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Poly (DL-lactide-co-glycolide) nanoparticle-based inhalable sustained drug delivery system for experimental tuberculosis

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***Objectives*: To improve the bioavailability of antitubercular drugs (ATDs) as well as to assess the feasibility of administering ATDs via the respiratory route, this study reports the formulation of three frontline ATDs, i.e. rifampicin, isoniazid and pyrazinamide encapsulated in poly (DL-lactide-co-glycolide) nanoparticles suitable for nebulization.**

***Methods*: Drug-loaded nanoparticles were prepared by the multiple emulsion technique, vacuum-dried and nebulized to guinea pigs. The formulation was evaluated with respect to the pharmacokinetics of each drug and its chemotherapeutic potential in *Mycobacterium tuberculosis* infected guinea pigs.**

***Results*: The aerosolized particles exhibited a mass median aerodynamic diameter of 1.88 ± 0.11 μm, favourable for bronchoalveolar lung delivery. A single nebulization to guinea pigs resulted in sustained therapeutic drug levels in the plasma for 6–8 days and in the lungs for up to 11 days. The elimination half-life and mean residence time of the drugs were significantly prolonged compared to when the parent drugs were administered orally, resulting in an enhanced relative bioavailability (compared to oral administration) for encapsulated drugs (12.7-, 32.8- and 14.7-fold for rifampicin, isoniazid and pyrazinamide, respectively). The absolute bioavailability [compared to intravenous (iv) administration] was also increased by 6.5-, 19.1- and 13.4-fold for rifampicin, isoniazid and pyrazinamide, respectively. On nebulization of nanoparticles containing drugs to *M. tuberculosis* infected guinea pigs at every 10th day, no tubercle bacilli could be detected in the lung after five doses of treatment whereas 46 daily doses of orally administered drug were required to obtain an equivalent therapeutic benefit.**

***Conclusions*: Nebulization of nanoparticles-based ATDs forms a sound basis for improving drug bioavailability and reducing the dosing frequency for better management of pulmonary tuberculosis.**

Keywords: poly(DL-lactide-co-glycolide), nanoparticles, antitubercular drugs, nebulization, tuberculosis

Introduction

Tuberculosis (TB) is a chronic communicable disease caused by the bacterium *Mycobacterium tuberculosis* that infects over 1.8 billion people worldwide and is responsible for 1.5 million deaths annually.[1] Although an effective therapeutic regimen is available, patient non-compliance [because of the need to take antitubercular drugs (ATDs) daily or several times a week] results in treatment failure as well as the emergence of drug resistance. Patient compliance can be improved by the use of ATD formulations, which reduce the dosing frequency of the drugs. For this purpose, ATDs encapsulated in liposomes[2] and polymers such as poly(DL-lactide-co-glycolide) (PLG)[3,4] have been successfully used as injectable preparations in experimental TB models.

In the last decade, nanoparticle-based drug delivery systems have been developed with the goal of better management of diverse clinical conditions.[5,6] The small size of nanoparticles can be used with advantage for direct pulmonary delivery.[7,8] Because PLG polymers are biodegradable and biocompatible, they have been the most commonly used drug carriers.[9] Pulmonary TB being the commonest form of TB and alveolar macrophages being the abode of *M. tuberculosis*, the present communication reports for the first time, on the nebulization of PLG-nanoparticles (PLG-NP) as carriers for three frontline ATDs, i.e. rifampicin, isoniazid and pyrazinamide and their chemotherapeutic potential against experimental TB in guinea pigs.

Materials and methods

Chemicals and drugs

Poly (DL-lactide-co-glycolide) (50:50) resomer RG 506, M_r 2500, was purchased from Boehringer Ingelheim (Germany), polyvinyl alcohol

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(PVA, 87–89% hydrolysed, average M_r 13 000–23 000), rifampicin, isoniazid and pyrazinamide were obtained from Sigma (St Louis, MO, USA). All other reagents were obtained from standard companies.

Animals

Dunkin Hartley guinea pigs of either sex (300–400 g), obtained from Hisar Agricultural University, Hisar (India) were used in the study. The animals were fed standard pellet diet and water *ad libitum*. The study was approved by the Institute's Ethics Committee.

Culture

The culture of *M. tuberculosis* H37Rv was originally obtained from the National Collection of Type Cultures (NCTC), London, UK, and was maintained on Youman's modified medium.

Preparation of PLG-nanoparticles (PLG-NP)

PLG-NP were prepared by the multiple emulsion technique described by Lamprecht *et al.*[10] with slight modifications. Briefly, 1 mL of an aqueous drug solution was first emulsified in 10 mL of dichloromethane (DCM) containing the polymer (drug/polymer: 1:1 by weight) by probe sonication (Misonix Inc., Farmingdale, NY, USA; Model XL 2020, 120 W, 20 kHz) for 1 min. In the case of rifampicin, the drug was directly added to DCM before sonication. The primary emulsion was poured into 8 mL of 1% aqueous PVA solution and sonicated for 3 min to form the second water-in-oil-in-water emulsion. The latter was stirred continuously overnight for complete removal of DCM. The PLG-NP were recovered by centrifugation (8000–10 000 rpm, 15 min), washed thrice with distilled water and vacuum-dried. Drug-free/empty PLG-NP were prepared by substituting normal saline for the ATD. The PLG-NP were resuspended in normal saline before each experiment. It should be noted that all three drugs were encapsulated simultaneously in PLG-NP.

Nanoparticle characterization

The drug-loaded PLG-NP were analysed for their size and polydispersity index (PI) on a Zetasizer 1000 HS (Malvern Instruments, UK), based on photon correlation spectroscopy. The amount of drugs entrapped within PLG-NP was determined by measuring the amount of un-encapsulated drug in the aqueous solution recovered after centrifugation and washing of the particles. Drugs were analysed using standard protocols—rifampicin by microbiological assay[11] (sensitivity 0.25 μg/mL), isoniazid by spectrofluorimetry[12] (sensitivity 0.01 μg/mL) and pyrazinamide by spectrophotometry[13] (sensitivity 5 μg/mL). The drug encapsulation efficiency was expressed as the percentage of drug entrapped with respect to the theoretical value, and the drug loading was expressed as the amount of drug entrapped per gram of polymer. Residual PVA was analysed colorimetrically[14] based on the reaction between two adjacent hydroxyl groups of PVA and an iodine molecule.

Nebulization

A compressor–nebulizer system (Medel Aerofamily, Italy; 250 kPa pressure, nebulizer airflow 5.5 L/min) was used in the study. Each animal received drugs suspended in 4 mL of 0.9% sodium chloride via a suitable face mask connected to the nebulizer, with an exposure time of 3–4 min/animal. The dose size was a function of the volume of saline suspension used, rather than the duration of administration.

Aerodynamic characterization of nebulized PLG-NP

Nebulized PLG-NP were sized on a 7-stage Andersen Cascade Impactor (Andersen Samplers, Inc., Atlanta, GA, USA) and evaluated for mass median aerodynamic diameter (MMAD) as well as geometric standard deviation (GSD).

In vivo drug disposition studies

Guinea pigs were divided into the following groups (6–8 animals per group)—Group 1, Drug-loaded PLG-NP, aerosol; Group 2, Free drugs, oral; Group 3, Free drugs, intravenous (iv); Group 4, Free drugs, aerosol; and Group 5, Drug-free/empty PLG-NP, aerosol (a positive control group to exclude the influence of PLG-NP on drug assay). In each case, drugs were administered once in therapeutic dose combinations, i.e. rifampicin 12 mg/kg, isoniazid 10 mg/kg, pyrazinamide 25 mg/kg body weight. Guinea pigs were bled at different time points. In addition,

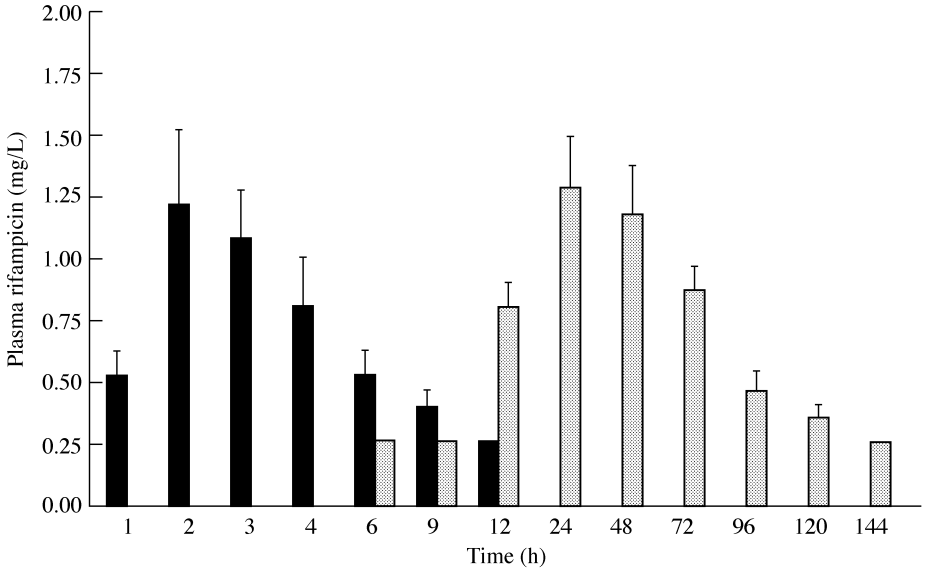

Figure 1. Plasma profile of rifampicin following the nebulization of drug-loaded PLG-NP, and oral administration of the parent drug. Values are mean ± S.D., $n = 6–8$. Black bars, oral rifampicin; grey bars, nebulized rifampicin-loaded PLG-NP.

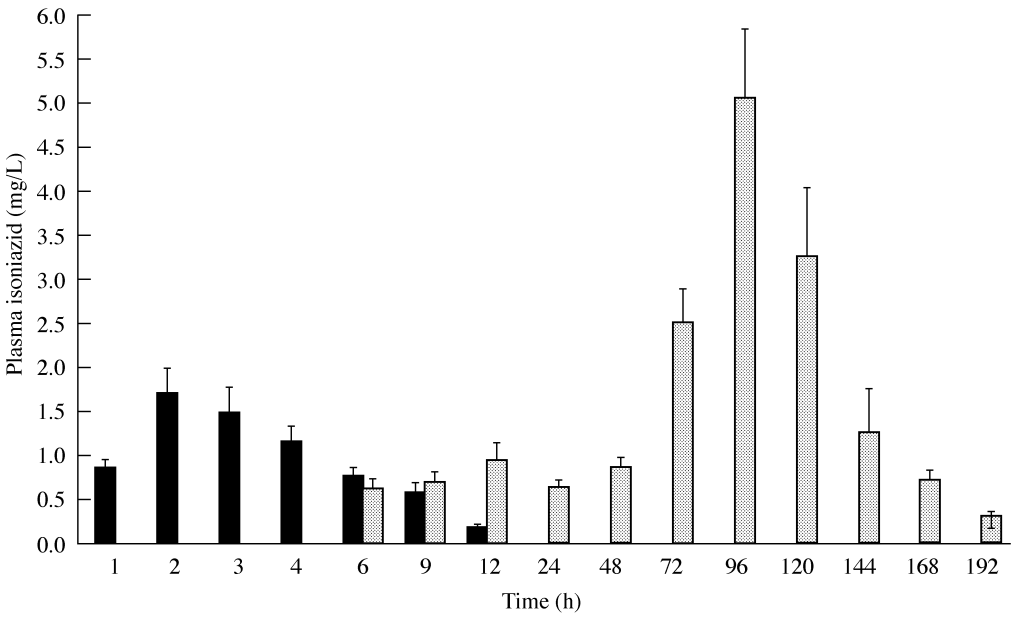

Figure 2. Plasma profile of isoniazid following the nebulization of drug-loaded PLG-NP, and oral administration of parent drug. Values are mean ± S.D., $n = 6–8$. Black bars, oral isoniazid; grey bars, nebulized isoniazid-loaded PLG-NP.

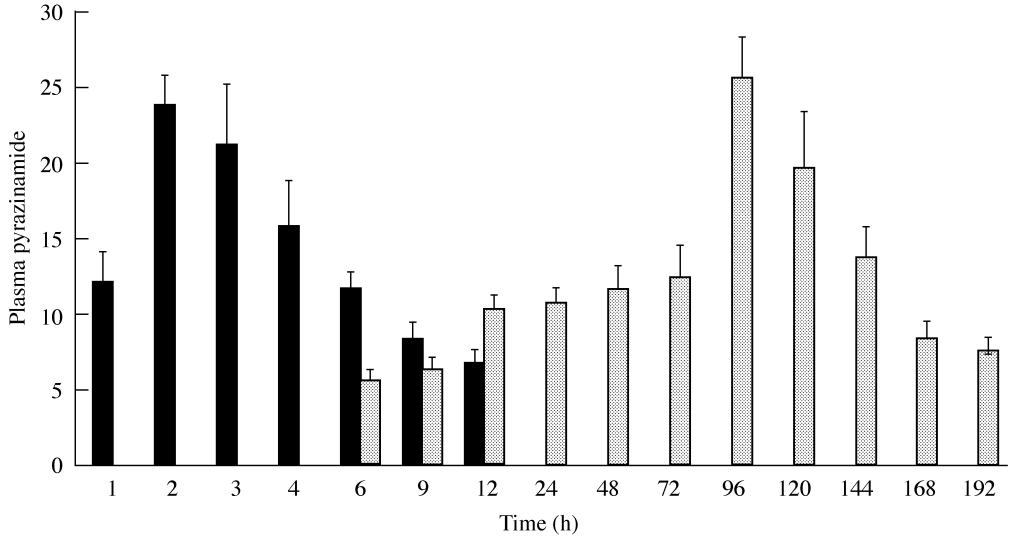

Figure 3. Plasma profile of pyrazinamide following the nebulization of drug-loaded PLG-NP, and oral administration of parent drug. Values are mean ± S.D., $n = 6–8$. Black bars, oral pyrazinamide; grey bars, nebulized pyrazinamide-loaded PLG-NP.

animals were killed at various intervals and lung homogenates were prepared. Drugs were determined in plasma and lung homogenates. The results were expressed as μg drug per mL plasma/g lung weight. The estimates of plasma drug concentration included only the drug available in the free form, at any given time-point.

Pharmacokinetic analysis

The plasma drug concentration versus time data was used to determine various pharmacokinetic parameters. Peak plasma concentration (C_{max}) and time taken to reach C_{max} (T_{max}) were obtained by visual data inspection. Elimination rate constant (K_{el}) was calculated by regression analysis whereas elimination half-life ($t_{1/2}$) was calculated from the equation $0.693/K_{el}$. The area under the concentration–time curve (AUC_{0-t}) was determined by the trapezoidal rule. The terminal $AUC_{t-\infty}$ was obtained by dividing the last measurable plasma drug concentration by K_{el}. AUMC/AUC, i.e. the area under the moment curve (AUMC)/area under curve (AUC), gave the mean residence time (MRT). Relative bioavailability (oral) of PLG-NP encapsulated drugs was computed by the formula $(AUC_{aerosol}/AUC_{oral}) \times (Dose_{oral}/Dose_{aerosol})$, whereas $(AUC_{aerosol}/AUC_{iv}) \times (Dose_{iv}/Dose_{aerosol})$ yielded the absolute bioavailability (iv).

Hepatotoxicity studies

Guinea pigs were divided into four groups with 4–5 animals in each group. Group 1 was administered drug-loaded PLG-NP via nebulizer

Table 1. Salient pharmacokinetic parameters following the nebulization of drug loaded PLG-NP compared to the parent drugs administered orally/iv to guinea pigs

	Rifampicin			Isoniazid			Pyrazinamide		
	oral	intravenous	nebulized PLG-NP	oral	intravenous	nebulized PLG-NP	oral	intravenous	nebulized PLG-NP
C_{max} (mg/L)	1.22 ± 0.30	25.40 ± 4.10	1.29 ± 0.20	1.71 ± 0.30	28.50 ± 3.10	5.06 ± 0.80	23.80 ± 2.10	71.10 ± 4.00	25.60 ± 2.80
T_{max} (h)	2.00 ± 0.00	0.02 ± 0.00	24.00 ± 0.00	2.00 ± 0.00	0.02 ± 0.00	96.00 ± 0.00	2.00 ± 0.00	0.17 ± 0.00	96.00 ± 0.00
K_{el}	–0.16 ± 0.03	–0.39 ± 0.05	–0.01 ± 0.00	–0.20 ± 0.00	–0.41 ± 0.06	–0.03 ± 0.00	–0.13 ± 0.02	–0.14 ± 0.02	–0.01 ± 0.00
$t_{1/2}$ (h)	4.30 ± 0.70	1.80 ± 0.30	69.30 ± 4.00	3.50 ± 0.70	1.69 ± 0.30	23.10 ± 2.00	5.30 ± 0.60	4.95 ± 0.70	69.00 ± 4.80
MRT (h)	6.20 ± 1.00	2.51 ± 0.60	60.30 ± 4.30	5.50 ± 1.10	2.41 ± 0.70	98.60 ± 10.20	6.60 ± 1.00	3.86 ± 0.73	101.20 ± 6.20
$AUC_{0-\infty}$ (mg h/L)	8.40 ± 1.10	16.50 ± 3.00	107.00 ± 8.20	10.90 ± 1.80	18.80 ± 2.90	359.00 ± 22.00	185.00 ± 6.50	202.00 ± 8.10	2715.00 ± 133.00
Relative bioavailability	1.00	–	12.70	1.00	–	32.80	1.00	–	14.70
Absolute bioavailability	0.51	1.00	6.50	0.58	1.00	19.10	0.92	1.00	13.40

Values are mean ± S.D., $n = 6–8$.

every 10 days (three doses); Group 2 was administered empty PLG-NP via nebulizer every 10 days (three doses); Group 3 was administered conventional therapy once daily by the oral route for 25 days; and Group 4 served as controls to calculate the normal range of biochemical parameters. On day 26, blood was collected from the animals in each group and the sera were analysed immediately for total bilirubin, alanine aminotransferase (ALT) and alkaline phosphatase (ALP) using standard kits.

Experimental infection and chemotherapy

Guinea pigs were infected intramuscularly with 1.5×10^5 viable bacilli of *M. tuberculosis* H37Rv in 0.1 mL of 0.9% sterile isotonic saline. Twenty days later, the animals were split into four groups with 5–6 animals per group—Group 1 served as untreated control, Group 2 was administered drug-free PLG-NP via nebulizer every 10 days (five doses), Group 3 was administered conventional therapy once daily by the oral route for 46 days and Group 4 was administered drug-loaded PLG-NP via nebulizer every 10 days (five doses). Drug doses were rifampicin 12 mg/kg + isoniazid 10 mg/kg + pyrazinamide 25 mg/kg body weight. In case of conventional administration, the latter were mixed and dissolved in isotonic saline just before administering to guinea pigs through the oral cavity. The preparations were made fresh daily. At day 46, the animals were killed. The caudal lobe of the right lung was taken out and homogenized in sterile PBS. The homogenates were inoculated on Middlebrook 7H10 Agar base in 1:100 and 1:1000 dilutions for enumeration of colony forming units (cfu). Colonies were counted on day 30 post-inoculation.

Statistical analysis

The cfu data were analysed by Student's unpaired *t*-test.

Results

Physicochemical characterization of drug-loaded PLG-NP

The majority (>80%) of the nanoparticles were in the size range of 186–290 nm with a polydispersity index of 0.38 ± 0.04. Drug encapsulation was 56.9 ± 2.7% for rifampicin, 66.3 ± 5.8% for isoniazid and 68 ± 5.6% for pyrazinamide. The drug loading (mg drug/g polymer) was 570 ± 27 for rifampicin, 663 ± 58 for isoniazid and 680 ± 56 for pyrazinamide. The results are mean ± S.D. of five nanoparticle preparations. The amount of residual PVA was 14.4–15.3% w/w of vacuum-dried nanoparticles.

Aerodynamic characterization of nebulized drug-loaded PLG-NP

Approximately 96% of the aerosolized particles were able to be respired (≤6 μm), the majority (33.5 ± 2.2% by mass) of the particles being in the range of 1.1–2.1 μm. The MMAD was 1.88 ± 0.11 μm with a GSD of 2 ± 0.2 μm ($n = 5$).

In vivo drug disposition studies

Plasma. Following the nebulization of drug-loaded PLG-NP, the drugs were detectable in the plasma from 6 h onwards. In the case of rifampicin, therapeutic drug levels were observed until day 6 (Figure 1) whereas isoniazid and pyrazinamide were present at therapeutic concentration up to day 8 (Figures 2 and 3), respectively. On the other hand, no drug was detectable in the plasma beyond 12–24 h or 6–10 h, following conventional oral or aerosol/iv administration of the drugs, respectively.

Pharmacokinetics. The C_{max} was comparable following oral administration of the parent drugs versus nebulized encapsulated drugs

Table 2. Biochemical hepatotoxicity studies following the nebulization of drug-loaded PLG-NP to guinea pigs

	Serum bilirubin (mg/100 mL)	Serum ALT (U/L)	Serum ALP (U/L)
Nebulized drug-loaded PLG-NP every 10 days (three doses)	0.40–0.71	21–36	31–50
Nebulized empty PLG-NP every 10 days (three doses)	0.40–0.62	25–36	28–42
Free drugs orally daily (25 doses)	0.38–0.66	29–44	30–47

Normal range in guinea pigs—total bilirubin: 0.1–1.0 mg/100 mL; ALT: 17–50 U/L and ALP: 19–70 U/L.

(Table 1) except for isoniazid. However, a longer time (T_{max}) was required to achieve peak plasma concentration in case of PLG-NP. The latter also exhibited significantly prolonged half-lives and MRT resulting in higher $AUC_{0-\infty}$ values compared to the parent drugs. The relative bioavailability was increased by 12.7-, 32.8- and 14.7-fold in case of rifampicin, isoniazid and pyrazinamide, respectively. Absolute bioavailability was also found to be increased by 6.5-, 19.1- and 13.4-fold for rifampicin, isoniazid and pyrazinamide, respectively.

Nebulization of the parent drugs resulted in a low MRT of 2.5–4 h for all three drugs. The $AUC_{0-\infty}$ were 2.1 ± 0.3, 18.0 ± 2.0 and 98.0 ± 4.0 mg h/L for rifampicin, isoniazid and pyrazinamide, respectively. Hence, given the same route of administration, nebulization of encapsulated drugs exhibited 51.0-, 20.0- and 28.0-fold higher bioavailability for rifampicin, isoniazid and pyrazinamide, respectively, compared to nebulization of the parent compounds.

Organs. All three drugs were present at therapeutic concentrations in the lungs till day 11 (rifampicin 1.25 ± 0.0 μg/g, isoniazid 0.55 ± 0.10 μg/g and pyrazinamide 35.5 ± 5.0 μg/g lung weight) in case of nebulized PLG-NP. However, the drugs were not detected in the lungs beyond 24 h after oral or aerosol administration of the parent compounds.

Hepatotoxicity studies

Serum analysis for total bilirubin, ALT and ALP showed that there was no evidence of biochemical hepatotoxicity in guinea pigs nebulized with drug-loaded PLG-NP every 10 days (three doses) (Table 2).

Chemotherapeutic efficacy

Nebulization of drug-loaded PLG-NP after every 10 days (five doses) and conventional daily oral administration (46 doses) exhibited no detectable cfu count in the lungs of infected guinea pigs (Table 3) based on <1 colony at the lowest dilution tested. Untreated animals and animals nebulized with drug-free PLG-NP showed comparable bacterial load in lungs (4.92 ± 0.39 $\log_{10}$ cfu/right caudal lung lobe and 4.7 ± 0.21 $\log_{10}$ cfu/right caudal lung lobe, respectively, $P > 0.05$).

Table 3. Chemotherapeutic efficacy of drug-loaded PLG-NP nebulized to guinea pigs

	$\log_{10}$ cfu/right caudal lung lobe
Untreated controls	4.92 ± 0.39
Nebulized empty PLG-NP (five doses)[a]	4.7 ± 0.21[ns]
Oral free drugs (46 doses)[b]	<1.0*
Nebulized drug-loaded PLG-NP (five doses)[c]	<1.0*

ns, non-significant ($P > 0.05$) compared to untreated controls.
[a]Nanoparticles administered every 10 days.
[b]Drugs administered daily.
[c]Nanoparticles administered every 10 days.
*No cfu detected at 1:100 and 1:1000 dilutions of lung homogenates on day 30 post-inoculation.

Discussion

Patient non-compliance has been a major obstacle in the successful management of TB, and has compelled researchers to develop sustained-release drug formulations so that the dosing frequency may be reduced. Further, direct pulmonary delivery of ATDs remains a therapeutic challenge and hence this study was designed to evaluate the potential of nebulized PLG-NP-based ATD delivery systems.

The PLG-NP prepared according to the multiple-emulsion and solvent-evaporation process yielded small size (186–290 nm) and low polydispersity (0.38 ± 0.04) nanoparticles, with higher drug encapsulation efficiency (57–68%) as well as drug loading (570–680 mg/g polymer), which is better than microparticles.[15] Aerodynamic particle characterization revealed that the majority of the aerosolized particles were respirable with an MMAD of 1.88 ± 0.11 μm suitable for delivering encapsulated drugs into the deeper pulmonary regions. This notion was supported by the observation that following nebulization, drugs could be detected in the plasma from 6 h onwards up to 144 h in case of rifampicin (Figure 1) and up to 192 h in case of isoniazid/pyrazinamide (Figures 2 and 3). The residual PVA (14.4–15.3% w/w) provides particle stability by forming a barrier to the diffusional release of macromolecules[16] resulting in slow and sustained release of drugs. A predilection of rifampicin for the lungs may be the reason for a relatively shorter stay of rifampicin in plasma compared to isoniazid/pyrazinamide. All three drugs were cleared from plasma within 24 h and 6 h, in case of oral or aerosol administration of the parent compounds, respectively. In either case, no drugs were detectable in the lungs beyond 24 h.

The C_{max} was comparable after oral administration of the parent compounds versus nebulized encapsulated drugs except for isoniazid (Table 1). The slow and sustained release of ATDs from PLG-NP accounts for the long time (T_{max}) required to attain C_{max}. The slow elimination rate (K_{el}) resulted in significantly prolonged $t_{1/2}$ of all three drugs compared to conventional oral administration of the drugs. Further, higher MRT and $AUC_{0-\infty}$ values gave an increase in the relative bioavailability (compared to oral) of rifampicin, isoniazid and pyrazinamide by 12.7-, 32.8- and 14.7-fold, respect-

ively. The absolute bioavailability (compared to iv) was also increased by 6.5-, 19.1- and 13.4-fold for rifampicin, isoniazid and pyrazinamide, respectively. These pharmacokinetic observations bear important therapeutic implications: the circulating levels of all three drugs were higher following nebulization of the PLG-NP than after conventional iv or oral administration, suggesting that there would be better delivery of drugs to the lung tissue via the circulation. It is known that nebulization yields better drug pharmacokinetics[17] which is further supported by our findings. It should also be emphasized that repeated aerosolization of PLG-NP did not result in biochemical hepatotoxicity (Table 2).

All the drugs were present above the therapeutic concentration in lungs till day 11 post-nebulization. This formed the basis of the chemotherapeutic schedule in which nebulized drug-loaded PLG-NP were administered every 10 days for 6 weeks (five doses) whereas the parent drugs were administered orally daily for 6 weeks (46 days). In either case, no cfu were detected in the lung homogenates at the indicated dilutions compared to untreated controls and animals administered with nebulized empty PLG-NP (Table 3). The results are better than our previous reports which showed that the subcutaneous[4]/oral[15] administration of drug-loaded PLG-microparticles significantly reduced the bacilli count in lungs of infected mice, but not to an undetectable level. This could be attributed to the use of three drugs (instead of two, i.e. rifampicin and isoniazid in subcutaneous studies)[4] as well as an enhanced bioavailability of nanoparticles compared to microparticles.[15] Although in this study, both conventionally administered and nebulized drugs were equally efficacious, it should be emphasized that 46 doses (oral drugs daily) have been reduced to five doses (nebulized PLG-NP every 10 days). The results bear important implications because reduction in dosing frequency would certainly enhance the patient compliance and hence, improve management of pulmonary TB.

In earlier studies, Dalencon *et al.*[18] reported the loading of rifabutin in nanocapsules and the preparation was evaluated in experimental toxoplasmosis. The encapsulation of ethionamide, a second-line ATD, was reported by Lopes *et al.*[19] but the authors did not carry out any *in vivo* studies. The successful use of PLG microparticles for pulmonary administration entails the encapsulation of at least a single drug.[20] Other authors[21] have demonstrated the encapsulation of two drugs (rifampicin and isoniazid) but chemotherapeutic efficacy studies were not carried out. Therefore, this study communicates for the first time, the detailed evaluation of three frontline ATDs (rifampicin, isoniazid and pyrazinamide) co-encapsulated in PLG nanoparticles suitable for pulmonary administration every 10 days and bearing significant therapeutic potential for the management of pulmonary TB.

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References

1. Fauci, A. S. (2001). Infectious diseases: considerations for the 21st century. *Clinical Infectious Diseases* **32**, 675–85.

2. Deol, P. & Khuller, G. K. (1997). Lung specific stealth liposomes: stability, biodistribution and toxicity of liposomal antitubercular drugs in mice. *Biochimica et Biophysica Acta* **1334**, 161–72.

3. Quenelle, D. C., Winchester, G. A., Staas, J. K. *et al.* (2001). Treatment of tuberculosis using a combination of sustained-release rifampin-loaded microspheres and oral dosing with isoniazid. *Antimicrobial Agents and Chemotherapy* **45**, 1637–44.

4. Dutt, M. & Khuller, G. K. (2001). Therapeutic efficacy of Poly (DL-Lactide-Co-Glycotide)-encapsulated antitubercular drugs against *Mycobacterium tuberculosis* infection induced in mice. *Antimicrobial Agents and Chemotherapy* **45**, 363–6.

5. Ahlin, P., Kristl, J., Kristl, A. *et al.* (2002). Investigation of polymeric nanoparticles as carriers of enalaprilat for oral administration. *International Journal of Pharmaceutics* **239**, 113–20.

6. Horisawa, E., Hirota, T., Kawazoe, S. *et al.* (2002). Prolonged anti-inflammatory action of DL-Lactide/Glycolide copolymer nanospheres containing betamethazone sodium phosphate for an intra-articular delivery system in antigen-induced arthritic rabbit. *Pharmaceutical Research* **19**, 403–10.

7. Jacobs, C. & Muller, R. H. (2002). Production and characterisation of a budesonide nanosuspension for pulmonary administration. *Pharmaceutical Research* **19**, 189–94.

8. Dailey, L. A., Schmehl, T., Gessler, T. *et al.* (2003). Nebulization of biodegradable nanoparticles, impact of nebulizer technology and nanoparticle characteristics on aerosol features. *Journal of Controlled Release* **86**, 131–44.

9. Anderson, J. M. & Shive, M. S. (1997). Biodegradation and biocompatibility of PLA and PLGA microspheres. *Advanced Drug Delivery Reviews* **28**, 5–24.

10. Lamprecht, A., Ubrich, N., Hombreiro, P. M. *et al.* (1999). Biodegradable monodispersed nanoparticles prepared by pressure homogenisation-emulsification. *International Journal of Pharmaceutics* **184**, 97–105.

11. Saito, H. & Tomioka, H. (1989).Therapeutic efficacy of liposome entrapped rifampicin against *Mycobacterium avium* complex infections induced in mice. *Antimicrobial Agents and Chemotherapy* **33**, 429–31.

12. Scott, E. H. & Wright, R. C. (1967). Fluorimetric determination of isonicotinic acid hydrazide in plasma. *Journal of Laboratory and Clinical Medicine* **70**, 355–60.

13. Gurumurthy, P., Nair, N. G. K. & Sarma, G. R. (1980). Methods for the estimation of pyrazinamide and pyrazinoic acid in body fluids. *Indian Journal of Medical Research* **71**, 129–34.

14. Joshi, D. P., Lan-Chun-Fung, Y. L. & Pritchard, J. W. (1979). Determination of poly (vinyl alcohol) via its complex with boric acid and iodine. *Analytica Chimica Acta* **104**, 153–60.

15. Ain, Q., Sharma, S. & Khuller, G. K. (2003). Chemotherapeutic potential of orally administered poly (lactide-co-glycolide) microparticles containing isoniazid, rifampicin and pyrazinamide against experimental tuberculosis. *Antimicrobial Agents and Chemotherapy* **47**, 3005–7.

16. Sahoo, S. K., Panyam, J., Prabha, S. *et al.* (2002). Residual poly vinyl alcohol associated with poly (D, L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. *Journal of Controlled Release* **82**, 105–14.

17. Dhuley, J. N. (1999). Aerosolized liposomal hamycin for treatment of systemic *Candida* infections in mice. *FEMS Immunology and Medical Microbiology* **25**, 231–6.

18. Dalencon, F., Amjaud, Y., Lafforgue, C. *et al.* (1997). Atovaquone and rifabutin-loaded nanocapsules, formulation studies. *International Journal of Pharmaceutics* **153**, 127–30.

19. Lopes, E., Pohlmann, A. R., Bassani, V. *et al.* (2000). Polymeric colloidal systems containing ethionamide, preparation and physicochemical characterization. *Pharmazie* **55**, 527–30.

20. Suarez, S., O'Hara, P., Kazantseva, M. *et al.* (2001). Airways delivery of rifampicin microparticles for the treatment of tuberculosis. *Journal of Antimicrobial Chemotherapy* **48**, 431–4.

21. Sharma, R., Saxena, D., Dwivedi, A. K. *et al.* (2001). Inhalable microparticles containing drug combinations to target alveolar macrophages for treatment of pulmonary tuberculosis. *Pharmaceutical Research* **18**, 1405–10.