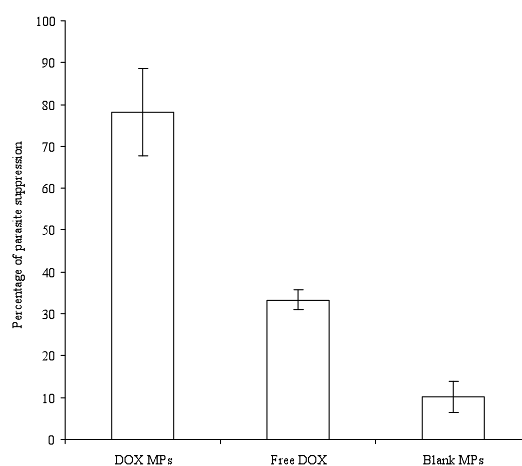

Figure 3 : *In vivo* studies on infected hamsters

CONCLUSIONS

In conclusion, our developed drug delivery system, for first time proved efficacy of DOX in micro particulate delivery system against treatment of VL using targeted drug delivery approach. Secondly, by exploiting passive and active targeting modes, total dose required for therapy was grossly cut down to few orders less, as evidenced from low dose administration (in µg), rendering the therapy favorable and affordable to common people.

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