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Article

Ameliorating the antitumor activity of lenalidomide using PLGA nanoparticles for the treatment of multiple myeloma

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Lenalidomide (LND) is an anti-cancer drug and an effective derivative of thalidomide used for multiple myeloma therapy. Because of its poor solubility in water, LND is known to cause low oral bioavailability (below 33%), and as a direct consequence of this, the dosing frequency is extended thus increasing risk of toxicity. To improve its bioavailability and sustained release, the present study aims to formulate polymeric nanoparticles (NPs) for LND using [Poly (lactic-co-glycolic acid)] (PLGA) as a polymer. The polymeric NPs were evaluated for particle size, SEM, XRD, drug content, entrapment efficiency (EE), *in vitro* release studies and *in vivo* bioavailability studies in rats. The formulated NPs possessed a size of 179±0.9 nm and a zeta potential of -24.4 ± 0.2 mV. The drug loading and EE of the optimized formulation was 32 ± 0.37 % and 78 ± 0.92% respectively. After oral administration of LND PLGA-NPs, the relative bioavailability was enhanced about 3.67-fold compared to LND. This study demonstrates the novel drug delivery for LND with PLGA-NPs as effective drug delivery system for sustained delivery of LND.

Uniterms: Multiple myeloma/treatment. Lenalidomide/uses. PLGA/sustained release. Polymeric nanoparticles/development. Solubility.

INTRODUCTION

Classified as a plasma cell malignancy, multiple myeloma (MM) is identified by the bone marrow accumulation of terminally differentiated B cells. Irrespective of therapeutic progression, MM remains without a cure till date. (Morgenroth *et al.*, 2011). Lenalidomide (LND), a thalidomide analogue is an immunomodulatory agent with antineoplastic and antiangiogenic properties which has been cleared for clinical application for the treatment of MM and transfusion dependent anemia (Kastritis, Dimopoulos, 2007; Richardson *et al.*, 2006). LND is off-white to pale yellow powder marketed with the trade name of Revlimid. Revlimid hemihydrate (commercial form) has poor oral bioavailability (< 33%) because of its inadequate solubility in water. LND has a shorter half-life of 3 hours (Song *et al.*, 2014). Novel therapeutic approaches are, therefore, urgently needed. Hence, the main target of this study was to formulate a nanoparticulated drug delivery system for LND which can enhance the solubility of LND, consequently improving its bioavailability.

In the past few decades, nanoparticulated drug delivery systems have remarkably ameliorated the efficacy of conventional therapies, by increasing the solubility and decreasing the drug associated systemic toxicities (Maeda *et al.*, 2000). Selective targeting and preferential accumulation of the drug at the tumor site can be achieved with nanoparticles existing within a diameter range of 20-200 nm, via greater permeability and retention (Egusquiaguirre *et al.*, 2012). PLGA is a biodegradable and biocompatible polymer that is hydrolytically degraded into nontoxic oligomer and monomer, lactic acid, and glycolic acid (Vega *et al.*, 2008). The higher surface area to volume ratio of PLGA NPs provides an amended pharmacokinetic and biodistribution profile of the therapeutic agents and thus reduces toxicity due to their specific aggregation at the target site (Noori Koopaei *et al.*, 2014). They also improve

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the solubility of hydrophobic compounds. Therefore, we expect that PLGA-NPs drug delivery system can improve poor oral bioavailability of LND along with sustained release to achieve intended therapeutic effect on level with the marketed products, while keeping its anti-neoplastic activity constant. The ultimate goal of this delivery system is to ensure the applicability to a wider population of cancer patients.

MATERIAL AND METHODS

Material

Lenalidomide (98.7%),and thalidomide (99.1%) (internal standard, IS) were obtained as a gift sample from NatcoPharmaLtd., (Hyderabad, India). PLGA, tetrahydrofuran, acetonitrile and acetone of HPLC grade was purchased from Sigma Aldrich, (Mumbai, India). The HPLC grade water was obtained by using Milli-Q Academic system, Millipore (Bangalore, India). All other chemicals used in this study were of analytical grade.

Methods

Preparation of lenalidomide nanoparticles (Venkatesh et al., 2015)

The nanoprecipation technique was the method of choice for the preparation of the nanoparticles as previously described (Venkatesh *et al., 2015*). Briefly, LND (20 mg) and PLGA (100 mg) were dissolved in dichlromethane (5 mL; Solution A) and were subjected to sonication for 5 min to dissolve all substances.125 mg of Poloxamer F-127 was dissolved in 50 mL of deionized water (Solution B). Solution A was then incorporated into solution B under magnetic stirring at varying rpm using a syringe with a flow rate of 1 mL/10 min. The schematic representation of fabrication of LND-PLGA-NPs is shown in Figure 1. The obtained nanosuspension was centrifuged, and lyophilized with cryoprotectant (2% sucrose) and tested for various characterization parameters.

Drug loading and entrapment efficiency

Drug loading and entrapment efficiency are assessed after freeze-drying and adding 10 mL of acetonitrile (a common solvent for drug and PLGA) to facilitate the coat of the NPs to get dissolved. The obtained suspension was diluted appropriately with mobile phase to determine drug content and entrapment efficiency. Samples were measured at an absorbance of 220 nm using high pressure liquid chromatography (Figure 2). The following equations

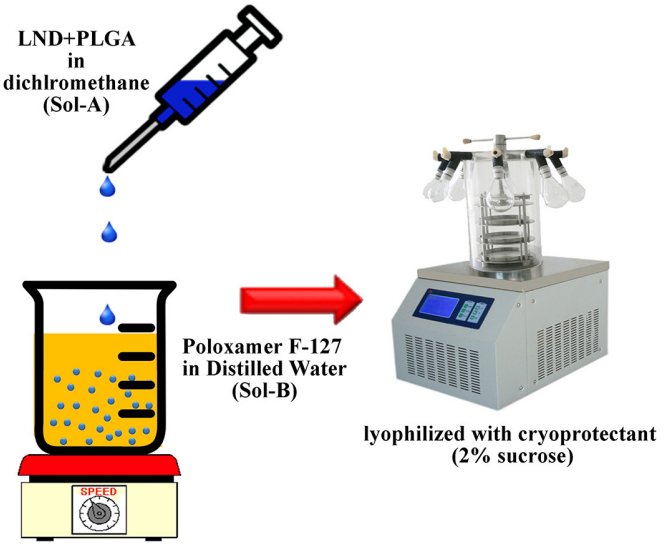

FIGURE 1 - Schematic representation of preparation of lenalidomide-PLGA-NPs.

were utilized for the determination of drug loading and entrapment efficiency of LND in NPs.

$$Drug\ loading\ (\%\ w/w) = \frac{Mass\ of\ drug\ in\ nanoparticle}{Mass\ of\ nanoparticle\ recovered} \times 100$$

$$Entrapment\ efficiency\ (\%) = \frac{Mass\ of\ the\ drug\ in\ nanoparticle}{Mass\ of\ the\ drug\ used\ in\ the\ formulation} \times 100$$

Characterization of nanoparticles

Particle size and zetapotential (Karri et al., 2015)

The prepared nanoparticles were washed with double distilled water (filtered through 0.22µm) several times before particle size analysis. The average particle size and zeta potential of the LND-PLGA-NPs were determined by Particle Size Analyzer (Malvern Instruments Ltd, UK) which allows sample measurement in the range of 0.020-2000µm.

Scanning electron microscopy (SEM)

The uniformity of the particles (size and shape) were verified using SEM. Lyophilized NPs were re-suspended in distilled water; subsequently they were placed on a silicon grid and left to dry at room temperature. The NPs suspension was then vacuum coated with gold for 3min before SEM analysis. Surface characteristics of the

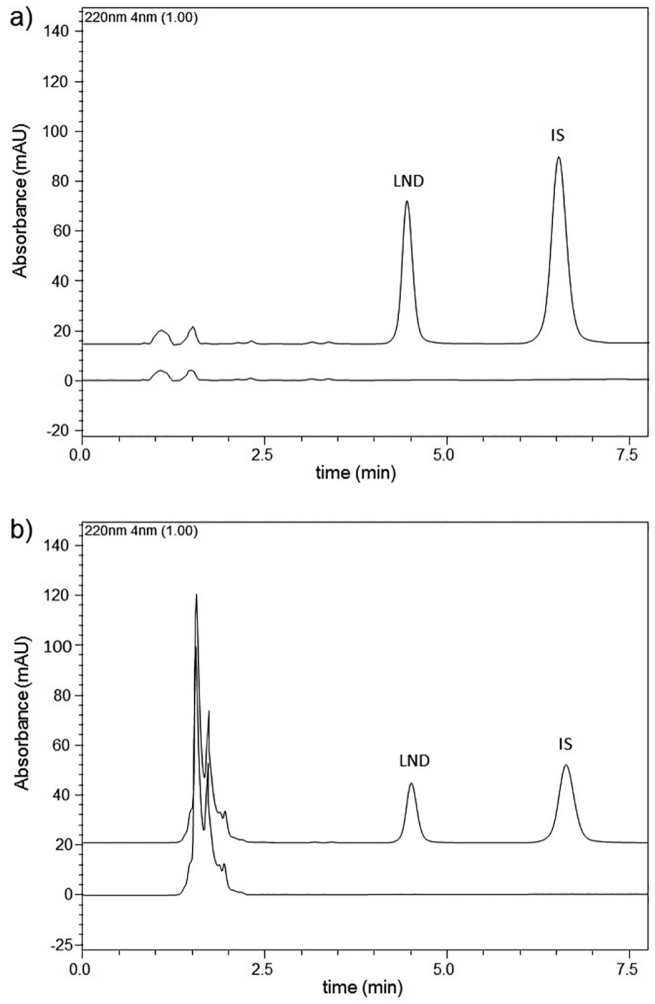

FIGURE 2 -Representative chromatograms corresponding to (a) lenalidomide loaded PLGA-NPs with IS (thalidomide) and blank PLGA-NPs and (b) lenalidomide spiked in rat plasma with IS (thalidomide) and processed blank rat plasma.

samples were observed under a SEM (JEOL Ltd, Japan) operated at 15-keV pulse under different resolutions.

Differential scanning calorimetry

DSC analysis was performed using DSC Q200. (TA instruments, U.S.A). The samples sealed in aluminum pans and heated at a rate of 10 °C per/min within a temperature range of 30 to 300 °C with constant nitrogen gas supply at a rate of 40 mL/min. DSC analysis was performed for PLGA, LND and LND-PLGA nanoparticles.

X-Ray diffraction (XRD) studies

An XRD peak mainly depends on the crystal size as they indicate the crystalline nature at particular value at 2θ range. Molecular arrangements of LND alone and in nanoparticulate formulations were performed on an X-ray diffractometer (PANalytical X'Pert Pro, The Netherlands) by applying CuKα radiation. The data was collected with an angular range from 3° to 50°2θ in continuous mode using a step size of $0.02^{0}2\theta$ and step time of 5 sec.

ANALYTICAL CONDITIONS

Quantification of LND in NPs and plasma samples was achieved using Shimadzu HPLC (LC 20 AD) (Kyoto, Japan) connected to a PDA detector (SPD-M20A). The chromatographic separation was performed on a Luna C_{18} column (150 × 4.6 mm) (Phenomenex, USA)with a mobile phase containing 20 mM KH_2PO_4: acetonitrile (86:14 v/v) at a pH of 4.2, supplied at a flow rate of 1 mL/min. Sample detection was carried out at 220 nm. Data collection and instrumental control was achieved by means of LC Solutions software (SP 1.1). Thalidomide was used as internal standard (IS), as it belongs to same class of LND and offered acceptable resolution with LND peak. Retention times of LND and IS were 4.5and 6.7 min respectively. The calibration curves were linear in the range of 0.5–30 µg/mL(r = 0.997) in NPs, and 20-600 ng/mL(r=0.998) in rat plasma, suggesting that the method was linear over the selected range (Mannemala, Nagarajan, 2015b). The intra and inter-day accuracy and precision were within CV%≤ 6%, indicating the method meets the acceptance criteria (FDA, 2001). The extraction efficiency in case of spiked plasma samples was 98.7±3.1%, suggesting that the procedure was consistent and robust (Mannemala, Nagarajan, 2015a).

In vitro release studies (Gomathi *et al.,* 2014)

The dialysis bag diffusion technique was incorporated to analyze the *in vitro* drug release of LND from NPs. The drug loaded NPs were placed in the dialysis bag (3 mL) and immersed into 250 mL of HCl buffer (pH 1.2) for a period of 48 h. The receptor phase was stirred and the temperature was maintained at 37 °C. At predetermined intervals (0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24 and 48 h), samples were drawn from the receptor compartment and equal volumes of fresh media were replaced to maintain sink equilibrium. The amount of drug dissolved was quantitated by high pressure liquid chromatography at 220 nm.

Cytotoxicity studies

Human multiple myeloma U266 cells were obtained from National center for cellular sciences (NCCS), Pune, India. Minimal essential medium (MEM) was used to culture the cells which was further supplemented with 10% fetal bovine serum (FBS), 3% L-glutamine,

100 U/mL pencillin-G and 100 µg/mL streptomycin (Himedia) in a humidified atmosphere of 5% CO_2:95% air at 37±2°C. A 96 well microtiter plate was seeded with 1lakh cells per milliliter of the U266 cell suspension for performing the cytotoxicity assay. After 24 h of seeding, fresh medium containing different concentrations of LND-PLGA-NPs suspension was added to the plate. A 10 mg/mL working concentration was freshly obtained by dissolving 0.5 mg of nanoparticles in 4.5 mL of DMSO and was filtered through a 0.22 µ filter prior to each assay. Blank cells (cells without test samples) were incubated with DMSO whose negligible presence in the wells was not found to be affecting the interfering with experiments.

In vivo bioavailability studies (Ma *et al.,* 2012)

All the animal investigations were performed as per the requisite protocol approved by the Institutional Animal Ethical Committee of JSS College of Pharmacy, Ooty, India. Approval letter no (JSSCP/IAEC/M.PHARM/PH.ANALYSIS/02/2012-13). *In vivo* studies were carried out in healthymale Wistar rats of 150–200g.The animals were housed in individual cages in the animal house for 10 days prior to the initiation of the study to facilitate environmental acclimatization and had access to feed and water *ad libitum*.12 h circadian rhythms were maintained and the temperature was kept constant throughout the study period. The animals were divided into 3 groups containing three animals in each group. The animal dose (30 mg/kg) was chosen based on the surface area ratio from human to rat. Group 1 pertained to the control, group 2 were treated with free drug and group 3 were treated with LNDPLGA-NPs.Blood samples, each not more than 500 µL were withdrawn from the tail vein at 0, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24 and 48 h period using a sterilized syringe. Fasting blood samples (0^{th} hour) were withdrawn early in the morning. The blood samples were collected in ria-vials containing anticoagulant (100 µL of 11% sodium citrate) and centrifuged at 4000 rpm for 15 min to separate the plasma and was stored at -20°C. The plasma samples were deproteinized by mixing the samples with equal volumes of 10% perchloric acid and vortexed for 2 min followed by centrifugation at 4000 rpm for 15 min. The supernatant liquid was separated and analyzed. The amount of LND in plasma samples by was estimated using HPLC at optimized chromatographic conditions.

Statistical analysis

Statistical data were analyzed using GraphPad Prism® 6 program (Graph pad Inc., USA).

In vitro

The experiments were replicated at least three times. The amount of each drug in the receptor compartment was estimated through HPLC. Data were shown as mean±S.D (n = 3). The differences between two samples were determined by student's t-test ($p < 0.05$).

In vivo

The experiments were replicated at least six times. The amount of drug in plasma was estimated through HPLC. Data was analyzed by one-way analysis of variance (ANOVA), and the mean differences between groups were considered to be significant at $p<0.05$. Data were shown as mean±S.D (n = 6). The relative oral bioavailability of NFV was calculated according to the equation:

$$\text{Relative BA (\%)} = 100 \times \frac{(AUC_A/dose_B)}{(AUC_B/dose_A)}$$

AUC_A and AUC_B represent the area under the blood concentration time curve of LND-PLGA-NPs and LND suspension, and $dose_A$ and $dose_B$ mean the dose of LND-PLGA-NPs and LND suspension following oral administration.

RESULTS AND DISCUSSION

Preparation

The nanoparticles containing LND were successfully obtained by the nanoprecipitation method. Different batches of LND-PLGA-NPs were prepared using different concentrations (100-500 mg) of polymer by keeping drug concentration constant with varying stirring rates (500-1500 rpm). During the procedure, polymer concentration increment caused a dramatic increase in size of the NPs (142-265 nm) and entrapment efficiency (16-78%). But after reaching a concentration of 300 mg, the saturation rate and entrapment of the drug to that of polymer were decreased. In the other hand, the stirring rate showed a significant influence on the size of NPs, i.e., at higher stirring rate (1500 rpm) the NPs particle size was reduced, whereas at lower stirring rate the particle size of NPs was not reduced, effectively. Drug loading has a very important influence in the polymeric NPs preference over others such as solid lipid NPs. The improper entrapment leads to the initial burst of the NPs, which hinders its sustained release property (Venkatesh *et al., 2015*). Hence, the batch with highest entrapment efficiency (78±0.92%) and drug loading (32±0.37%) was chosen as formulation of choice for further characterization.

Particle size and zeta potential analysis

The mean particle size of LND NPs was found to be179.4±0.9nm (Figure 3a) and the zeta potential were found to be -24.4±0.2mV (Figure 3b). The experiment was performed in triplicate (n=3) in order to ensure reproducibility and minimize the error. PDI values were found to be 0.464, which indicates that the system has a relatively narrow distribution. This narrow distribution ensures good stability of PLGA-NPs suspension by avoiding problems such as Ostwald ripening. Zeta potential reports had showed that the prepared PLGA nanoparticles had typical negative zeta potential attributed to PLGA preparations due to carboxyl groups present in their structure. The Zeta potential of the prepared NPs is also a prominent factor to ensure stability. Highly charged NPs are capable to remain stable as colloidal suspension. Since the prepared NPs have shown high zeta potential which ensure the stability of the formulation. The morphological evaluation (size, shape and morphology) of the LND-PLGA-NPs was performed using SEM (Figure 4). SEM studies confirm that nanoparticles are in the range of 50-300 nm with smooth surface and spherical shape which ensures the drug release in sustained manner.

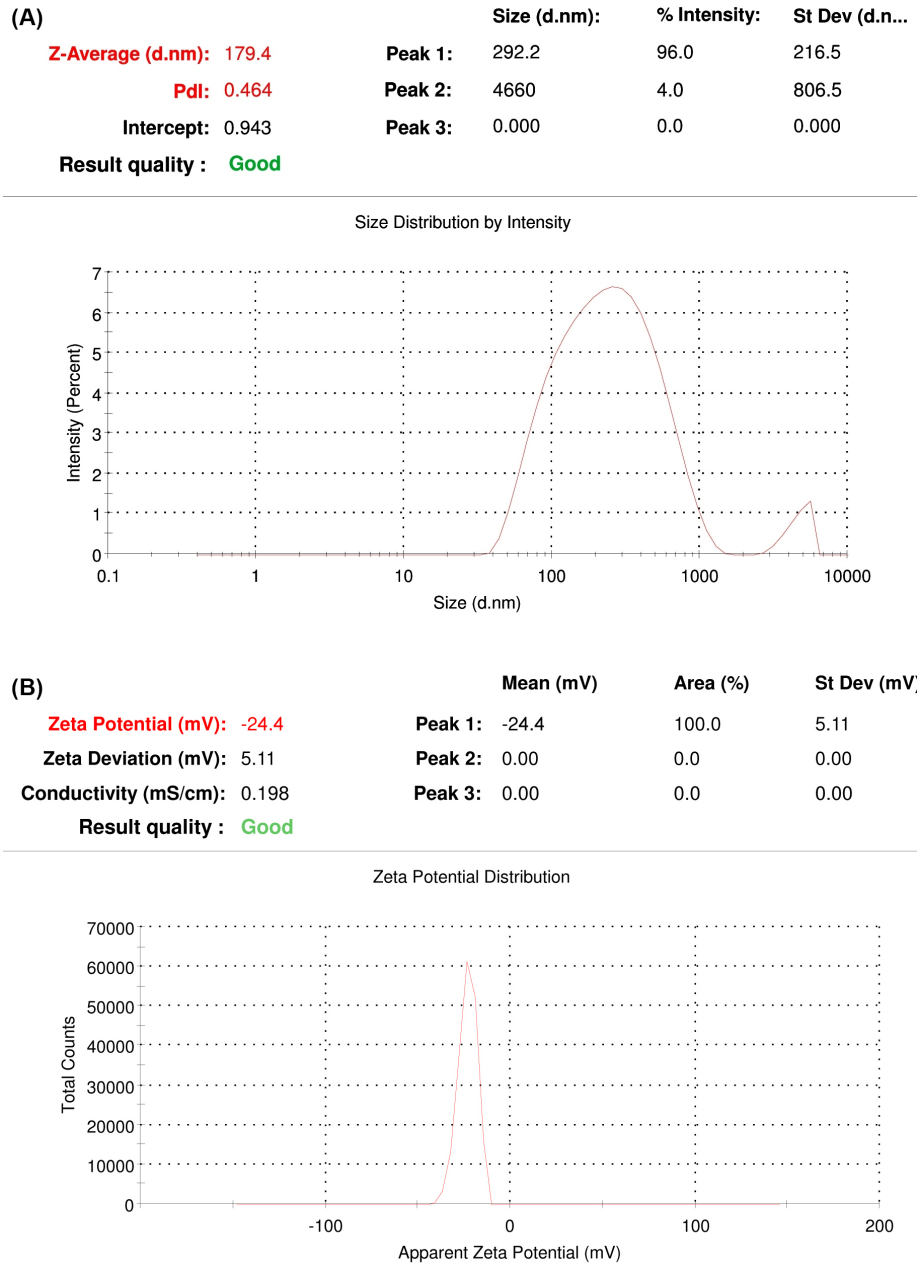

FIGURE 3 - (a) Particle Size; (b) Zeta potential of lenalidomide-PLGA-NPs.

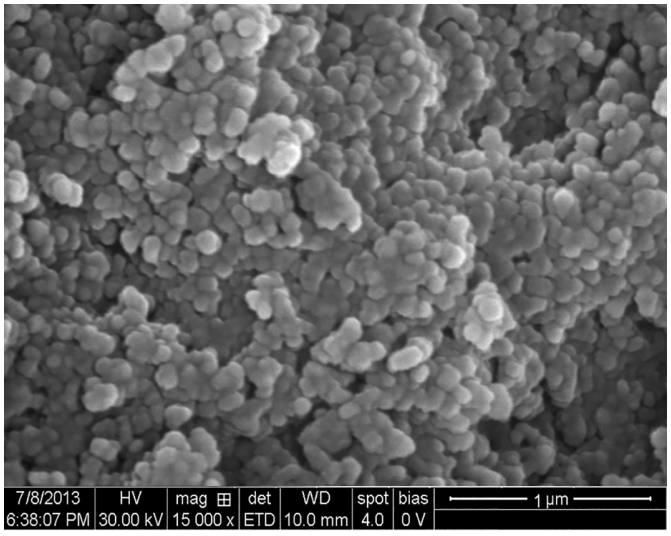

FIGURE 4 - SEM photograph of lenalidomide -PLGA-NPs.

DSC and XRD studies

The DSC thermograms of LND, PLGA, and NPs are shown in Figure 5. The DSC curve of LND exhibit an exothermic peak at peak temperature of 265 °C corresponding to its melting point. The polymer, PLGA had shown peak at a temperature of 65.12. As per the DSC graph of LND-PLGA-NPs, the characteristic exothermic peak of LND was observed with minimal intensity and shifted to lower temperature range. This indicates the conversion of crystalline nature of LND to amorphous form which ensures the better stability. Furthermore, it also confirms that LND was entrapped into PLGA-NPs. Reported findings have suggested that with an increase in the amorphous nature of the therapeutic system, a corresponding increase in the efficiency of delivery is observed (Abdelwahed *et al.*, 2006).

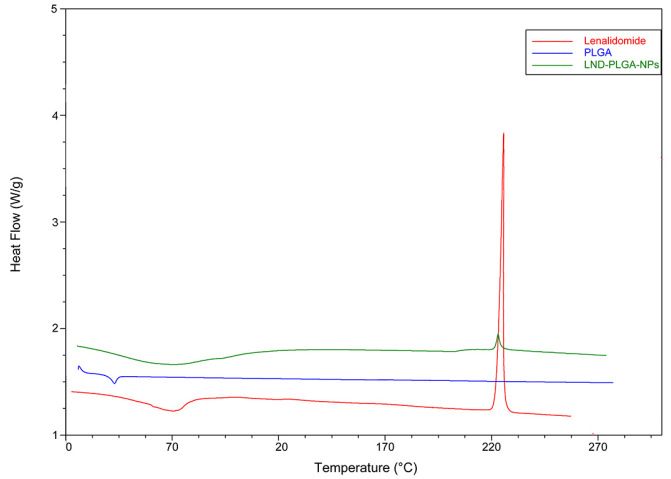

FIGURE 5 -DSC curves of lenalidomide, PLGA, and lenalidomide -PLGA-NPs.

The physical nature of pure LND, PLGA-NPs and LND loaded PLGA-NPs were observed using X-ray diffraction analysis (Figure 6). Pure LND possessed a crystal structure when compared to LND-PLGA-NPs, which were in amorphous form. Pure LND has shown multiple peaks at different 2θ values indicating crystalline form of pure LND. However, the intensity of LND peaks were greatly reduced in PLGA-NPs signifying the conversion of crystalline form LND to amorphous form and LND loading in to PLGA-NPs.

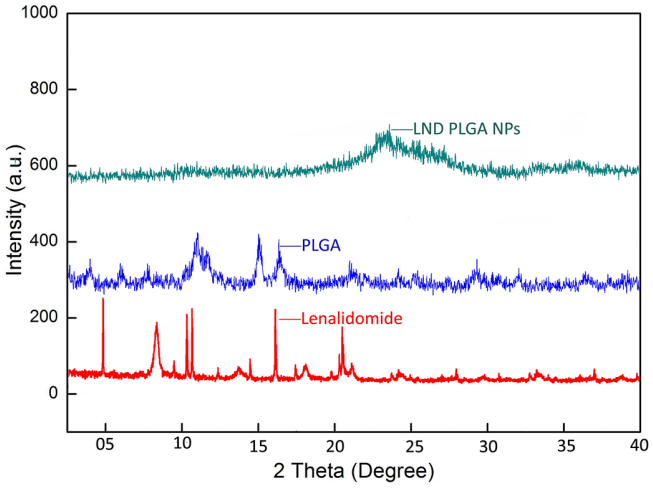

FIGURE 6 -XRD of lenalidomide, PLGA and lenalidomide-PLGA-NPs.

In vitro drug release

In vitro release studies of LND loaded PLGA-NPs were studied up to 48 h in HCl buffer (pH 1.2) for a period of 48 h. The summarized *in vitro* release of LND-PLGA-NPs is shown in Figure 7, as cumulative percentage drug release. The NPs has shown a biphasic pattern of drug release. Initially, a burst release of drug was observed (39%) followed by sustained release. 40% of LND was released within 6 hours and about 76.89 ± 2.99% and 83 ± 3.90% was released in 24 and 48 h respectively. This clearly depicts the pronounced time prolongation of the drug release. The initial burst release of drug may be due to drug desorption of surface entrapped or adhered LND from the particle surface, and the sustained release can be characterized by the drug diffusion through the polymeric matrix and subsequent dissolution of drug or erosion of the polymeric matrix. In dissolution media, the release of LND from PLGA-NPs occurred either by the formation of holes in the particles or by their disintegration. A constant rate of release of the drug from the NPs will facilitate better drug control *in vivo*.

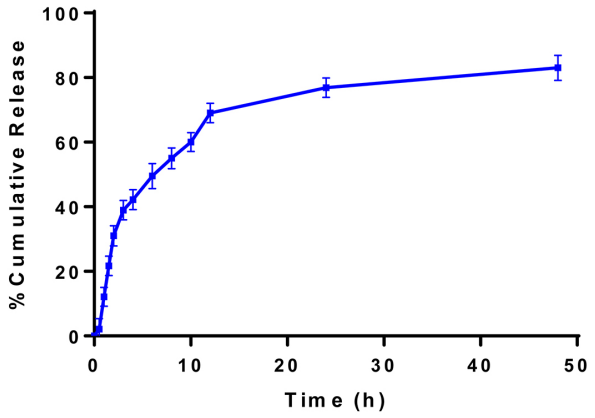

FIGURE 7 -Release rates of lenalidomide from PLGA-NPs *in vitro* in HCl buffer pH 1.2 (mean±SD, n = 3).

Cytotoxicity studies

The extent of cell viability due to LND-PLGA-NPs was examined by MTT assay in U266 cells. Eight concentrations (1-100 µg/mL) of LND-PLGA-NPs were prepared and tested yielding proof that the NPs exhibited significant toxicity against U266 cells. Cytotoxicity concentration (CTC_{50}) value for NPs was calculated from the concentration and subsequent response; it was found to be34.09 µg/mL (Figure 8). This proves the cytotoxic potential of the PLGA-NPs and lends further credence to the viability if this concept.

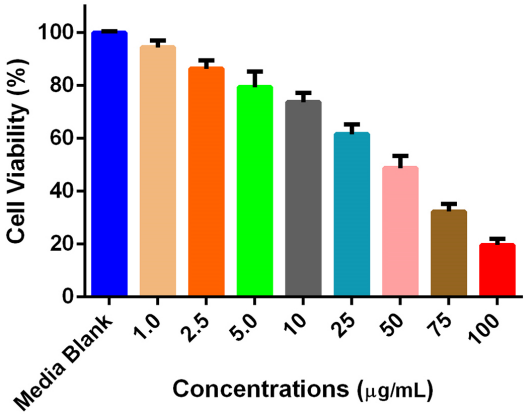

FIGURE 8 - MTT assay of lenalidomide - PLGA-NPs.

In vivo bioavailability studies

The formulations (LND aqueous suspension, dispersion of the LND-loaded PLGA NPs) were administered by oral gavage with a single dose of 30 mg/kg. The nanoparticles were dispersed in ultra-pure water. Table I and Figure 9 shows the pharmacokinetic data of the drug suspension and nanoparticles. Figure 9 depicts a remarkable difference in bioavailability between LND PLGA NPs and LND suspension. After oral administration of LND suspension, the drug was rapidly absorbed and a C_{max} of 0.57±0.003 µg/mL was reached in 1.5 h. Consequently, the plasma concentration decreased abruptly, as the drug was rapidly distributed and metabolized and was detected up to 48 h after administration, resulting in low AUC_{0-t} (4.9695 ± 0.036 h.µg/mL) and low $t_{½}$ (9.762±1.9 h). Figure 9 shows that peak plasma concentration for LND PLGA NPs (C_{max}) of approximately1.37±0.007 µg/mL was achieved at 4 h.

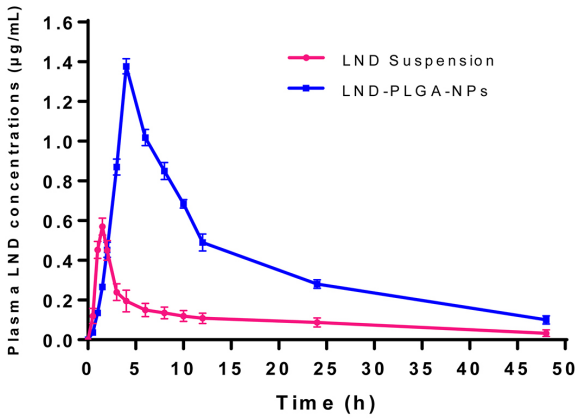

FIGURE 9 - Mean plasma concentration time profiles of lenalidomide in wistar rats: (a) lenalidomide suspension (b) lenalidomide PLGA-NPs with (mean±SD, n=6).

Table I depicts the pharmacokinetic parameters C_{max}, AUC_{0-t}, $t_{½}$and t_{max} after oral administration of LND loaded PLGA-NPs and suspension. From Table I, it can be perceived that, the C_{max} value for LND PLGA NPs (1.377±0.007) was significantly higher than LND suspension (0.57±0.003). In the same way, the $t_{½}$ was rapid for LND-PLGA-NPs (20.2183±2.2), when compared to LND suspension (9.762 ±1.9). From the data in Table I, it is apparent that, the t_{max} was also rapid for LND PLGA NPs (4.0±0.026), when compared to LND suspension (1.5±0.024). This may be due to the primarily related prolonged absorption phase and sustained release of PLGA-NPs. Likewise, the area under curve AUC for LND PLGA NPs was 3-fold higher than the LND suspension. Finally, the relative bioavailability for LND PLGA NPs was computed and found to be 367.80%, suggesting better absorption of LND-PLGA-NPs compared to the suspension. On the contrary, particles belonging to nanoscale are easily absorbed into the intestinal folds, while larger particle surface area to volume offers faster

TABLE I - Pharmacokinetic parameters of lenalidomide after oral administration of LND loaded PLGA-NPs and suspension at the dose of 30 mg/kg

Parameters	LND Suspension	LND-PLGA-NPs
C_{max}(µg/mL)	0.57±0.003	1.377±0.007
AUC_{0-t}(h.µg/mL)	4.9695±0.036	18.276±0.042
$t_{½}$(h)	9.762±1.9	20.2183±2.2
t_{max}(h)	1.5±0.024	4.0±0.026
F_{rel}(%)	-	367.80

drug dissolution. In current study, there was a significant improvement in bioavailability with LND-PLGA-NPs in contrast to LND suspension. The reasons may be many, but it is assumed that nano based drug delivery system enhances the bioavailability by modulating the physiochemical properties of poorly soluble drugs. Overall, our results suggest that LND-PLGA-NPs are most likely to have great potential as a therapeutic, by enhanced pharmacokinetic profiles.

CONCLUSION

Nanoparticles of a poorly soluble drug LND were successfully formulated by nano-precipitation method using PLGA as polymer. The particle size, zeta and SEM reports confirmed that the size of LND-PLGA-NPs was below 200 nm with spherical shape and uniform size distribution. DSC and XRD analysis of NPs confirmed the conversion of the crystalline nature of LND to amorphous form and drug entrapment within the NPs. Cytotoxic studies suggested that LND-PLGA-NPs were toxic towards U266 cancer cells. *In vitro* and *in vivo* studies confirmed that LND-PLGA-NPs demonstrate increased bioavailability with a sustained release up to 48 h. Due to their hydrophobic nature, conventional chemotherapeutics of LND suffer from poor solubility and an inability to penetrate the tumors (poor bioavailability). This results in grave side effects that include immune system depletion and metastasis to neighboring organs. Hence, the present PLGA nanoparticles can effectively solve the solubility problem and enhance the bioavailability of LND.

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