Poly (D,L-lactic-co-glycolide) Nanoparticles for the Improved Therapeutic Efficacy of All-trans-retinoic Acid: A Study of Acute Myeloid Leukemia (AML) Cell Differentiation *In Vitro*

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Abstract: All-trans-retinoic acid reverses malignant cell growth and induces cell differentiation and apoptosis. Poor aqueous solubility and uncertain bioavailability are the limiting factors for using all-trans-retinoic acid for tumor therapy. The objective of present study was to encapsulate the hydrophobic drug all-trans-retinoic acid in the polymer poly (lactide-co-glycolide). The encapsulation was expected to improve the bioavailability and solubility of the drug. Oil in water single emulsion solvent evaporation technique used for the preparation efficiently encapsulated about 60% of the drug. The drug release profile showed a biphasic pattern with 70% of the drug being released in first 48 hrs and the residual drug showing a slow controlled release reaching up to 8 days. The particle size of 150-200 nm as determined with TEM was ideal for tumor targeting. All-trans-retinoic acid loaded nanoparticles were efficient to induce differentiation and blocked the proliferation of HL-60 cells invitro. These studies also revealed that the dosage of drug required for the therapeutic effects have been reduced efficiently. Our studies thereby demonstrate that Poly (lactide-co-glycolide) based nanoparticles may be efficient for parenteral administration of the drug.

Keywords: All-trans-retinoic acid, Controlled release, Nanoparticles, cancer, Myeloid leukemia, HL-60.

INTRODUCTION

All-trans-Retinoic acid (ATRA), a potent regulator of gene transcriptions, is produced in the body by the oxidation of retinol (active form of vitamin A) or retinal [1,2]. It had been shown to regulate important biological processes such as embryo development, control and maintenance of organ homeostasis and cellular growth, differentiation and apoptosis [3]. It also reverses malignant cell growth *in vitro* and in vivo, by inducing cell differentiation and apoptosis [4-8]. ATRA was also proved to have antiangiogenic effects in several systems [3]. Therefore, it is now increasingly used in cancer differentiation therapy and chemoprevention therapy [9]. The treatment of Acute Promyelocytic Leukemia with ATRA has shown a very high rate of complete remission. The drug was found to induce differentiation of the leukemic cells into mature granulocytes [10].

Poor aqueous solubility is a restraining factor in the parenteral formulation of ATRA and to date no commercial parenteral formulation is available [11]. On the other hand oral administration of ATRA induces its metabolism by activating cytochrome P450 enzyme [12]. Even a small amount of ATRA administration is enough to induce cytochrome P450. Repeated administration accelerates metabolism by the enzyme and it would be impossible to maintain effective level of drug in the blood. Uncertain bioavailability of the drug results from its variable absorption from the gastrointestinal tract. Fatty acid composition and pH of intraluminal bile also affects its intestinal absorption [13]. Increase in the drug dosage would give rise to severe toxic side effects. Therapeutical dosage of drug would result in retinoic acid syndrome and neurotoxicity, especially in children [12]. Several other side effects of ATRA include teratogenicity, retinoid acute resistance, hypertriglyceridemia, mucocutaneous dryness, headache, hepatotoxicity, nausea, vomiting, and abdominal pain [14-16].

A controlled release system of ATRA is required for maintaining the required therapeutic level and reaching the target pathological sites in the body. Different formulation strategies have been investigated in an attempt to overcome the accelerated metabolism of ATRA that leads to a progressive decline in plasma ATRA concentrations during a chronic daily schedule. These include the use of inhibitors of cytochrome P450 enzymes, administration of oral ATRA on an intermittent schedule, and encapsulation of ATRA into microspheres, microemulsions, nanodisks, liposomes, micelles and nanoparticles [17-22]. The nano dimension of the carriers would play a critical role in achieving optimized therapeutic regimen. Nanosized carriers are shown to possess better ability to penetrate through the physiological barriers and accumulate at the target site.

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Liposomal formulations of ATRA were developed a number of years ago. But unfortunately they have not passed beyond clinical trials [23,24]. Different polymers were also utilised to formulate appropriate drug delivery system for ATRA. Poly lactide and poly lactide-co-glycolide were the most sought of materials in those investigations [25, 26]. PLGA (Poly-DL-lactide-co-glycolide) is a FDA approved polymer. These polymers being polyesters in nature would undergo hydrolysis inside the body and form biocompatible products that could be removed by the citric acid cycle.

The aim of this work was to prepare PLGA nanoparticles encapsulating all-trans- retinoic acid and to characterise the drug loaded nanoparticles. The encapsulation efficiency of the carriers was calculated and an invitro release study in physiological pH was performed. An invitro assay of drug entrapped polymeric nanoparticle induced cell differentiation was performed using HL60 cells, a leukemic cell line derived from a 36-year-old woman with acute promyelocytic leukemia at the National Cancer Institute. Differentiation of the HL60 cells is accompanied with morphological changes characteristic of granulocyte. This was determined on Wright Giemsa stained Cytospin slide preparations [27]. The arrest of proliferation was confirmed by Fluorescence activated cell sorting (FACS) technique [28]. Percentage of viable cells was assessed by dye exclusion methods using trypan blue as the vital stain [29].

MATERIALS AND METHODS

Materials

PLGA (50:50), All trans Retinoic acid, Poly vinyl alcohol (Mw 1, 25,000), Trypan blue and Wright Giemsa stain were purchased from Sigma-Aldrich, USA. Dimethyl sulphoxide, Dichloromethane and Acetone were of reagent grade. HL 60 cell line was purchased from the National Centre for Cell Science, Pune. RPMI medium, Fetal bovine serum, penicillin G and Streptomycin were purchased from Gibco, Invitrogen.

Preparation of Drug Loaded Nanoparticles

PLGA nanoparticles were prepared by single emulsion solvent evaporation technique. PLGA (50:50) was dissolved in dichloromethane (DCM), 75 mg in 2 ml, and mixed with Retinoic acid solution in acetone, 5 mg in 3 ml. This mixture was made upto 10 ml with DCM and poured into 50 ml water containing poly vinyl alcohol (PVA - 1% w/v). The resultant oil in water emulsion was then carefully probe sonicated with an ultrasonicator (Vibracell – VCX – 130) for 3 min. The emulsion was stirred overnight and the organic solvent was allowed to evaporate. The nanoparticles were recovered by repeated centrifugation (10,500 rpm) and washing with water. The pellets were resuspended in water and kept for lyophilization. The lyophilization was completed at the end of 36 hrs and the product was stored at -20^{o} C till use.

Morphology and Particle Size Determination Studies

The morphology and particle size of the nanoparticles was determined by observation of the samples on a transmission electron microscope (TEM, JEOL 1011, Japan). 20µl of nanoparticles resuspended in water was loaded onto copper coated grids for TEM. Particle size was also measured using particle size analyzer (Beckman Coulter).

Determination of Retinoic Acid Encapsulated in Nanoparticles

The encapsulation efficiency of nanoparticles was determined by spectrophotometric analysis. A known quantity of the lyophilized product was dissolved in dichloromethane and measured at 365nm with UV spectrophotometer (Perkin Elmer, USA). ATRA concentration in the solution was calculated by comparing the absorption of the sample solution to standard curve relating light absorption and ATRA concentration. The encapsulation efficiency was determined using the following formula,

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Amount of retinoic acid in nanoparticles}}{\text{Initial quantity of drug taken}} \text{ X } 100$$

In Vitro Drug Release Studies

Retinoic acid loaded nanoparticles, 20 mg were dispersed in 20ml of PBS (phosphate buffered saline, pH 7.4) and incubated at 37^{o}C under continuous shaking at 250 rpm. At predetermined intervals, a known volume was removed and centrifuged to obtain a clear solution free of any particles. The amount of drug released into the medium was measured at 365 nm with UV spectrophotometer as already described. The medium was replaced each time to maintain the sink conditions. The experiment was performed in triplicate at regular time intervals from 0-192 hrs.

In Vitro Cell Studies

Cell Line and Culture Conditions

HL 60 cell line was purchased from the National Centre for Cell Science, Pune and routinely passaged at a density of 2 X 10^5 cells/mL in RPMI medium (Gibco) containing 10% Fetal bovine serum (Gibco), 100 U/mL penicillin G (Gibco) and 100 mg/L Streptomycin (Gibco) in a 5% CO_2 automatically controlled atmosphere and incubated at 37°C.

Induction of Differentiation

In a 6 well plate, HL 60 cells were seeded at a density of 1,00,000 cells/well. The culture medium was RPMI 1640 supplemented with 10% FBS. The first well was maintained as control. Well 2 was incubated with the nanoparticles uniformly dispersed in Dulbecco's Phosphate buffered saline solution at a concentration 27 ng/mL(DL3) while well 3 was incubated with free drug at concentrations 1µg/mL(designated as FD1). The cells were incubated for 7 days under 5% CO_2 at 37^{o}C.

Cell Viability Assay

The viability of cells treated with free ATRA and ATRA entrapped nanoparticles were compared using membrane integrity analysis by trypan blue dye exclusion method. Two batches of cells were treated independently with free retinoic acid and retinoic acid incorporated nanoparticles from 12 hr to 196 hrs. Thereafter cells were incubated with 0.1% trypan blue (Sigma) in 0.9% NaCl for 10 minutes. Trypan blue exclusion was studied by scoring 200 cells using a light microscope.

In Vitro Evaluation of Activity of the Encapsulated Retinoic Acid

HL 60 cells were seeded in 6 multi-well plates (2×10^6 cells/well) and maintained in RPMI 1640 containing 10% fetal calf serum, and 100U/mL:100µg/mL penicillin: streptomycin at 37°C in 5% CO_2 for 24 hrs. Cells were treated with 1µg/ml free-RA and 27 ng/ml of encapsulated RA for 7 days. Cytocentrifuge slides were prepared and morphological differentiation was assessed by Giemsa stain, by using a Leica DM 100 inverted microscope [30].

Flow Cytometric Analysis

After treating with free retinoic acid and retinoic acid nanoparticles for 1-7 days, cells were collected, pelleted and washed with phosphate buffered saline (PBS). They were resuspended in 300µl PBS. 700µl of ice cold ethanol (70%) was added while vortexing. The cells were then incubated at 4°C for 1 hour, washed with PBS and resuspended in 250 µl of PBS together with 10 µg of RNase. Incubation was continued for 1 hour at 37° C. The cellular DNA was stained with 10 µl of propidium iodide (10 mg/ml) for 15 minutes at room temperature. The stained cells were analyzed by FACS (Becton-Dickinson FACS II Flow Cytometer). Data of cytometric analysis are displayed as graphs of cell number versus relative fluorescence intensity.

RESULTS AND DISCUSSION

Emulsion solvent evaporation is a well established technique for the preparation of nanoparticles. The hydrophobic drug, retinoic acid was encapsulated in Poly L-lactide-co-glycolide polymer using oil-in-water emulsion technique. The polymer-drug organic solution is emulsified in aqueous phase to yield oil in water emulsion. Once the emulsion is formed, organic solvent is removed by means of evaporation to solidify the polymer droplets. After lyophilization, the nanoparticles were resuspended in dichloromethane to determine the encapsulation efficiency of the particles.

The encapsulation efficiency of PLGA nanoparticles determined by measuring the absorbance of the particles dissolved in DCM was 60% approximately. Being lipophilic, retinoic acid might have partitioned with the hydrophobic polymeric phase on the evaporation of organic solvent within an aqueous emulsion. Emulsification solvent evaporation is thus an ideal technique for loading ATRA into a hydrophobic polymer.

The particle size as observed under transmission electron microscope was found to be ~150-200 nm as shown in Fig. (**1**). The size of nanoemulsions would decrease with increasing concentrations of surfactants in the preparation. High concentration of PVA in the aqueous phase might have contributed the reduced particle size. Particle size trivially influences the distribution of the particles in the body after intravenous injection. Size less than 200 nm would be ideal for drug targeting and site specific delivery by means of nanoparticles. The DSC thermogram of pure ATRA is characterized by an endotherm melting peak at 187°C. The thermogram of ATRA encapsulated nanoparticle did not reveal the ATRA melting peak indicating that the drug exists in an amorphous state within the particle.

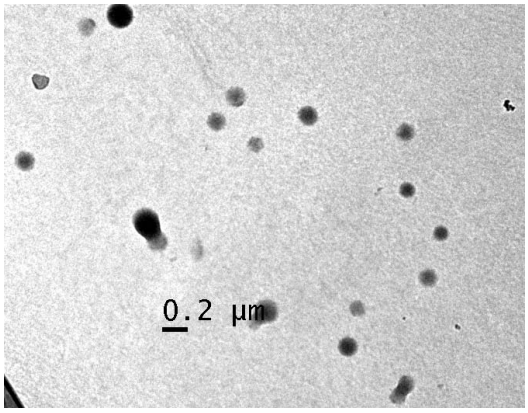

Fig. (1). Transmission Electron Microscopy images of ATRA entrapped PLGA nanoparticles.

The *in vitro* release profile of the drug from nanoparticles was studied by dispersing the particles in PBS. Fig. (**2**), illustrates the percentage wise release of ATRA from the particles at regular time intervals. The drug release shows a biphasic pattern. Approximately 70% of the drug was released in 48 hrs and almost 100% was released into the medium by the 8th day. Since retinoic acid is hydrophobic in nature, it would have agglomerated on the polymeric surface during solvent evaporation. This surface bound ATRA is released in a sudden burst initially. Smaller particle size that increases the surface area would also accelerate the release of the drug from the particles. This initial burst release contributes the logarithmic phase of drug release profile. Relatively small proportion of the drug would be entrapped within the polymeric phase. This entrapped portion of the drug would be released by diffusion through the polymer or hydrolytic degradation of the polymer. So there will be a slow down in the drug release pattern. The latter lag phase in the release profile indicates drug release by diffusion and degradation of the polymer.

Analysis of HL-60 Cells Treated with ATRA Loaded Nanoparticles

Viability of the HL 60 cells was analyzed by membrane integrity study using trypan blue dye exclusion method. The result of the assay is shown in Fig. (**3**). No significant difference in viability was observed between control and nanoparticle treated cells, which ranges from 80-100%. There was a greater amount of cytotoxicity associated with the free drug at any point of time and cell population exhibited a lower viability range between 73-94%. Numerous studies have proved that retinoic acid induces morphological and functional differentiation of the human promyelocytic leukemia cell line, HL-60. The morphological differentiation of the treated cells was studied using Giemsa staining method as shown in Fig. (**4**). After the cells were treated for 196 hrs with the free drug, about 50 – 60 % cells were of the granulocytic phenotype. In the case of cells exposed to retinoic acid encapsulated nanoparticles, the number of cells showing granulocytic phenotype was about 40-60%.

Antiproliferative and differentiation inducing capabilities of ATRA was not affected when entrapped in polymeric nanoparticles. Cytotoxicity of ATRA associated with its ability to induce differentiation of cells that eventually result in

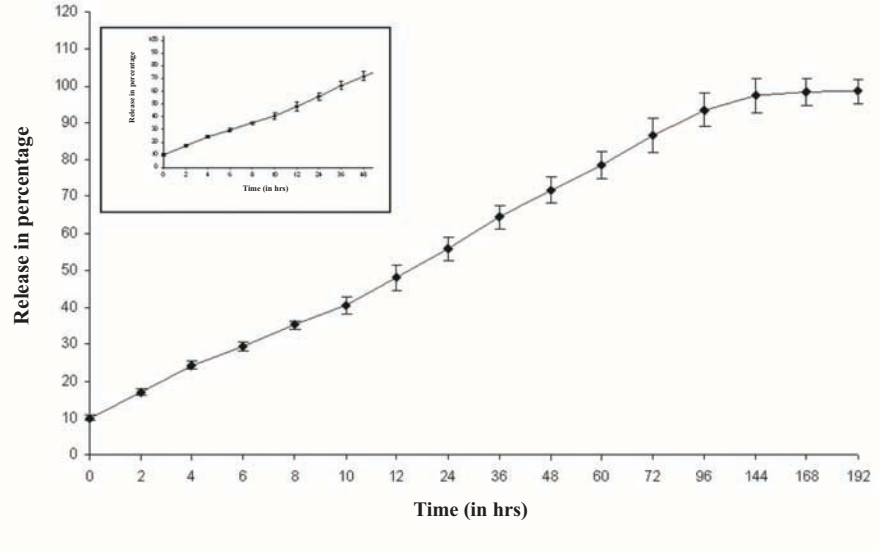

Fig. (2). Graph showing the release profile of retinoic acid encapsulated nanoparticles in Phosphate buffered saline (pH 7.4). The figure inset illustrates the logarithmic bulk release profile of ATRA from the particles extending up to 48 hrs.

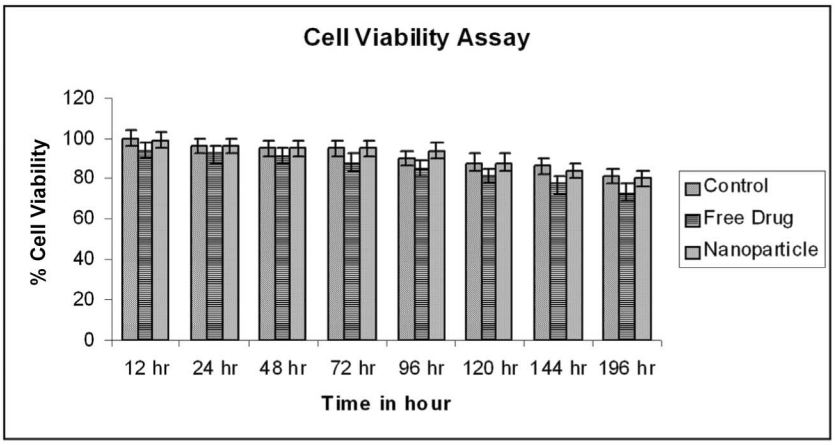

Fig. (3). Graphical comparison of the percentage of viable cells in control cell population and after treatment with free drug and drug entrapped PLGA nanoparticles determined by trypan blue dye exclusion method.

apoptosis also would not be reduced when loaded on polymeric carriers. The dosage of ATRA containing nanoparticles used to treat the cell population was low compared to the free drug dose used in the experiment. But the corresponding difference in number of differentiated cells and viable cells between the two was negligibly small. Hence the drug carrying nanoparticles would be more efficient for the treatment. There is an evident reduction in drug dosage required for the therapeutic effect. Improved solubility and better bioavailability of the nanodimensional formulation of the drug accounts for the enhanced therapeutic index of the drug entrapped in nanocarriers. They would be having an additional advantage of passive targeting and accumulation at the tumor site, *in vivo*.

The flow cytometric analysis (FACS) performed after 24 hrs also reveals that the drug induced differentiation of the cells. Fig. (**5**), shows the results of FACS analysis. 14.5 % of the cell population belonged to G2-M phase which is comparatively less than that of the control population. In the case of nanoparticles it was about 19.3% which is much similar to control population. After 196 hrs, there was a hike in the G1 population in case of cells exposed to free drug (85.6%) as well as to nanoparticles (74.2%) indicating the onset of differentiation. Thus, the observation revealed that retinoic acid encapsulated nanoparticles induce differentiation of HL 60 cells slowly but steadily.

In an asynchronous cell culture, cells would be distributed in different phases of the cell cycle. Percentage of cells in different phases would be proportional to the duration of these phases in the cell cycle. Increase in number of cells in a particular phase therefore indicates the lengthening of that phase in cell cycle. G1 phase may lengthen either due to cell proliferation arrest or cell differentiation. Differentiated cells would remain in a quiescent state which would appear to be in the G1 phase. Results of the FACS investigation of HL 60 cells treated with retinoic acid loaded nanoparticles thus indicates proliferation arrest and differentiation compared to

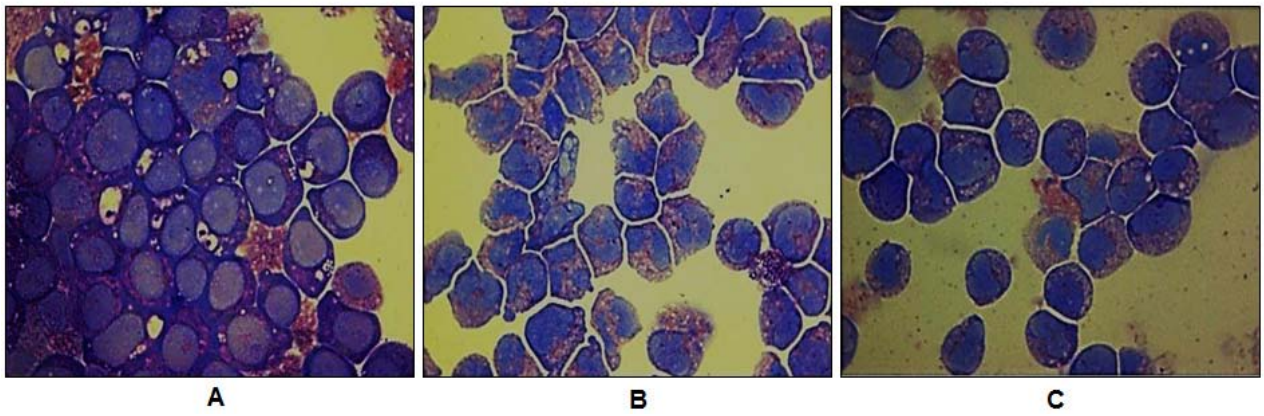

Fig. (4). Wright-Geimsa stained images showing morphological differentiation of (**A**) control cell population, (**B**) cells treated with free ATRA and (**C**) cells treated with ATRA entrapped PLGA nanoparticles.

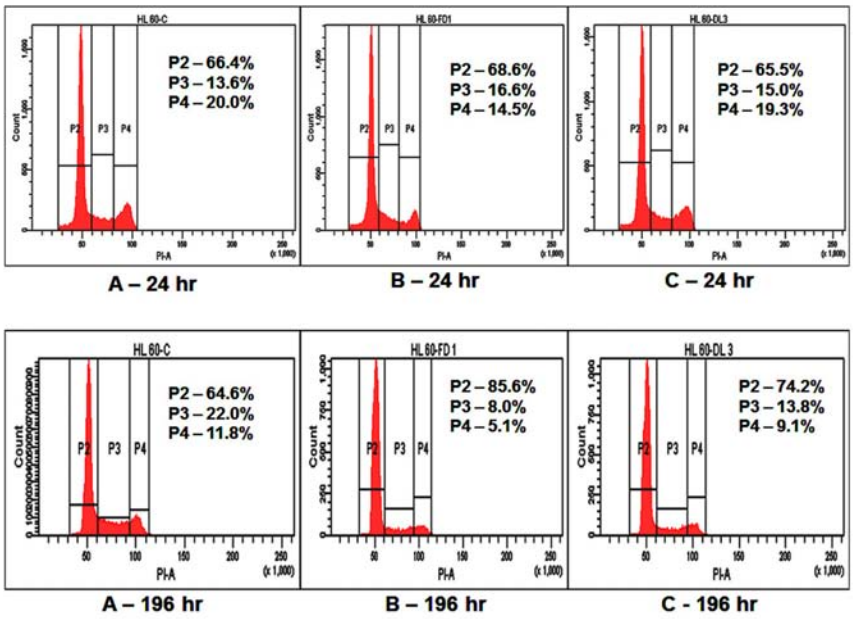

Fig. (5). Flow Cytometric analysis of (**A**) control cells (**B**) cells exposed to free drug & (**C**) cells exposed to retinoic acid nanoparticles. (P2- G1 phase, P3- S Phase & P4 – G2-M Phase).

the untreated cells (negative control). Thus it could be confirmed that antiproliferative and differentiation inducing activity of retinoic acid was not impaired by PLGA used in the preparation of nanoparticles. Comparatively slow differentiation induction and antiproliferative activity might be the result of lower dosage of drug loaded nanoparticles in the treatment. But this difference in activity was negligible. Thus these polymeric carriers undeniably comprise an improved therapeutic index.

CONCLUSIONS

PLGA nanoparticles prepared using the single emulsion solvent evaporation technique showed about 60% entrapment efficiency. High concentration of PVA in emulsion decreased the particle size to about ~150-200 nm. The drug was found to exist in an amorphous state inside the nanoparticles. Hydrophobic drug agglomerated on the surface of the polymer resulted in an initial burst release. Further release of ATRA was by diffusion through the polymer or by erosion of the polymer materials by the degradation of ester linkages. PLGA composition in the carrier system was not found to be associated with any cytotoxic activity. Slow and steady induction of cell differentiation indicated controlled release of the drug from the particles. Lower concentration of drug loaded nanoparticles induced almost same antiproliferative and differentiation inducing capability when compared with a higher dose of free drug. Hence the all-trans-retinoic acid entrapped nanoparticles have a higher therapeutic index compared with free all-trans-retinoic acid. Antiproliferative activity and differentiation inducing activity of ATRA was not impaired by PLGA used in carrier preparation. All these characters suggest that PLGA nanoparticles be competent for the controlled release and parenteral administration of ATRA.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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