

## Oral nanoparticle-based antituberculosis drug delivery to the brain in an experimental model

Rajesh Pandey and G. K. Khuller\*

Department of Biochemistry, Postgraduate Institute of Medical Education & Research,  
Chandigarh-160 012, India

Received 2 December 2005; returned 10 February 2006; revised 19 February 2006; accepted 13 March 2006

**Objectives:** To evaluate the potential of orally administered poly-lactide-co-glycolide (PLG, a synthetic polymer) nanoparticle encapsulated antituberculosis drugs (ATDs) (rifampicin + isoniazid + pyrazinamide + ethambutol) for cerebral drug delivery in a murine model.

**Methods:** The formulation was prepared using the multiple emulsion technique and administered orally to mice for biodistribution, pharmacokinetic and chemotherapeutic studies.

**Results:** A single oral dose of the formulation to mice could maintain sustained drug levels for 5–8 days in the plasma and for 9 days in the brain. There was a significant improvement in the pharmacokinetic parameters such as mean residence time and relative bioavailability as compared with free drugs. The pharmacodynamic parameters such as the ratio of area under the curve to minimum inhibitory concentration (AUC/MIC) and the time up to which MIC levels were maintained in plasma ( $T_{MIC}$ ) were also improved. In *Mycobacterium tuberculosis* H<sub>37</sub>Rv infected mice, five oral doses of the nanoparticle formulation administered every 10th day resulted in undetectable bacilli in the meninges, as assessed on the basis of cfu and histopathology.

**Conclusions:** Polymeric nanoparticles bear significant potential for ATD delivery to the brain.

Keywords: poly-lactide-co-glycolide, bioavailability, pharmacodynamics, chemotherapy

### Introduction

Cerebral tuberculosis (TB) can present in several different ways including tubercular meningitis, tubercular encephalopathy, encephalitis, arteritis, abscess etc.<sup>1</sup> It is the most serious extrapulmonary complication associated with TB.<sup>2</sup> The condition is often fatal, especially in children despite the availability of potent antituberculosis drugs (ATDs).<sup>3</sup> Furthermore, neurological sequelae are fairly common and add to the complexity of the disease.<sup>3</sup> The ATDs employed for the treatment of cerebral TB are the same as those required against pulmonary TB, i.e. rifampicin, isoniazid and pyrazinamide. Ethambutol and streptomycin are also useful adjuncts in the initial phase of treatment.<sup>4,5</sup> However, the ATDs need to be administered on a regular basis for a prolonged time period. Hence, the development of a drug carrier that can efficiently deliver its encapsulated contents (ATDs) to the brain would be welcomed as a therapeutic strategy. Nanoparticle-based systems for brain drug delivery are being developed.<sup>6,7</sup> However, to date, no ATD delivery system has been evaluated against cerebral TB. We recently demonstrated the

chemotherapeutic potential of poly-lactide-co-glycolide (PLG, a synthetic, biodegradable/biocompatible polymer approved by US FDA for human use)<sup>8</sup> nanoparticles encapsulating three<sup>9,10</sup> or four<sup>11</sup> frontline ATDs (rifampicin + isoniazid + pyrazinamide + ethambutol). The formulation clearly showed the ability to release the encapsulated drugs over a prolonged time period in plasma, lungs and spleen of mice/guinea pigs and thereby to replace daily conventional chemotherapy with fewer doses of the formulation in experimental TB (assessed in the lungs and spleen). Our findings encouraged us to evaluate the potential of oral PLG-nanoparticle encapsulated ATDs for drug delivery to the brain in a mouse model, and the results are presented in this article.

### Materials and methods

#### Chemicals and drugs

Rifampicin, isoniazid, pyrazinamide, ethambutol and polyvinyl alcohol (PVA;  $M_r$  13 000–19 000, 85% hydrolysed) were obtained from

\*Corresponding author. Tel: +91-0172-2744401; Fax: +91-0172-2745078; E-mail: gkkhuller@yahoo.co.in

Sigma Chemical Co. (St Louis, MO, USA). PLG (50:50 resomer, RG 506,  $M_n$  97400) was purchased from Boehringer Ingelheim, Germany. HPLC grade solvents and water were obtained from Merck Ltd, Mumbai, India. All other chemicals used in the study were of analytical grade.

### Culture

The culture of *Mycobacterium tuberculosis* H<sub>37</sub>Rv originally obtained from the National Collection of Type Cultures (NCTC, London) was maintained on Youman's modified medium.

### Animals

Laca mice of either sex weighing 20–25 g obtained from the Central Animal House, Postgraduate Institute of Medical Education and Research, Chandigarh, India, were used in the study. Animals were housed in biosafety cabinets (Nuair Instruments, NU 605-600E, Series 6) and provided with pellet diet/water *ad libitum*. The study was approved by the Institute's Animal Ethics Committee.

### Preparation of ATD-loaded PLG-nanoparticles

ATD-loaded PLG-nanoparticles were prepared by the multiple emulsion and solvent evaporation technique as described previously.<sup>9–11</sup> Briefly, 10 mg of isoniazid and 10 mg of pyrazinamide were dissolved in distilled water, which was then added to dichloromethane (DCM) containing 10 mg of rifampicin and 30 mg of PLG [total drug/polymer 1:1 (w/w); water/DCM 1:10 (v/v)]. The mixture was sonicated for 1 min to form the primary emulsion, which was poured into 1% (w/v) aqueous PVA and re-sonicated for 3 min. The secondary emulsion formed was stirred overnight for the removal of DCM and centrifuged (8000–10 000 rpm for 15 min) to harvest the nanoparticles, which were washed with distilled water and vacuum dried. Nanoparticles of ethambutol were prepared as a distinct formulation, by dissolving the drug in the initial aqueous phase.<sup>11</sup>

### Characterization of ATD-loaded PLG-nanoparticles

The particle size ranged from 186 to 290 nm (Zetasizer, Malvern Instruments, Malvern, UK), as reported previously.<sup>9–11</sup> The dried formulations were put in 5 mL of 5% (w/v) SDS in 0.1 M NaOH (the formulation lysis reagent) for 30 min at 50°C to release the encapsulated drugs. The percentage drug encapsulation efficiency was determined by the formula<sup>11</sup> [amount of drug (mg) released from the nanoparticles/amount of drug (mg) initially taken to prepare the nanoparticles] × 100. The drugs were analysed using an HPLC system comprising a dual-piston reciprocating pump, an online degasser, a UV-visual dual wavelength detector (each of Series 200) and a 600 Series Link Interface for data acquisition/processing, all from Perkin Elmer Instruments LLC (Shelton, CT, USA). In the case of rifampicin/isoniazid/pyrazinamide, the three drugs were separated and quantified by employing a USP gradient program<sup>11</sup> with sodium phosphate buffer (pH 6.8)/acetonitrile as the mobile phase (at 1.5 mL/min), 238 nm as the detection wavelength and a reversed phase C18 column (Cosmosil 5C18-MS-II from Waters; 250 × 4.6 mm; 5 µm particle size). Ethambutol was analysed by employing a USP isocratic program<sup>11</sup> with triethylamine buffer (pH 7.0)/acetonitrile as the mobile phase (at 1 mL/min), 200 nm as the detection wavelength and a cyan column (Zorbax CN from Agilent; 150 × 4.6 mm; 5 µm particle size). The sensitivity of the methods was as follows: rifampicin, 0.4 mg/L; isoniazid, 0.2 mg/L; pyrazinamide, 1.0 mg/L; and ethambutol, 0.5 mg/L.

### Preparation of drug doses for in vivo studies

The drug doses used throughout the study were rifampicin 10 mg/kg, isoniazid 25 mg/kg, pyrazinamide 150 mg/kg and ethambutol 100 mg/kg body weight.<sup>12,13</sup> These doses are reported to result in pharmacodynamic correlates that are similar to those observed in humans.<sup>12</sup> The dose being different for each drug, the initial amount of drug taken to prepare the formulations was calculated by the formula (amount of drug required per animal/mean drug encapsulation efficiency) × 100, as described previously.<sup>11</sup> Once the total amount of drugs required was known, an equivalent amount of PLG was used in the preparation process. For a 20 g mouse, 6.80 mg of the PLG-nanoparticle formulation encapsulating three drugs (containing 0.20 mg rifampicin + 0.50 mg isoniazid + 3.00 mg pyrazinamide) and 2.70 mg of the PLG-ethambutol formulation (containing 2.00 mg ethambutol) comprised a therapeutic dose combination, which was suspended in 100 µL of distilled water just before oral dosing in each experiment. Free drugs were freshly dissolved in distilled water/methanol (5:1, v/v) immediately before dosing.

### In vivo drug disposition studies

Mice were administered either a single oral dose of free ATDs or PLG-nanoparticle encapsulated ATDs (four-drug combination in each case with  $n = 15$  per group). The animals were bled (four mice per time point) at 1 h, 2 h, 3 h, 4 h, 6 h, 12 h and day 1–11. The plasma obtained from each mouse was divided into two parts. The first part (100 µL) was deproteinized with 100 µL of acetonitrile, vortexed for 5 min and centrifuged at 7338 rpm for 20 min at 4–8°C. The supernatant was used for the analysis of rifampicin and ethambutol. The second portion of plasma (50 µL) was deproteinized with 50 µL of 10% (v/v) trichloroacetic acid, processed as above, and analysed simultaneously for isoniazid and pyrazinamide. The drugs were analysed using validated HPLC techniques<sup>11</sup> and compared with calibration graphs (obtained by analysing pooled blank mouse plasma spiked with known drug amounts) to obtain the plasma drug concentration versus time profile. The area under the concentration over time curve ( $AUC_{0-\infty}$ ) was determined by the data analysis tool on SigmaPlot software (version 8.0). The ratio  $AUMC/AUC$  [area under moment curve (AUMC)/area under curve (AUC)] yielded the mean residence time (MRT). The AUC was further used to compute the relative bioavailability of ATDs: ( $AUC_{0-\infty}$  of oral encapsulated drugs/ $AUC_{0-\infty}$  of oral free drugs) × (dose of oral free drugs/dose of oral encapsulated drugs). In addition, the pharmacodynamic parameters such as  $C_{max}/MIC$  (peak plasma concentration/minimum inhibitory concentration) and  $AUC/MIC$  ratios were also determined. The pharmacokinetic/pharmacodynamic data were analysed using Student's unpaired *t*-test.

The animals were sacrificed (three mice per time point) at day 1 and 2 in the case of free drugs and on day 3, 6, 9 and 10 in the case of PLG-encapsulated drugs. Each mouse was laid on its ventral aspect on the dissection tray. The skin over the head was removed in order to completely expose the skull surface. A midline incision was made over the skull with a sharp knife and the bony flaps were retracted on either side by blunt forceps. The entire brain was dissected out, weighed, homogenized in 3 mL of isotonic saline and divided into two equal parts. In the first part, the drug levels were determined by following the same analytical procedure as described for plasma. To the second part, formulation lysis reagent was added (1:1, v/v) and the mixture was incubated for 2 h at 37°C followed by centrifugation at 7338 rpm for 10 min. The supernatant was processed for protein precipitation and drug analysis as described above. The addition of formulation lysis reagent was expected to release the

## Nanoparticle-based drug delivery for brain tuberculosis

encapsulated drugs from the intact nanoparticles (if present in the sample). For each drug, the values obtained by analysing the two parts were compared using Student's unpaired '*t*-test'. The entire drug disposition study was repeated to confirm the reproducibility of results.

### Experimental infection and chemotherapy

Mice were infected via the lateral tail vein with  $1.5 \times 10^7$  bacilli of *M. tuberculosis* H<sub>37</sub>Rv. A similar inoculum was used in other reports and is known to provide bacillary loads similar to those found in human TB cases.<sup>14</sup> Fifteen days later, 15 mice were sacrificed, and the brain from each was removed aseptically, homogenized in 3 mL of isotonic saline and 100  $\mu$ L of 1 in 1000 diluted homogenates was plated on Middlebrook 7H11 agar plates supplemented with oleic acid albumin dextrose catalase for the enumeration of basal cfu. Subsequently, mice were grouped as follows (*n* = 14 per group): group 1, untreated control; group 2, oral free four-drug combination daily; and group 3, oral four-drug PLG formulation every 10 days.

Five animals each in groups 2 and 3 were treated until 4 weeks (28 doses of free drugs or 3 doses of PLG-nanoparticles) and sacrificed (along with half of the animals in group 1) on day 32. The entire brain from each mouse was removed aseptically and homogenized in 3 mL of sterile isotonic saline. In the case of group 1, 100  $\mu$ L of 1 in 1000 diluted homogenates was plated on Middlebrook agar plates whereas in the case of groups 2 and 3, 100  $\mu$ L of undiluted homogenates was plated. The remaining animals were treated until 6 weeks (46 doses of free drugs or 5 doses of PLG-nanoparticles) and sacrificed (along with the rest of the animals in group 1) on day 52. The entire brains from five mice per group were removed and homogenized; each homogenate of 3mL volume was equally divided and 750 $\mu$ L was plated in four milk bottles containing slopes prepared from the same Middlebrook medium. The cfu were counted after 28 days of plating. The cfu data were analysed by one-way ANOVA followed by Student's unpaired *t*-test to compare the untreated and treated groups.

### Histopathology

The remaining mice (four from each group) at the completion of 6 weeks of chemotherapy were sacrificed (on day 52 as mentioned above), and immediately the brains including the meninges were lightly smeared on glass slides. Each specimen of brain was then fixed in 10% formalin. The smears were stained with Ziehl–Neelsen stain whereas the brain specimens were subjected to routine histological processing. The sections were stained with haematoxylin and eosin followed by microscopic examination.

## Results and discussion

The era of nanotechnology has led to the emergence of entirely new research strategies in the field of drug delivery.<sup>15</sup> The argument that polymers could be employed as drug carriers with the advantage of providing a controlled release of drug(s) is well documented. Our previous reports<sup>9–11</sup> demonstrated the encapsulation of four frontline ATDs (rifampicin, isoniazid, pyrazinamide and ethambutol) in PLG-nanoparticles. The drug encapsulation efficiency ranged  $55.91 \pm 2.70\%$  for rifampicin,  $67.34 \pm 3.8\%$  for isoniazid,  $68.32 \pm 5.50\%$  for pyrazinamide and  $43.11 \pm 4.21\%$  for ethambutol. Following a single oral dose of ATD-loaded PLG-nanoparticles to mice, therapeutic drug concentrations (MIC<sub>90</sub> of rifampicin = 0.2 mg/L, isoniazid = 0.1 mg/L, pyrazinamide = 8.0 mg/L, ethambutol = 1.5 mg/L)<sup>11</sup>

**Table 1.** Salient pharmacokinetic/pharmacodynamic parameters of antituberculous drugs following the oral administration of PLG-nanoparticle encapsulated drugs as compared with oral free drugs in mice

	Ethambutol		Rifampicin		Isoniazid		Pyrazinamide	
	oral free four-drug combination	oral ATD-loaded PLG-nanoparticles	oral free four-drug combination	oral ATD-loaded PLG-nanoparticles	oral free four-drug combination	oral ATD-loaded PLG-nanoparticles	oral free four-drug combination	oral ATD-loaded PLG-nanoparticles
MRT (h)	4.50 ± 0.70	38.40 ± 5.00*	4.20 ± 1.00	51.00 ± 5.40*	4.50 ± 0.70	42.40 ± 5.00*	6.90 ± 1.00	53.10 ± 4.50*
AUC <sub>0-∞</sub> (mg/L/h)	7.40 ± 0.31	226.60 ± 27.70*	6.37 ± 1.10	120.48 ± 10.10*	12.62 ± 2.10	318.28 ± 15.90*	211.00 ± 15.00	3372.00 ± 158.00*
Relative bioavailability	1.00	30.54*	1.00	18.84*	1.00	25.20*	1.00	15.98*
AUC/MIC	4.93 ± 0.20	150.67 ± 18.50*	25.48 ± 4.00	480.00 ± 40.00*	126.20 ± 21.00	3180.00 ± 150.00*	26.38 ± 1.90	421.50 ± 20.00*
T <sub>MIC</sub> (h)	3 ± 0	72 ± 0*	6 ± 0	120 ± 0*	8 ± 0	192 ± 0*	10 ± 0	192 ± 0*

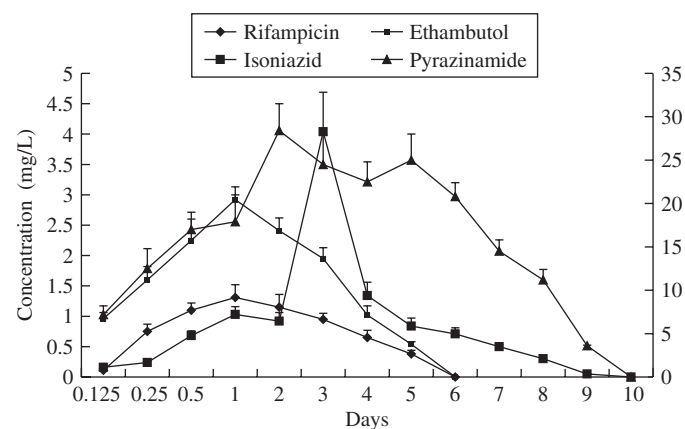
MRT, mean residence time; AUC<sub>0-∞</sub>, area under plasma concentration curve; MIC, minimum inhibitory concentration; T<sub>MIC</sub>, time for which drug levels were maintained above MIC in plasma.

Values are means ± SD, *n* = 8.

\**P* < 0.001 compared with free drugs.

were maintained in the plasma for 3 days, 5 days and 8 days in the case of ethambutol, rifampicin and isoniazid/pyrazinamide, respectively (Figure 1), confirming our previous observations.<sup>9–11</sup> Free drugs, on the other hand, were not detectable in the plasma beyond 12 h of oral administration. Owing to the slow but prolonged release of drugs from the PLG formulation, the MRT and AUC/relative bioavailability were enhanced by 9- to 14-fold and 15- to 30-fold, respectively, as compared with free drugs (Table 1). The pharmacodynamic parameters were also in favour of the PLG formulation, for ATDs that exhibit concentration-dependent killing (rifampicin) as well as time-dependent killing (isoniazid and pyrazinamide) of mycobacteria.<sup>16</sup> The results suggested that the PLG formulation was likely to provide therapeutic drug levels in the brain. Nevertheless, we substantiated the hypothesis by analysing the drugs in brain homogenates at various time points. It was observed that therapeutic drug concentrations were maintained in the brain until day 9 in the case of PLG-nanoparticles; however, ethambutol was observed until day 6 only (Table 2). Drugs (except ethambutol) were detected until day 1 in the case of animals dosed with oral free drugs, which was in agreement with the clinical reports documenting the cerebral distribution of free ATDs, especially isoniazid and pyrazinamide.<sup>17</sup>

Two intriguing questions remained to be answered before proceeding for the chemotherapeutic studies. First, in the case



**Figure 1.** Plasma drug profile following a single oral administration of PLG-nanoparticle encapsulated ATDs to mice. Values are means  $\pm$  SD,  $n = 8$  at each time point. The curve for pyrazinamide is with reference to the secondary axis. Free drugs were not detectable beyond 12 h of oral administration.

of mice dosed with PLG-nanoparticles, it was important to know whether the drug analysis in brain was restricted to free drugs (i.e. drugs released from nanoparticles) or encapsulated drugs (drugs still incorporated in the nanoparticles) or both. To address this issue, brain homogenate from a normal mouse was divided into two parts and separately spiked with free drugs or PLG-nanoparticle encapsulated drugs. Protein-free filtrates were prepared as usual and injected for HPLC analysis. In the case of free drugs, peaks were detected at the expected retention times whereas samples containing PLG-loaded drugs failed to elicit any detector response (data not shown). This proved that our analytical technique was restricted to free drugs only, which is the therapeutically active form. The second issue was the possibility of ATD-loaded PLG-nanoparticles localizing to the brain after crossing the blood–brain barrier. It was observed that when the brain homogenates were incubated with formulation lysis reagent, there was a significant increase in drug levels on day 3 compared with the values obtained prior to the addition of the reagent (Table 2). The ingredients present in the reagent (an alkaline solution of SDS) are known to accelerate the degradation of the polymer, thereby releasing the encapsulated contents.<sup>18</sup> Our results clearly indicate the presence of nanoparticles in the brain up to day 3 following oral dosing with the PLG formulation; subsequently, drug levels continued to be maintained, possibly owing to the release from partly degraded nanoparticles as well as being drawn from the circulation. Studies from other laboratories have also demonstrated the localization of polymeric nanoparticles to the brain following intravenous<sup>19</sup> or oral<sup>20</sup> administration. The process is likely to be influenced by the targeting process<sup>21</sup> as well as by the surface charge on the nanoparticles; neutral particles (PVA-stabilized PLG in our case) stand not only better chances of cerebral uptake but also a lower risk of toxicity to the blood–brain barrier.<sup>22</sup>

The data from tissue biodistribution studies suggested a chemotherapeutic schedule comprising daily administration of oral free drugs or intermittent (every 10th day) administration of ATD-loaded PLG-nanoparticles in *M. tuberculosis* infected mice. Several experimental approaches have been described to induce mycobacterial infection of the brain. Intracisternal inoculation of bacilli in rabbits<sup>23</sup> is well suited for this purpose; however, there were technical difficulties in applying this to small animals such as mice. The presence of mycobacteria in the meninges was reported following intravenous challenge with *Mycobacterium bovis* and *Mycobacterium avium* complex to swine<sup>24</sup> and mice,<sup>25</sup> respectively. Further, our recent findings

**Table 2.** Drug levels in brain following the single oral administration of PLG-nanoparticle encapsulated drugs to mice

Day	Rifampicin ( $\mu\text{g/g}$ )		Isoniazid ( $\mu\text{g/g}$ )		Pyrazinamide ( $\mu\text{g/g}$ )		Ethambutol ( $\mu\text{g/g}$ )	
	pre-lysis	post-lysis	pre-lysis	post-lysis	pre-lysis	post-lysis	pre-lysis	post-lysis
3	1.55 $\pm$ 0.29	2.31 $\pm$ 0.33*	1.90 $\pm$ 0.35	2.52 $\pm$ 0.20*	30.58 $\pm$ 3.60	46.77 $\pm$ 3.51*	1.77 $\pm$ 0.24	2.16 $\pm$ 0.11*
6	0.95 $\pm$ 0.13	0.92 $\pm$ 0.11**	1.32 $\pm$ 0.22	1.36 $\pm$ 0.25**	23.92 $\pm$ 3.00	24.05 $\pm$ 3.13**	1.51 $\pm$ 0.20	1.50 $\pm$ 0.12**
9	0.33 $\pm$ 0.05	0.34 $\pm$ 0.04**	0.13 $\pm$ 0.02	0.13 $\pm$ 0.01**	10 $\pm$ 1.10	9.77 $\pm$ 1.00**	not detectable	not detectable
10	not detectable	not detectable	0.10 $\pm$ 0.00	0.10 $\pm$ 0.00**	not detectable	not detectable	not detectable	not detectable

Values are means  $\pm$  SD,  $n = 6$  at each time point. Formulation lysis was carried out with the addition of 5% SDS in 0.1 M NaOH to the brain homogenates. \* $P < 0.05$  and \*\* $P > 0.05$  compared with pre-lysis values. Free drugs were not detectable in the brain beyond 24 h of oral administration: rifampicin 0.27  $\pm$  0.03  $\mu\text{g/g}$ , isoniazid 0.82  $\pm$  0.10  $\mu\text{g/g}$  and pyrazinamide 4.53  $\pm$  0.62  $\mu\text{g/g}$ . Free ethambutol was undetectable at 24 h.

## Nanoparticle-based drug delivery for brain tuberculosis

suggested that nanoparticle-based ATD therapy is highly efficacious irrespective of the route of infection.<sup>26</sup> Hence, we chose to infect via the intravenous route.

Isolation of *M. tuberculosis* from cerebral tissue is diagnostic of brain TB.<sup>27</sup> A high bacillary load of  $\sim 6.8$  log cfu in brain at 2 weeks post-challenge confirmed the establishment of infection (Table 3). In the control animals, the cfu at 4 weeks or 6 weeks were comparable and not significantly different ( $P > 0.05$ ) from the basal load at the start of treatment (Figure 2). This was attributed to the fact that once the infection was established,

**Table 3.** Basal mycobacterial counts in individual mouse brains on day 15 following intravenous challenge with *M. tuberculosis* H<sub>37</sub>Rv

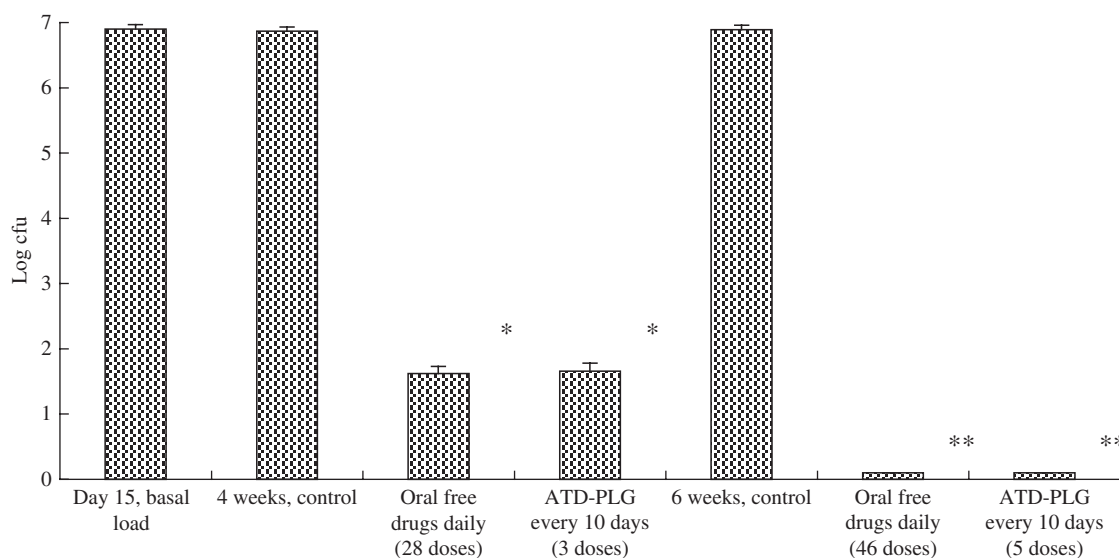
Animal number	Total mycobacterial counts in brain	Log <sub>10</sub> cfu/brain
1	$810 \times 10^4$	6.91
2	$645 \times 10^4$	6.81
3	$690 \times 10^4$	6.84
4	$774 \times 10^4$	6.89
5	$588 \times 10^4$	6.77
6	$810 \times 10^4$	6.91
7	$600 \times 10^4$	6.78
8	$522 \times 10^4$	6.72
9	$615 \times 10^4$	6.79
10	$675 \times 10^4$	6.83
11	$561 \times 10^4$	6.75
12	$615 \times 10^4$	6.79
13	$561 \times 10^4$	6.75
14	$615 \times 10^4$	6.79
15	$537 \times 10^4$	6.73

Mean basal load =  $6.80 \pm 0.06$  log cfu,  $n = 15$ . The brain from each mouse was homogenized in 3 mL of isotonic saline and 100  $\mu$ L of 1 in 1000 diluted homogenates was plated on Middlebrook medium. Therefore, the total mycobacterial counts (C) were obtained by the formula  $C = (\text{total countable colonies} \times 10 \times 1000 \times 3)$ , i.e.  $C = (\text{total countable colonies} \times 30000)$ .

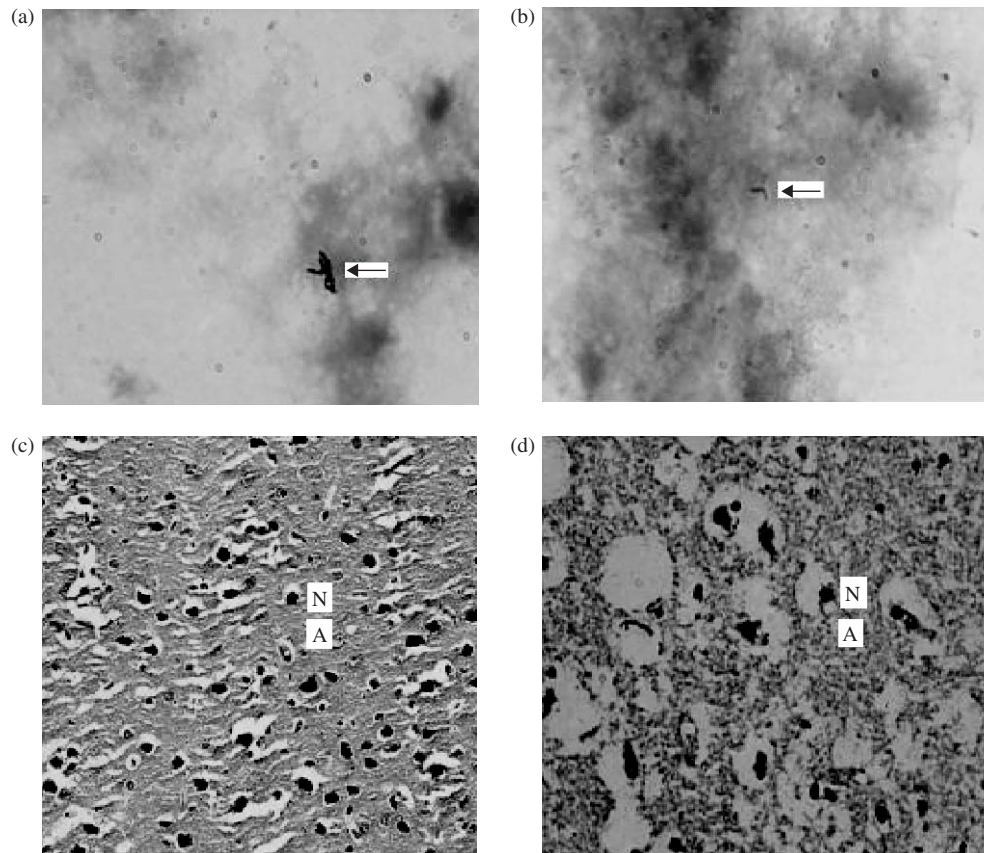
the immune response served to ensure a balance between mycobacterial replication and death.<sup>28</sup> Four weeks of chemotherapy resulted in a significant ( $P < 0.001$ ) reduction in cfu in the case of free drugs as well as ATD-loaded PLG-nanoparticles. A further 2 weeks of treatment resulted in undetectable bacilli in the brain, thereby proving that 46 doses of conventional free drugs could be replaced with 5 doses of PLG-encapsulated drugs. It is emphasized that the animals were sacrificed at day 32 and day 52 to exclude the possibility of any residual drugs remaining in the tissues at the time of sacrifice. However, to confirm whether the cfu detected on agar plates were derived from the meninges or the cerebral parenchyma, we resorted to histopathological studies.

The meningeal smears from untreated control mice were positive for acid-fast bacilli although no inflammatory cells were detected (Figure 3a and b). Animals treated with free drugs or PLG-encapsulated drugs for 6 weeks were smear-negative and the cortical sections showed normal neurons as well as the supporting glial tissue similar to that observed in a healthy mouse (Figure 3c). On the other hand, the sections from untreated mice showed features of shock and early autolysis; the cells lost their function, some of them were fragmented, the cytoplasm was hazy and the background myelin was granular all over (Figure 3d). No inflammatory reaction of TB was detected. From these observations it appears that (i) the cfu detected on agar plates were a result of the growth of bacilli within the meninges since the bacteria do not seem to have crossed the blood-brain barrier (a longer time might be required to achieve the same) and (ii) the bacilli did not seem to evoke any inflammatory response and the degenerative changes in the control animals were presumably because of the moribund condition of the animals suffering from systemic TB. Although the experimental design did not produce a clear-cut picture of cerebral TB, our results certainly indicate the ability of PLG-based therapy to target mycobacteria localized in the meninges; however, further studies are warranted.

This is the first report documenting the role of nanotechnology-based ATD delivery to the brain. The approach highlights



**Figure 2.** Mycobacterial counts in the brain following chemotherapy with oral ATD-loaded PLG-nanoparticles in *M. tuberculosis* H<sub>37</sub>Rv infected mice. Values are means  $\pm$  SD of five animals. \* $P < 0.001$  compared with 4 weeks control. \*\*No detectable cfu following the plating of undiluted tissue homogenates.



**Figure 3.** Representative light photomicrographs of meningeal smears and brain sections from mice intravenously infected with *M. tuberculosis* H<sub>37</sub>Rv. Ziehl–Neelsen staining shows the presence of acid-fast bacilli (marked with arrows) in the meningeal smears, either in bunches (a) or arranged in their typical V or L shape (b) (1000×, under oil-immersion). The animals treated with free drugs or PLG-encapsulated drugs were found to have an unremarkable histology similar to that seen in a normal mouse brain (c): the section of cerebral cortex shows neurons of different size and shape as dark cells (marked as ‘N’). The space around each cell is a histological artefact. Astrocytes (marked as ‘A’) are distributed within the background (200×). However, the section from untreated control mice (d) shows oedema of the brain, as seen by increased intercellular myelin. The cells are uneven and disrupted with a number of fragments. The empty space around each cell has increased in size as compared with normal brain (c) (400×). Sections were stained with haematoxylin and eosin.

the potential for simplified and intermittent therapy against life-threatening forms of extrapulmonary TB and merits evaluation in a higher animal model.

### Acknowledgements

We are grateful to Dr B. N. Datta (former Professor and Head, Department of Pathology, Postgraduate Institute of Medical Education & Research, Chandigarh, India) for carrying out the histopathology studies. This work was supported by a grant from the Department of Science and Technology, Government of India, New Delhi.

### Transparency declarations

None to declare.

### References

1. Katti MK. Pathogenesis, diagnosis, treatment, and outcome aspects of cerebral tuberculosis. *Med Sci Monit* 2004; **10**: RA215–29.
2. Tsenova L, Ellison E, Harbacheuski R *et al.* Virulence of selected *Mycobacterium tuberculosis* clinical isolates in the rabbit model of meningitis is dependent on phenolic glycolipid produced by the bacilli. *J Infect Dis* 2005; **192**: 98–106.
3. Saitoh A, Pong A, Waecker NJ Jr *et al.* Prediction of neurologic sequelae in childhood tuberculous meningitis: a review of 20 cases and proposal of a novel scoring system. *Pediatr Infect Dis J* 2005; **24**: 207–12.
4. Byrd T, Zinser P. Tuberculosis meningitis. *Curr Treat Options Neurol* 2001; **3**: 427–32.
5. Thwaites GE, Tran TH. Tuberculous meningitis: many questions, too few answers. *Lancet Neurol* 2005; **4**: 160–70.
6. Garcia-Garcia E, Gil S, Andrieux K *et al.* A relevant *in vitro* rat model for the evaluation of blood-brain barrier translocation of nanoparticles. *Cell Mol Life Sci* 2005; **62**: 1400–8.
7. Olivier JC. Drug transport to brain with targeted nanoparticles. *NeuroRx* 2005; **2**: 108–19.
8. Bala I, Hariharan S, Ravi Kumar MNV. PLGA nanoparticles in drug delivery: the state of the art. *Crit Rev Ther Drug Carrier Syst* 2004; **21**: 387–422.
9. Pandey R, Zahoor A, Sharma S *et al.* Nanoparticle encapsulated antitubercular drugs as a potential oral drug delivery system against murine tuberculosis. *Tuberculosis (Edinb)* 2003; **83**: 373–8.
10. Sharma A, Pandey R, Sharma S *et al.* Chemotherapeutic efficacy of poly (DL-lactide-co-glycolide) nanoparticle encapsulated antitubercular

## Nanoparticle-based drug delivery for brain tuberculosis

drugs at sub-therapeutic dose against experimental tuberculosis. *Int J Antimicrob Agents* 2004; **24**: 599–604.

11. Pandey R, Sharma S, Khuller GK. Chemotherapeutic efficacy of nanoparticle encapsulated antitubercular drugs. *Drug Deliv* 2006 (In Press).

12. Lalonde V, Truffot-Pernot C, Paccaly-Moulin A *et al.* Powerful bactericidal activity of sparfloxacin (AT-4140) against *Mycobacterium tuberculosis* in mice. *Antimicrob Agents Chemother* 1993; **37**: 407–13.

13. Lounis N, Truffot-Pernot C, Bentoucha A *et al.* Efficacies of clarithromycin regimens against *Mycobacterium xenopi* in mice. *Antimicrob Agents Chemother* 2001; **45**: 3229–30.

14. Lounis N, Maslo C, Truffot-Pernot C *et al.* Impact of iron loading on the activity of isoniazid or ethambutol in the treatment of murine tuberculosis. *Int J Tuberc Lung Dis* 2003; **7**: 575–9.

15. Moghimi SM, Hunter AC, Murray JC. Nanomedicine: current status and future prospects. *FASEB J* 2005; **19**: 311–30.

16. Nuernberger E, Grosset J. Pharmacokinetic and pharmacodynamic issues in the treatment of mycobacterial infections. *Eur J Clin Microbiol Infect Dis* 2004; **23**: 243–55.

17. Kobayashi N, Toyota E, Takahara M *et al.* Tuberculosis of the central nervous system experienced at the International Medical Center of Japan. *Kekkaku* 1998; **73**: 513–7.

18. Jain R, Shah NH, Malick AW *et al.* Controlled drug delivery by biodegradable poly (ester) devices: different preparative approaches. *Drug Dev Ind Pharm* 1998; **24**: 703–27.

19. Tosi G, Rivasi F, Gandolfi F *et al.* Conjugated poly (D,L-lactide-co-glycolide) for the preparation of *in vivo* detectable nanoparticles. *Biomaterials* 2005; **26**: 4189–95.

20. Das D, Lin S. Double-coated poly (butylcyanoacrylate) nanoparticulate delivery systems for brain targeting of dalargin via oral administration. *J Pharm Sci* 2005; **94**: 1343–53.

21. Kabanov AV, Batrakova EV. New technologies for drug delivery across the blood brain barrier. *Curr Pharm Des* 2004; **10**: 1355–63.

22. Lockman PR, Koziara JM, Mumper RJ *et al.* Nanoparticle surface charges alter blood-brain barrier integrity and permeability. *J Drug Target* 2004; **12**: 635–41.

23. Tsenova L, Sokol K, Freedman VH *et al.* A combination of thalidomide plus antibiotics protects rabbits from mycobacterial meningitis-associated death. *J Infect Dis* 1998; **177**: 1563–72.

24. Bolin CA, Whipple DL, Khanna KV *et al.* Infection of swine with *Mycobacterium bovis* as a model of human tuberculosis. *J Infect Dis* 1997; **176**: 1559–66.

25. Wu HS, Kolonoski P, Chang YY *et al.* Invasion of the brain and chronic central nervous system infection after *Mycobacterium avium* complex infection in mice. *Infect Immun* 2000; **68**: 2979–84.

26. Johnson CM, Pandey R, Sharma S *et al.* Oral therapy with poly (DL-lactide-co-glycolide) nanoparticle encapsulated anti-tuberculosis drugs against *Mycobacterium tuberculosis* infected guinea pigs. *Antimicrob Agents Chemother* 2005; **49**: 4335–8.

27. Renard JF, Onnient Y, Proust F *et al.* Neuroaspergillosis and brain tuberculosis in an immunocompetent patient with good outcome. *J Neurol Neurosurg Psychiatry* 1998; **64**: 411–12.

28. Munoz-Elias EJ, Timm J, Botha T *et al.* Replication dynamics of *Mycobacterium tuberculosis* in chronically infected mice. *Infect Immun* 2005; **73**: 546–51.