ORIGINAL ARTICLE

Inhaled microparticles of antitubercular antibiotic for *in vitro* and *in vivo* alveolar macrophage targeting and activation of phagocytosis

Rajesh Parikh¹, Sonali Dalwadi², Pooja Aboti¹ and Leena Patel³

Tuberculosis (TB) is a chronic infectious disease with increasing incidence of drug resistance. Oral treatment for TB and multidrug resistance-TB can have serious side effects. The causative agent of TB, *Mycobacterium tuberculosis*, resides in alveolar macrophages (AMs). Pulmonary administration of antitubercular (anti-TB) antibiotic can help in delivery of high concentration to AM. The ability of AM to phagocytose can also be utilized to generate mycobactericidal nitric oxide (NO) to improve efficacy of anti-TB antibiotics. The objective in this investigation was made to prepare isoniazid microparticles (IM) and polymeric microparticles of isoniazid (INH-PM) using poly-ε-caprolactone as polymer and to evaluate *in vitro* through cell culture techniques and *in vivo* through pulmonary administration of IM and INH-PM for uptake of isoniazid by AM. The hepatotoxicity was determined through serum glutamate oxaloacetate transferase (SGOT) and serum glutamate pyruvate transferase (SGPT) levels and histological examination. The results depicted that the significantly higher ($P < 0.05$) concentration of isoniazid was found in AM with INH-PM *in vitro* and *in vivo*. NO production was also significantly higher but less than toxic level. SGOT and SGPT levels, uptake of INH by liver and histological examination were indicative of no hepatotoxicity with INH-PM and IM. Phagocytosis of IM and INH-PM leads to significantly higher drug level in AM as well as production of significantly higher levels of NO without compromising the viability of cells. The administration of IM and INH-PM as dry powder inhalation formulation may reduce the treatment time of TB and chances of drug-resistant TB.

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INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease. Lung is the primary site of infection. The causative agent *Mycobacterium tuberculosis* resides in alveolar macrophage (AM). The conventional treatment of TB involves systemic delivery of antitubercular drugs through oral route. The major disadvantage associated with oral treatment is undesirable side effects and toxic effects due to high doses. Delivery of antitubercular drugs directly to the primary infection site through pulmonary route may help in reduction of side effects as well as toxic effects and provide an advantage of dose reduction.^{1–6} Antitubercular drugs are always to be used in combination to avoid the chances of drug resistance.⁷

In any combination of antitubercular drugs, isoniazid (INH) is always recommended as first line agent as per WHO guidelines. The major side effect associated with INH is hepatotoxicity. So, it may be anticipated that AM targeting through pulmonary administration of INH may improve patient compliance through reduction in dose, hepatotoxicity and shortening of treatment period. INH can be

delivered through pulmonary route either as polymeric or non-polymeric micro/nano particles^{8,9} or pro-liposome formulation.¹⁰

Spray drying is one of the most preferred techniques for development of particles for nasal and pulmonary delivery.^{11,12} Few reports are available for spray-dried polymeric and non-polymeric microparticles of INH which show the drug targeting, activation of macrophage for phagocytosis and toxicity study. So, in the present investigation spray-dried isoniazid microparticles (IM) and isoniazid polymeric microparticles (INH-PM) using poly-ε-caprolactone as polymer were prepared and evaluated for AM targeting with activation of phagocytosis process which help to reduce toxicity and to reduce the treatment period with improved patient compliance.

The cell culture technique is an important tool to access efficacy as well as cell viability *in vitro*.¹³ In this investigation, IM and INH-PM were evaluated and compared with pure INH by *in vitro* cell culture method with respect to the ability of alveolar macrophages to internalize the particles and to generate microbicidal reactive nitrogen species.^{14,15} The *in vivo* drug uptake and phagocytosis

¹Department of Pharmaceutics and Pharmaceutical Technology, Ramanbhai Patel College of Pharmacy, Charotar University of Science and Technology, Gujarat, India;

²Department of Pharmaceutical Technology, Ramanbhai Patel College of Pharmacy, Charotar University of Science and Technology, Gujarat, India and ³Department of Pharmacology, Ramanbhai Patel College of Pharmacy, Charotar University of Science and Technology, Gujarat, India

Correspondence: S Dalwadi, Department of Pharmaceutical Technology, Ramanbhai Patel College of Pharmacy, Charotar University of Science and Technology, CHARUSAT Campus, Changa, Taluka Petlad, District Anand, Gujarat 388 421, India.

E-mail: sonali.dalwadi@yahoo.com

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activation has been studied for IM and INH-PM and compared with pure INH by bronchoalveolar lavage fluid (BALF) method. The liver toxicity study and histological examination were obtained showing the safety of formulation *in vivo*.

EXPERIMENTAL PROCEDURE

Materials

Isoniazid is gifted by IPCA, India. Poly-caprolactone was purchased from Sigma-Aldrich, St Louis, MO, USA. Fetal Bovine Serum (FBS) and F12K medium were purchased from Invitrogen (Bangalore, India). Percoll, N-(1-naphthyl)-ethylenediamine dihydrochloride (NED), MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye and Geimsa's stain was purchased from Sigma-Aldrich. Polyvinyl alcohol, sulfanilamide, phosphoric acid, methanol, dichloromethane, and phosphate buffer saline pH 7.4 (PBS) were purchased from Merck Chemicals, Bangalore, India. Serum glutamate oxaloacetate transferase (SGOT) and serum glutamate pyruvate transferase (SGPT) kits were purchased from Crest Biosystems, Goa, India. All the chemicals and ingredients utilized for the study were of AR grade. Double-distilled water was utilized throughout the study.

Preparation of isoniazid microparticles (IM) through spray drying process

Isoniazid solution (5% w/v) in double-distilled water was prepared and subjected to drying using spray dryer with high efficiency cyclone separator (LU-222 advanced, Labultima, Mumbai, India) under following conditions: inlet temperature 45 °C, aspiration 45 N m³ h⁻¹, feed rate of 1 ml min⁻¹. The powder in high efficiency cyclone separator was collected, weighed (AUX220, Shimadzu, Kyoto, Japan) and stored in light resistant container till further analysis.

Preparation of polymeric microparticles of isoniazid (INH-PM) using poly-ε-caprolactone

The polymeric microparticles of isoniazid were prepared by double emulsification method and the microparticles were obtained using spray drying process. The weighed amount of polymer (500 mg) was dissolved completely in 5 ml of dichloromethane. Isoniazid (500 mg) was dissolved in double-distilled water (3 ml). The solution of INH was dispersed in polymer solution using syringe with 23 G needle to obtain w/o emulsion. This emulsion was again dispersed in 1.5% w/v polyvinyl alcohol solution (75 ml) and homogenized for 1 min using high-speed homogenizer at 10 500 r.p.m. The prepared w/o/w emulsion was spray dried to prepare INH-PM at following conditions: Inlet temperature 120 °C, feed rate 2 ml min⁻¹ and aspiration rate 60 N m³ h⁻¹. The INH-PM were collected from high efficiency cyclone separator, weighed and stored at room temperature and in air tight light resistant container till further analysis.

Particle size and inhalation characteristics

The particles size measurement IM and INH-PM were observed using high-resolution microscope (Axio Lab.A1, Carl Zeiss MicroImaging, GmbH, Gottingen, Germany) and images were taken. Particle size was measured through image analysis process performed using software (Biovis Image Plus (P+) V4.56 (Expert Vision Labs Pvt Ltd, Mumbai, India)). The inhalation characteristics of particles in terms of mass median aerodynamic diameter, respirable fine particle fraction (FPF_{<4μm}) and emitted dose (%ED) were determined by cascade impaction study using 8-Stage Anderson Cascade Impactor (ACI, Copley Scientific Ltd, Nottingham, UK).

In Vitro studies using primary alveolar macrophages cell culture

Alveolar macrophage isolation. The Institutional Animal Ethics Committee (IAEC), registered with the Government of India approved the protocol (RPCP/IAEC/2012-2013/R-22) for animal experiments. Albino rats (Adult Wistar) were taken for the study. The rat was anesthetized by intraperitoneal injection of 100–200 μl of ketamine/xylazine cocktail. Euthanized by exsanguinations. The use of cervical dislocation is not indicated as it can damage the trachea, contaminating the sample with blood. The area of incision was cleaned using 70% isopropyl alcohol or ethanol. The trachea on the ventral

side of the neck was surgically exposed and an 18-G needle attached to a 5-ml syringe containing 4 ml of pre-warmed (37 °C) phosphate buffer saline (PBS) containing 0.5 mM EDTA was inserted through the tracheal wall into the lumen just below the larynx. The surgical suture material was tied around the trachea to secure the needle in place. Gently the pre-warmed (37 °C) PBS with 0.5 mM EDTA was infused into the lung. A total volume of not more than 2 ml of saline can generally be instilled into the lung at any one time. Gently the fluid was withdrawn into the syringe and re-infused back into the lung three times in succession. The final lung fluid was expressed from the syringe into a 50-ml conical polypropylene tube on ice. The above steps were repeated on several rats and fluid was pooled into a 50-ml conical centrifuge tube.¹⁶ Centrifugation was done for 10 min at 400 × g, 4 °C. The cells were counted in a hemocytometer using trypan blue exclusion. The cells were plated after re-suspension in complete media containing F12K medium with 4% FBS. The re-suspension volume should be determined and that gives 2 × 10⁵ cells per well in 24 well plates or 10⁶ cells per well in 96 well plates. Incubation was done for 60 min at 37 °C in CO₂ incubator (Thermo-Fisher Scientific, Mumbai, India). The nonadherents were removed by washing twice with 500 μl warm PBS, using gentle swirling action. Following an adhesion step, the cells were typically greater than 90% macrophages.

Drug Uptake Studies. The isolated primary alveolar macrophage cells suspended in F12K medium containing 4% FBS were plated in 24 well plates (1 × 10⁶ cells ml⁻¹ wells⁻¹) and incubated for 60 min at 37 °C. The nonadherents were removed by washing twice with 500 μl warm PBS, using gentle swirling action. The samples were prepared by dispersing INH, IM and INH-PM in F12K medium containing 4% FBS to prepare concentrations – 25, 50 and 100 μg per well and added to the plates. The plates were incubated for treatment for 3 h. Cells from the plates were collected by treating with warm PBS. The collected cells were stained with Geimsa's staining and were fixed over the slides using 10% formalin. The slides were observed under high-resolution microscope at 45 × magnification. The photomicrograph was obtained for the drug taken up by primary macrophages using high-resolution microscope using 3 MP camera attached to high-resolution microscope.¹⁷ Drug taken up by primary macrophages were also to be determined quantitatively.¹⁸ The cells adhered to the well were collected in 1.5 ml tubes for each samples – INH, IM and INH-PM after treatment. A total of 500 μl of cell suspension was layered over 70% Percoll and centrifuged for 10 min at 20 000 r.p.m. and 4 °C. The ring formed at the interface was collected, washed with PBS and centrifuged at 20 000 r.p.m. and 4 °C for 10 min. The supernatant was discarded and replaced with 500 μl of water and twice subjected to freeze thaw at –80 °C. The 500 μl of methanol was added and sonication was done for 1 min to precipitate the cell matrix and dissolve the drug. The suspension formed was centrifuged at 12 000 r.p.m. and 4 °C for 10 min.^{17,19} The supernatant was analyzed at 263 nm using UV spectrophotometer for determination of drug taken up by alveolar macrophages.

Cell viability study. MTT assay is colorimetric determination of cell viability. In MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, cells are exposed to drug. MTT enters the cells and in mitochondria by succinic dehydrogenase reduced to insoluble dark purple formazan product.²⁰ Cells are then solubilised and released reagent is measured. MTT reduction is possible only in metabolically active cells so the level of activity is the measure of viability of cell. The isolated primary alveolar macrophage cells (1 × 10⁶ cells ml⁻¹ per well) suspended in F12K medium containing 4% FBS were plated in 96 well plates and incubated for 60 min at 37 °C. The nonadherents were removed by washing twice with 500 μl warm PBS, using gentle swirling action. After incubation of 96 well plates, the cells were washed with warm PBS. The cells were treated with 500 μl each of solutions of INH, IM and INH-PM in drug concentration range of 0, 5, 10, 20, 40, 60, 80, 100, 500, 1000 μg ml⁻¹. The plates were incubated for 24 h in CO₂ incubator. After incubation the supernatant of each column were removed and used for nitrite measurement. The columns were washed with PBS twice. A total of 150 μl of media and 50 μl of MTT solution (5 mg ml⁻¹ in PBS) were added to each column 2–11. The plates were kept for incubation for 2–3 h. After incubation the supernatant was removed and 200 μl of DMSO was added to dissolve the

formazan crystals.^{21,22} The absorbance at 570 nm was taken with microplate reader (ELx800-MS, Biotek, VT, USA).²⁰ The cell viability was calculated.

Nitrite measurement for activation of phagocytosis. This method was used to investigate nitric oxide (NO) in the form of nitrite (NO_2^-), which is one of two primary, stable and non-volatile breakdown products of NO. This measurement relies on diazotization reaction with Griess reagent. Griess reagent was prepared by mixing 1% sulfanilamide, 0.1%NED and 2.5% phosphoric acid in water at volume ratio of 1:1:1. The plates treated with INH, IM and INH-PM were incubated for 24 h in CO_2 incubator as described in cell viability study. After incubation the supernatant was collected and mixed with an equal volume of Griess reagent for 10 min. The absorbance of solution was measured at 540 nm, 10 min after mixing. The amount of NO generated during the activation of macrophages was determined against the calibration spectra of sodium nitrite at 0.01–5 $\mu\text{g ml}^{-1}$ concentration range.²²

In Vivo animal experiments

Treatment to animals and drug uptake by AM. The Institutional Animal Ethics Committee (IAEC), registered with the Government of India approved the protocol (RPCP/IAEC/2012-2013/R-12) for animal experiments. Albino rats (Wistar) were taken for the study. They were divided in three groups, each group was consisting of six rats weighing between 300 and 340 g. Each rat in group I, group II and group III was subjected to the following treatment:

Group I: From the tail vein of each rat, 0.5 ml of blood was collected, centrifuged at 3000 r.p.m. and 4 °C for 10 min and the serum was separated and stored at –80 °C till further analysis of SGOT and SGPT. Each rat in the group was administered INH at a dose of 25 mg kg^{-1} body weight via oral route, as a dispersion prepared in 10% w/v carboxymethyl cellulose in water. The rats were anesthetized by intraperitoneal administration of phenobarbital at a dose of 50 mg kg^{-1} body weight and the trachea was cannulated. Bronchoalveolar lavage (BAL) fluid was collected from each animal at an interval of 15 min, 30 min, 60 min, 90 min, 120 min, 150 min, 180 min and 240 min. For the collection of BAL fluid at each time point, 2 ml of PBS was first introduced through cannula and then BAL fluid was sampled. The BAL fluid was centrifuged at 20 000 r.p.m. and 4 °C for 10 min to obtain a cell pellet. The supernatant was utilized for NO determination. The pellet was collected and mixed with water (1 ml) subjected twice through freeze thaw at –80 °C. The 500 μl of methanol was added and sonication was done for 1 min. Then the suspension was centrifuged at 20 000 r.p.m. and 4 °C for 10 min. The aqueous phase was filtered through a 0.22- μm cellulose acetate membrane filter. The absorbance of filtrate was measured at 263 nm using UV spectrophotometer (UV-1800 240 V, Shimadzu). Each rat was sacrificed after the last sample of BAL fluid was collected. Immediately, 1 ml of blood was collected, centrifuged at 3000 r.p.m. 4 °C for 10 min and serum was separated and stored at –80 °C till further analysis. The lung and liver were isolated. The total weight of the lung and liver was measured.

Group II and group III: From the tail vein of each rat in both the groups, 0.5 ml of blood was collected, centrifuged at 3000 r.p.m. and 4 °C for 10 min and the serum was separated and stored at –80 °C till further analysis. Each rat in the groups was anesthetized by intraperitoneal administration of pentobarbital at 50 mg kg^{-1} body weight. The trachea was cannulated and each 1 ml dispersion of IM and INH-PM in PBS was administered to deliver a dose of equivalent to 5 mg of INH per kg of body weight.²³ Bronchoalveolar lavage fluid was collected from each animal at an interval of 15 min, 30 min, 60 min, 90 min, 120 min, 150 min, 180 min and 240 min. For the collection of BAL fluid at each time point, 2 ml of PBS was first introduced through cannula and then BAL fluid was sampled. The AM containing IM and INH-PM were separated from the BAL fluid as per the described method.¹⁹ The BAL fluid was centrifuged at 20 000 r.p.m. and 4 °C for 10 min to obtain a cell pellet. The supernatant was utilized for nitric oxide (NO) determination in the form of nitrite. The pellet was dispersed in 1 ml of PBS. The cell suspension (500 μl) was layered over 500 μl of 80% Percoll present in 1.5 ml capacity Eppendorf tube. The centrifugation was done at 20 000 r.p.m. and 4 °C for 10 min. The IM that have not been internalized in AM migrated to the lower Percoll layer whereas AM in which drug is not internalized remained as the upper layer and AM containing IM formed the middle layer.^{5,8,24,25} The middle layer was

collected and mixed with water (500 μl) and subjected twice through freeze thaw at –80 °C. The 500 μl of methanol was added and sonication was done for 1 min. Then it was centrifuged at 20 000 r.p.m. and 4 °C for 10 min. The aqueous phase was filtered through a 0.22- μm cellulose acetate membrane filter. The absorbance of filtrate was measured at 263 nm using UV spectrophotometer (UV-1800 240 V, Shimadzu). For collection of blood and isolation of lung and liver, the procedure as outlined for group I was followed.

Lung and liver accumulation of INH. The bigger lobe of liver and left lobe of lung of treatment groups were used for estimation of drug accumulated in the liver and lung. The homogenates (20%w/v) in double-distilled water were prepared using tissue homogenizer (RQ-127-A, Remi Elektrotechnik Ltd, Mumbai, India).² The homogenates were centrifuged at 20 000 r.p.m. and 4 °C for 10 min. The supernatant was treated with equal volume of methanol to precipitate the cell metrics. Centrifugation was again carried out at 20 000 r.p.m. and 4 °C for 10 min. The absorbance of INH in the supernatant was measured at 263 nm using UV spectrophotometer (UV-1800 240 V, Shimadzu).

Measurement of nitric oxide (NO) in BALF supernatant. NO produced in AM is instantly oxidized to nitrite.¹⁹ Hence, the NO was determined in terms of nitrite using Griess reagent.¹⁰ The supernatants collected as previously described in INH estimation in AM were utilized for nitrite measurement. Equal volumes of supernatant and Griess reagent were mixed and kept aside for 10 min. The absorbance was measured at 540 nm using UV-visible spectrophotometer (UV-1800 240 V, Shimadzu). The amount of NO generated were calculated using standard calibration curve of sodium nitrite.

Study of liver function test. The SGOT and SGPT levels were determined for liver function test. The serum samples collected for treatment groups were evaluated for SGOT and SGPT levels using SGOT kit and SGPT kit.

Histological examination. This histological examination was performed to check for any irritation and lesions produced with IM and INH-PM administration to the target tissues. The isolated lung and liver tissues from the treatment groups were subjected to histological examination using H&E staining and the images were obtained using high-resolution microscope (Axio Lab.A1, Carl Zeiss MicroImaging, GmbH, Gottingen, Germany).

Data analysis. Comparison for the two groups was made by *t*-test. The significant level was defined by $P < 0.05$.

RESULTS AND DISCUSSION

Particle size and aerosol characteristics

IM and INH-PM with the median particle size of $4.3 \pm 0.77 \mu\text{m}$ (560 particles) and $4.1 \pm 0.57 \mu\text{m}$ (570 particles) were obtained through spray drying process. Through the cascade impaction study, the following parameters were found: The mass median aerodynamic diameter: $1.9 \pm 0.061 \mu\text{m}$ for IM and $3.0 \pm 0.021 \mu\text{m}$ for INH-PM, respirable fine particle fraction ($\text{FPF}_{<4 \mu\text{m}}$): $54.85 \pm 1.05\%$ for IM and $51.83 \pm 1.00\%$ for INH-PM and emitted dose (%ED): $57.17 \pm 0.74\%$ for IM and $26.9 \pm 0.54\%$ for INH-PM. These characteristics indicate that the IM and INH-PM were suitable for administration as a dry powder inhalation as well as for deep lung deposition where the target cells AM resides.^{23,26}

In vitro cell culture study for INH, IM and INH-PM

Drug uptake study using primary rat alveolar macrophage cells. The photomicrographs taken for INH, IM and INH-PM using high-resolution microscope are shown in Figure 1. The arrows in photomicrographs indicate the AM with drug after treatment with 100 $\mu\text{g ml}^{-1}$ of INH, IM and INH-PM for 3 h. The uptake of IM may be due to the favorable particle size and sphericity favorable for AM uptake.²⁷ The Figure 2a shows the uptake of drug by primary alveolar macrophage cells treated with INH, IM and INH-PM at three

Figure 1 Photomicrograph (at $40\times$ magnification) of AM (stained with Giemsa's stain) treated with equivalent to $100\mu\text{g ml}^{-1}$ of (a) INH, (b) IM and (c) INH-PM for 3 h. Arrows indicate AM with drug particle. AM, alveolar macrophages; IM, isoniazid microparticles; INH, isoniazid; INH-PM, polymeric microparticles of isoniazid. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

different concentrations for 3 h. The results show that the uptake amount (%) of INH from INH-PM (8.28 ± 0.33) and IM (6.15 ± 0.52) is significantly higher ($P < 0.05$) as compared with that of pure INH (1.74 ± 0.69) by AM treated with each INH equivalent to $25\mu\text{g ml}^{-1}$ concentrations. The maximum fivefold and sevenfold increased uptake was found with each INH equivalent to $25\mu\text{g ml}^{-1}$ of IM and INH-PM respectively as compared with that found with $25\mu\text{g ml}^{-1}$ INH. The uptake amount (%) of INH from INH-PM and IM (6.15 ± 0.52) is also significantly higher ($P < 0.05$) as compared with that of pure INH by AM treated with each INH equivalent to $50\mu\text{g ml}^{-1}$ and $100\mu\text{g ml}^{-1}$ concentrations. The intracellular uptake may be affected by the size and shape of particles. The reports showed that the intracellular uptake decreases with increasing size and non-sphericity of particles.^{28–30} The uptake of INH-PM was higher as compared with IM, which may be due to porous and hydrophobic nature of poly- ϵ -caprolactone biodegradable aliphatic polyester type polymer.¹⁷ In addition to that, distinction should be made for the mode of intracellular uptake which may occur either by phagocytosis or by fluid phase endocytosis.³⁰

Cell viability Study. The results of MTT assay are shown in Table 1. The cytotoxicity induced by free INH was substantially higher compared with that induced by IM and INH-PM.²¹ With increase in drug concentration, the % cell viability reduces with free INH as compared with that of IM and INH. Viability of cell reduced almost 50% when treated with $1000\mu\text{g ml}^{-1}$ concentrations of INH. Despite low drug uptake of INH by AM (Figure 2a), INH showed low cell viability as compared with that of IM and INH-PM. Due to lower uptake of INH by AM, the concentration increases outside the cell leading to cell membrane disruption, cell necrosis and cell death. This indicated that INH is toxic to AM cells as compared with that of IM which may be due to slow internalization of INH to AM leading to

cell toxicity. For AM, cell viability was not compromised for 24 h by treatment with up to $1000\mu\text{g ml}^{-1}$ concentration of IM and INH-PM. It can be inferred that the absence of toxic effect of phagocytosed IM and INH-PM is advantageous for obtaining an efficient microbicidal effect in AM.²⁷

Nitric oxide measurement for activation of phagocytosis. Figure 2b shows the NO level obtained for 0 – $1000\mu\text{g ml}^{-1}$ concentration of INH, IM and INH-PM. The results show that as the concentration of INH, IM and INH-PM increased, the NO levels were also increased. This depicted the activation of AM by treatment with INH, IM and INH-PM.¹⁵ The NO obtained for INH-PM ($20.1 \pm 0.56\mu\text{g ml}^{-1}$) and IM ($1.73 \pm 0.18\mu\text{g ml}^{-1}$) at $1000\mu\text{g ml}^{-1}$ concentration was found significantly higher ($P < 0.05$) as compared with that of INH ($1.003 \pm 0.30\mu\text{g ml}^{-1}$). The MTT results showed that the NO generated after treatment with INH-PM and IM was not affecting the cell viability as compared with that of INH. This confirmed that the NO generated by treatment with INH-PM was the highest and was found to be non-toxic to AM. It can be inferred that phagocytosis may occur with INH-PM as compared with that with IM and that with INH without compromising cell viability.

In vivo study for INH, IM and INH-PM

Drug uptake study. The uptake of INH by AM is shown in Figure 3a. The maximum two times and three times higher uptake was found with IM and INH-PM respectively as compared with that of INH at 15 min of administration. The uptake amount (%) of INH by AM was found significantly higher ($P < 0.05$ at every sampling point) after administration of INH-PM (3.26 ± 0.46 at 15 min) and IM (0.83 ± 0.5 at 15 min) as compared with that of INH oral (0.25 ± 1.7 at 15 min). For INH-PM, the hydrophobic nature of spray-dried polymeric particles favors the phagocytic uptake as compared with IM.

Figure 2 (a) Uptake of INH after treatment with INH, IM and INH-PM at concentrations of 25, 50 and 100 $\mu\text{g mL}^{-1}$ for 3 h. (b) NO ($\mu\text{g mL}^{-1}$) generated after treatment of INH, IM and INH-PM to AM in concentration range of 0–1000 $\mu\text{g mL}^{-1}$. AM, alveolar macrophages; IM, isoniazid microparticles; INH, isoniazid; INH-PM, polymeric microparticles of isoniazid. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Table 1 Percent viable cells after treatment of INH, IM and INH-PM to AM for concentration range of 0–1000 $\mu\text{g mL}^{-1}$

Concentration of INH for treatment ($\mu\text{g mL}^{-1}$)	Percent viable cells		
	INH	IM	INH-PM
5	101	112	111
10	98.4	108	99.3
20	98.04	99.5	99
40	89	98	98
80	81	95	98
100	72	92	92.88
500	60	85	92
1000	51	73	90

The deep lung deposition and sphericity of INH-PM and IM bring them in close proximity with AM and this in turn may lead to ready internalization through phagocytosis.³¹

Phagocytosis activity and generation of NO. The levels of NO in BAL fluid are shown in Figure 3b. The presence of NO in BAL fluid is an indication of uptake of INH-PM and IM by AM through phagocytosis and activation of phagolysosome fusion process.³¹ For the

Figure 3 (a) INH uptake by AM for each time interval after administration of INH (oral), IM (I.T.) and INH-PM (I.T.) to the rat. (b) NO [$(1 \times 10^{-3}) \mu\text{M}$] generation by AM for each time interval after administration of INH (oral), IM (I.T.) and INH-PM (I.T.) to the rat. AM, alveolar macrophages; IM, isoniazid microparticles; INH, isoniazid; INH-PM, polymeric microparticles of isoniazid; I.T., intratracheal. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

treatment of TB, it is important to stimulate the phagocytes for favorable therapeutic outcome.³² Phagocytosis is accompanied with activation of key macrophage enzymes such as the inducible nitric oxide synthase and phagocyte oxidase (phox) or NADPH oxidase complex. Such activation leads to accumulation of reactive nitrogen intermediates in the forming phagosome.^{14,33,34} These intracellular reactive nitrogen intermediate are directly mycobactericidal.^{14,35,36}

NO in concentration of 4–5 μM is toxic to lung epithelial cells.³⁷ In the present investigation the maximum concentration of NO was found to be 0.0055 μM after 15 min of administration of INH-PM. The NO generation was found to be 0.003 μM after 30 min of IM administration. Thus it can be inferred that levels of NO generated are not toxic to epithelium. The NO generation was higher with INH-PM and IM as compared with that of INH. Significant uptake of INH by AM as well as generation of mycobactericidal NO is an indication to infer that efficacy of INH may significantly improve by IM and INH-PM through pulmonary route.

Lung and liver accumulation. Figure 4 shows the accumulation of INH in lung and liver after IT administration of INH-PM and IM as

Figure 4 INH accumulation in the lung and liver after 4 h of administration of INH (oral), IM (I.T.) and INH-PM (I.T.) to the rat ($*P < 0.05$ INH accumulation in liver is significantly lower for IM (I.T.) and INH-PM (I.T.) as compared with that after INH (oral), $\#P < 0.05$ for INH accumulation in lung is significantly higher for IM (I.T.) and INH-PM (I.T.) as compared with that after INH (oral)). IM, isoniazid microparticles; INH, isoniazid; INH-PM, polymeric microparticles of isoniazid; I.T., intratracheal. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

well as after oral administration of INH. Significantly lower uptake of INH by AM may be attributed to the fact that uptake of INH by liver is very high. Hence, only a small fraction of the administered dose is available for distribution to AM through diffusion process after oral administration. The result also suggested that the higher lung accumulation with INH-PM and IM leads to less systemic availability of drug. It can be depicted from the result that by pulmonary administration of INH-PM and IM, the systemic side effects and toxicity may be reduced which is the major drawback of anti-TB treatment.

Liver toxicity. Increased levels of SGPT and SGOT are observed in conditions such as hepatitis, cirrhosis, obstructive jaundice and other hepatic diseases.^{38,39} Significant increase in SGPT levels and SGOT levels were found post oral administration of INH (Figures 5a and b). In addition, the uptake of INH by liver is 60.17% of the dose after 4 h of oral administration (Figure 4). Thus, it can be inferred that hepatotoxicity may occur post oral administration of INH. INH uptake by liver after IT administration of INH-PM and IM is significantly low compared with oral administration. No significant difference in levels of SGOT and SGPT were found when levels were measured before and after IT administration of INH-PM and IM. Thus it can also be inferred that with INH-PM and IM, the hepatotoxicity may not occur.

Histological examination. Figures 6a and b show the histological images of lung and liver tissue isolated after 4 h of administration of INH, IM and INH-PM. The results show no change in the lungs and liver tissue integrity or irritation found with INH, IM and INH-PM administration as compared with that of blank tissue. Further, the IM and INH-PM administered through pulmonary route had neither produced any irritation nor lesions to lung

Figure 5 (a) SGPT level before and after (4 h) administration of INH, IM and INH-PM (b) SGOT level before and after (4 h) administration of INH, IM and INH-PM ($*P < 0.05$ shows SGPT and SGOT levels are significantly higher after INH oral administration as compared with SGPT and SGOT levels before INH oral administration, $\#P > 0.05$ shows that there is no significant difference obtained for SGPT and SGOT levels after IM (I.T.) and INH-PM (I.T.) administration as compared to SGPT and SGOT levels before IM (I.T.) and INH-PM (I.T.) administration). IM, isoniazid microparticles; INH, isoniazid; INH-PM, polymeric microparticles of isoniazid; I.T., intratracheal; SGOT, serum glutamate oxaloacetate transferase; SGPT, serum glutamate pyruvate transferase. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

epithelium which depicted that there was no toxicity found to lung epithelium. Though the histological examination showed no irritation to the liver tissue, the SGOT and SGPT levels (Figures 5a and b) revealed the change in liver physiology on INH oral administration as compared with that with IM and INH-PM intratracheal administration.

Figure 6 Histological images for (a) lung tissues ($40\times$) and (b) liver tissues ($100\times$) of treatment groups and of blank. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

CONCLUSION

IM and INH-PM suitable for deep lung deposition post pulmonary administration may provide improved efficacy over INH oral administration through significantly higher uptake as well as activation of

AM to produce mycobactericidal NO without compromising the cell viability *in vitro* and *in vivo*. The liver toxicity of IM and INH-PM may be almost negligible as indicated by lower liver uptake, non-significant levels of SGOT and SGPT as well as histological

examination. Further, INH-PM provided the rapid uptake and activation of AM due to hydrophobic nature of poly- ϵ -caprolactone polymer favorable for AM uptake as compared with that of IM. Though the uptake was found with non-polymeric and polymeric microparticles of isoniazid formulations, the distinction should be made for the mode of intracellular uptake which may occur either by phagocytosis or by fluid phase endocytosis by AM. It can be concluded that IM and INH-PM formulated as dry powder inhalation for alveolar macrophage targeting and activation of phagocytosis may improve patient compliance through improved efficacy and reduced hepatotoxicity which in turn may reduce the treatment period as well as the chances of drug-resistant tuberculosis.

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