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Tuberculosis: from molecular pathogenesis to effective drug carrier design

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In the past two decades, tuberculosis has gone from being a forgotten disease to a modern and recrudescent pathology. Tuberculosis is a curable infection and most of the negative therapeutic outcomes are related to poor patient compliance, which could be solved by new drug delivery approaches. By using such approaches the technological drawbacks of the currently used therapeutic agents could be addressed. In addition, optimum effectiveness of the drug by targeting the infection reservoirs could be achieved. In this article we compile the general physiological aspects of the infection along with new research updates especially on novel carriers used in the prevention of tuberculosis which might enhance therapeutic efficacy and patient compliance.

Tuberculosis

Tuberculosis (TB), a ubiquitous and highly contagious chronic granulomatous bacterial infection, is a leading killer of young adults worldwide. According to the World Health Organization (WHO), globally, there were an estimated 9.4 million new incidents of TB in 2008. Most of the cases occurred in South-East Asia (55%) and the African (30%) regions. The five countries with the largest numbers of cases include India, China, South Africa, Nigeria and Indonesia. Of the 9.4 million new TB cases in 2008, about 15% were HIV positive; 78% of these HIV-positive cases were in the African and 13% were in the South-East Asia regions [Global Tuberculosis Control 2011 (http://www.who.int/tb/publications/global_report/2011/gtbr11_full.pdf)]. The identification of multi-drug resistant (MDR) strains, defined as mycobacteria resistance to at least rifampicin (RIF) and isoniazid (INH) (two first line anti-TB drugs) and extensively drug resistant (XDR) strains, defined as MDR mycobacteria with additional resistance to fluoroquinolones and at least one of the injectable second-line antituberculosis drugs has worsened the condition. Notably, MDR- and XDR-TB have been recognized by WHO as the major challenge to be addressed in the fight against tuberculosis.

In spite of potentially curative treatments, TB remains the leading cause of deaths in the world today. Emergence of resistance to drugs used in the treatment of TB and particularly MDR-TB has become an obstacle to effective global TB control. Antimycobacterial therapy is available; however drugs are only partially effective because of the impermeable nature of the *Mycobacterium* cell wall and the propensity of *M. tuberculosis* to develop resistance [1]. Additionally *M. tuberculosis* has the capacity to remain viable within infected hosts for a prolonged time. Box 1 illustrates causes of death of treatable people.

TB pathogenesis

Infection of a host with *M. tuberculosis* is initiated following the inhalation of droplets (aerosols) containing a small number of bacilli [2]. The bacilli spread from the site of initial infection in the lung through the lymphatic or blood to other parts of the body, the apex of the lung and the regional lymph node being favored sites. Extrapulmonary TB of the pleura, lymphatics, bone, genitourinary system, meninges, peritoneum, or skin occurs in approximately 15% of TB patients.

Once in the lung, bacilli are subjected to phagocytosis by the resident macrophages of the lung, the alveolar macrophages, which in turn activated by the appropriate stimuli can effectively transfer the phagocytosed *M. tuberculosis* to the destructive environment of lysosomes, but some bacilli are able to escape lysosomal delivery and survive within the macrophage [2–4]. Infected macrophages can then either remain in the lung or are disseminated to other organs in the body. However, only a minority (approximately 10%) of

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BOX 1

Various factors responsible for the failure of curative treatment of TB.

Reasons why people die from TB: a curable disease

- *M. tuberculosis* finds its victims in developing countries with degraded social and health conditions where access to medicines is limited.
- Therapeutic regimen of long duration, patients usually does not take the prescribed medications with sufficient regularity and duration to achieve a cure.
- Treatment with a multi-drug regimen. Combined therapies are more effective than single ones, but their fulfillment becomes more difficult for patients and this leads to a poor patient compliance.
- Patients have to consume a large number of tablets (up to eight at one time), which is a common cause for non-compliance.
- Concomitant presence of conditions compromising the immune system functionality, such as HIV infection.

infected people develop TB, because in most healthy individuals, the immune defense system is sufficient to keep *M. tuberculosis* in such a check that the disease cannot develop.

Phagocytosis of *M. tuberculosis* by macrophages proceeds through a series of membrane invagination, budding, and fusion events, resulting in the formation of the phagosome [5]. Internalized material is distributed further within the cell through a series of vesicle trafficking events delivering the material to the antigen processing and presentation pathway. The lysosome is the final destination of both the phagosomal and endosomal cargo, where extensive degradation occurs resulting in the clearance of potentially harmful material and subsequent destruction of pathogens. Pathogenic mycobacteria have evolved mechanisms to interfere with mechanisms that regulate the transfer of cargo to lysosomal organelles for destruction.

Complement receptors (CR1, CR2, CR3 and CR4), mannose receptors (MR), dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN), and Fc receptors have an important role in binding of the organisms to the phagocytes [6–8]. The complex structure of the cell surface is responsible for the ability of multiple receptor molecules to internalize *M. tuberculosis* [9]. Recent reviews discuss the role of the mycobacterial lipids in host pathogen interactions [7,8,10–12].

M. tuberculosis residing within macrophages is kept in check within structures termed granulomas [13,14]. Although the precise biology of granulomas remain only partially understood, it is believed that granulomas are structured clusters containing mycobacteria infected macrophages in the center, surrounded by different types of immune cells, particularly macrophages and T lymphocytes [15]. Figure 1 illustrates the formation of granuloma. Within granulomas, both non-activated and activated macrophages coexist, where the activated macrophages process and present mycobacterial antigens to the surrounding T lymphocytes [16]. After presentation of mycobacterial antigens, the triggering of T cell receptors occurs and the T cells become activated [17]. Activated T cells secrete cytokines and chemokines keeping the macrophages in an activated state and ensuring the recruitment of other immune cells to the site of infection. Mycobacteria might exist as actively dividing bacilli or in a so-called 'dormant' state and both can occur within the same infected individual depending on the stage of the disease [18,19]. As long as host immunity is effective, there is usually no adverse effect of the *M. tuberculosis* on the health of the host even up to the lifetime [20]. Thus, the granuloma structure probably represents a balance. The delicacy of this balance is illustrated by the observation that any deterioration of host immunity results in a potentially life-threatening condition of individual harboring live *M. tuberculosis* [21].

Hart and co-workers hypothesized that prevention of phagolysosomal fusion is a mechanism by which *M. tuberculosis* survives inside macrophages [3,22]. Over the past few years several mechanisms have been unraveled that are used by *M. tuberculosis* to survive within hosts and to avoid immune defense mechanisms. To describe these mechanisms in detail is beyond the scope of this review. However, exhaustive information might be found in some recent compilations [12,23–31].

Drug therapy and its current limitations

The goals of treatment include cure without subsequent relapse, prevention of death, impediment to transmission, and prevention of the emergence of drug resistance. For these purposes long-term treatment with a combination of drugs is required. Various drugs which are used in the treatment of TB are tabulated in Table 1. Directly observed treatment, [(DOTS) short-course], the current recommended TB chemotherapy, consists of a 6-month therapy of four co-administered drugs. Treatment of TB and drug resistant cases requires multi-drug therapy, comprising:

(i) An initial intensive phase of RIF, INH, pyrazinamide (PZA), and ethambutol (ETB) daily for 2 months.
(ii) A continuation phase of RIF and INH for a further 4 months, either daily or three times per week.

In the case of MDR-TB, the WHO recommends the use of DOTS-Plus, which is DOTS plus second-line antitubercular drugs [Global Tuberculosis Control 2011 (http://www.who.int/tb/publications/global_report/2011/gtbr11_full.pdf)].

TB is treated with a multi-drug regimen and current treatment protocols are lengthy, usually 6–9 months, thus exceptionally vulnerable to the incidences of side effects, unsatisfactory patient compliance and slow improvement of patients. Therefore, despite the availability of highly effective treatments for TB, cure rates remain low. Present efforts in improving treatment focus on shortening the length of treatment or using innovative drug delivery strategies in addition to alternative administration routes, which might have a fundamental role in improving antitubercular chemotherapy efficacy, thereby enhancing patient compliance.

Shegokar [32] explored nanoparticulate systems for anti-TB therapy and briefly discussed new drugs under clinical trial. Sosnik *et al.* [33] reviewed the state-of-the-art in the development of nano-based drug delivery systems for encapsulation and release of antituberculous agents. Blasi *et al.* [34] provided a crucial report on micrometric and nanometric particulate systems designed and investigated to improve TB chemotherapy. Other reviews include du Toit *et al.* [35] and Pandey and Khuller [36]. In this article we have tried to provide a comprehensive account of recent advances in targeting and novel routes and means of drug delivery for the treatment of tuberculosis. The carrier systems have been

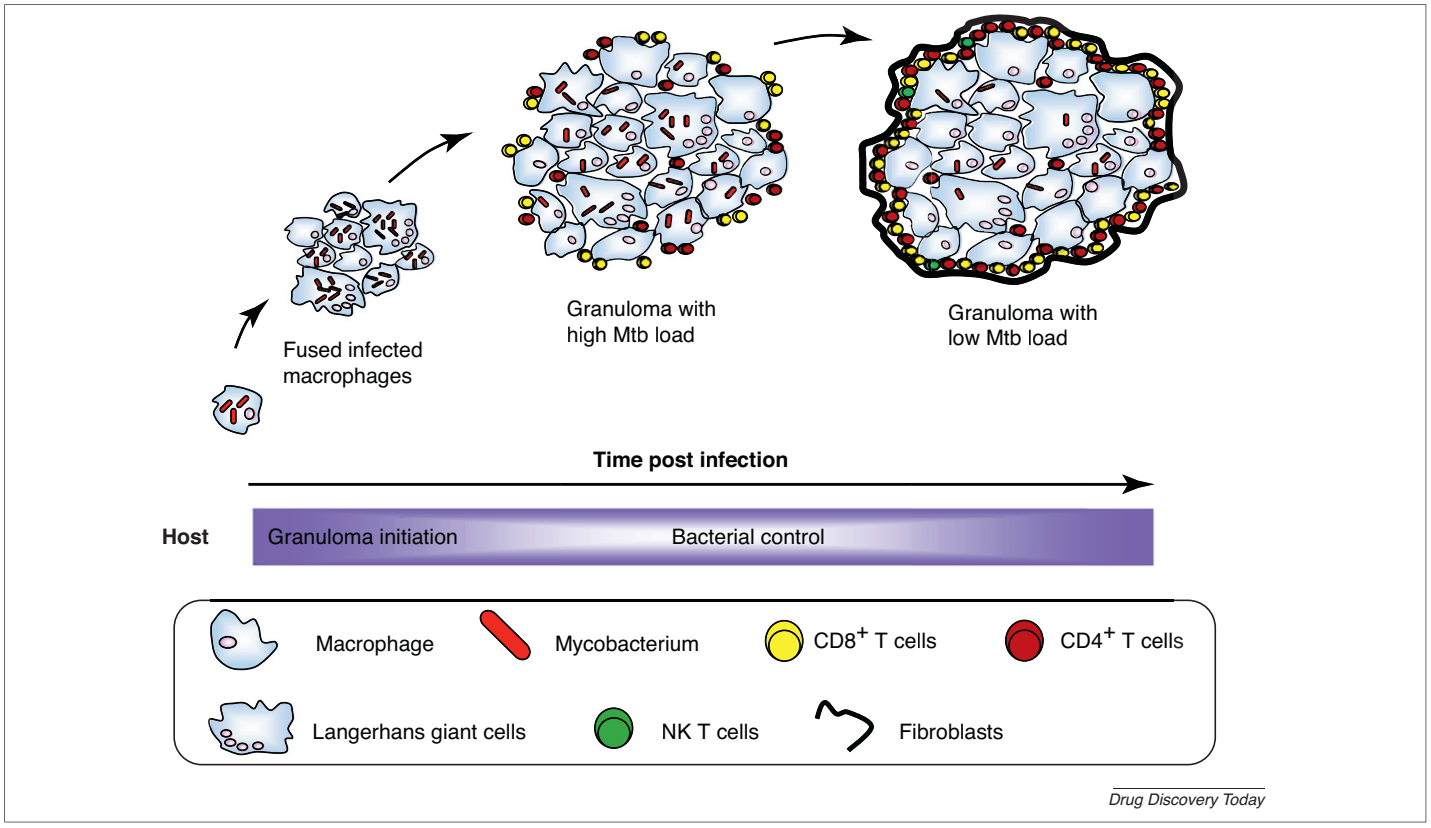

FIGURE 1

Stages of granuloma formation in tuberculosis (TB). The initial stage of TB is characterized by expansion of the bacterial population in the absence of adaptive immunity. When initiation of adaptive immunity eventually occurs, $CD4^+$ and $CD8^+$ effector T lymphocytes are recruited to infected tissue and curtail bacterial growth. The mature granuloma represents equilibrium between virulent mycobacteria and the host immune response. *Abbreviations*: Mtb, *Mycobacterium tuberculosis*; NK, Natural killer.

categorized according to the targeting strategies (i.e. passive, active or local delivery to lungs).

Novel drug delivery systems for antitubercular drugs

The need for new tools to fight TB, particularly MDR- and XDR-TB, is pushing towards new strategies and drug moieties to improve therapy. So far, TB therapies have exploited conventional routes of administration with various pharmaceutical dosage forms, such as tablets, capsules and injectable solutions. However, high dosages and frequent administrations are required to maintain the drug therapeutic concentration over an extended period of time. Novel drug delivery systems have recently emerged as an alternative to conventional oral dosage forms to solve this issue. Nanotechnology-related rational drug delivery might improve therapeutic success by constraining adverse drug effects and requiring less frequent administration regimes, ultimately resulting in patients who are more compliant and thus, attain higher adherence levels. Furthermore, such systems can enhance the effectiveness of approved drugs and extend their applicability by overcoming technological limitations, such as low bioavailability, resistance, cellular and anatomical barriers, among others [37]. The advantages and disadvantages of these systems have been outlined in Fig. 2.

Moreover, despite the emergence of new antibiotics, treatment of such intracellular pathogens still remains difficult because infections are localized within phagocytic cells and most antibiotics, although highly active *in vitro*, do not actively pass through cellular membranes, and hence, it is difficult to achieve the relatively high concentrations of the drugs within the infected cells [38–40]. The main challenge for intracellular chemotherapy is to design and develop a carrier system for antibiotics that could be efficiently endocytosed by phagocytic cells and, once inside the cells should prolong release of the antibiotics so that the number of doses and associated drug toxicity can be reduced. The problems associated with delivering free antibiotics to the intracellular space have led to the investigation of improved drug carriers for treating intracellular pathogens, including antibiotics loaded into liposomes, microspheres, polymeric carriers, and nanoplexes [41].

Polymeric and lipidic drug delivery systems are well suited as vehicles for the delivery of antimicrobial agents because they usually provide a sustained drug release effect, minimize the toxicity associated with the encapsulated drugs and increase the overall drug efficacy. Moreover, these systems protect the drug from premature immunological and enzymatic attacks and, in some cases, might act synergistically with cellular bactericidal mechanisms. Also such systems can synergize with the bactericidal mechanisms of the phagocyte, further improving the treatment of intracellular infections. The stimulation of the intracellular Reactive oxygen intermediates (ROI) might act synergistically with the antibiotic activity to kill intracellular bacteria, thus increasing treatment efficiency.

TABLE 1

Drugs used for the treatment of TB

Drug	Mechanism of action
First-line agents	
Rifampicin	Inhibits bacterial RNA synthesis by binding to the β-subunit of bacterial DNA-dependent RNA-polymerase leading to blocking of the initiation chain formation in RNA synthesis.
Isoniazid	A pro-drug activated by katG, exerts its lethal effect through inhibition of synthesis of mycolic acids, through formation of a covalent complex with an acyl carrier protein and β-ketoacyl carrier protein synthetase.
Pyrazinamide	Converts to active pyrazanoic acid by pyrazinamidase in susceptible organisms. Pyrazanoic acid inhibits growth of bacterium by lowering pH in the immediate surroundings. PZA inhibits trans-translation, a key cellular process for managing damaged proteins and 'rescuing' nonfunctioning ribosomes in *M. tuberculosis*. Also acts as an antimetabolite of nicotinamide thereby interfering NAD synthesis, inhibiting short-chain, fatty-acid precursors' synthesis.
Ethambutol	Inhibits mycobacterial arabinosyl transferases involved in the polymerization of D-arabinofuranose to arabinoglycan, an essential cell wall component.
Aminoglycosides	Irreversible inhibitors of protein synthesis through binding to specific 30S-subunit ribosomal proteins.
	Inhibits bacterial topoisomerase II (DNA gyrase) and topoisomerase IV and thus inhibiting bacterial DNA synthesis.
Bacteriostatic second-line drugs	
P-aminosalicylic acid	Anti-metabolite interfering with incorporation of para-aminobenzoic acid into folic acid – folate synthesis antagonist.
Cycloserine	Structural analogue of D-alanine, inhibits incorporation of D-alanine into peptidoglycan pentapeptide through inhibition of alanine racemase.
Other drugs	
Clofazimine	Unknown, but might involve DNA binding. Possesses direct antimycobacterial and immunosuppressive properties.
Amoxicillin and/or clavulanic acid	Amoxicillin inhibits cell wall synthesis. Clavulinic acid is a β-lactamase inhibitor.
Clarithromycin	Inhibition of protein synthesis through binding to 50S ribosomal RNA as aminoacyl translocation reactions and the formation of initiation complexes are blocked.
Rifabutin	Activity is similar to that of RIF. Inhibits bacterial RNA synthesis by binding strongly to the β-subunit of bacterial DNA-dependent RNA-polymerase.
Thiacetazone	Not clearly elucidated.

Abbreviations: katG: catalase peroxidase, NAD: nicotinamide adenine dinucleotide.

Approximately 80% of TB cases affect the lungs; the site of entrance of the bacilli, making inhalable dosage forms extremely useful in obtaining high drug concentrations in the lungs and targeting directly the alveolar macrophages, the site of residence of the *M. tuberculosis*. Consequently it is anticipated that the required dose would decrease notably along with significant reduction in systemic side effects. Although identifying novel anti-TB agents remains a priority, no new drug has reached the market for approximately 40 years, shifting the paradigm towards the development of new formulations with old drugs. The development of novel formulations for currently used agents might represent a cost-effective and promising alternative. Achievement of high local drug concentrations, targeting of alveolar macrophages and the possibility of prolonging residence time by modifying drug release are the main features which support the use of such systems. The goal of this article is to highlight the potential advantages of novel drug delivery strategy relevant in the treatment of TB.

Various carriers for passive drug delivery for the treatment of TB

Liposomes. Liposomes are the most widely studied carriers for macrophage-specific antibacterial drug delivery. Because compared with drug solution, intravenous injection of Streptomycin loaded liposomes [42] to infected mice led to a significant decrease of the *Mycobacterium* count in the spleen, but not in the lungs. Moreover, a prolonged mouse survival and reduced acute drug toxicity were observed with respect to free drug. Klemens *et al*. incorporated gentamicin into liposomes and compared the antibacterial activity with that of the free drug in a mouse model of disseminated *M. avium* complex infection [43]. The encapsulated drug significantly reduced the bacterial count in spleen and liver. In addition, a dose-related reduction of the bacterial load, without sterilization was found. Additional studies have been tabulated in Table 2.

It can be concluded through these studies that at equivalent doses, antibiotic-loaded liposomes were more significantly effective in reducing the number of bacteria in lungs, liver and spleen than the combination of free oral drugs. In addition, no sign of liposome-induced hepatotoxicity was observed during the treatment. Thus, liposome-mediated treatment of TB might be a promising approach to obtain a therapy with good patient compliance, low cost and reduced dosage frequency and toxic effects.

Niosomes. Micron-sized RIF-loaded niosomes containing Span 85 as the surfactant were prepared by Jain and Vyas [44]. Up to 65%

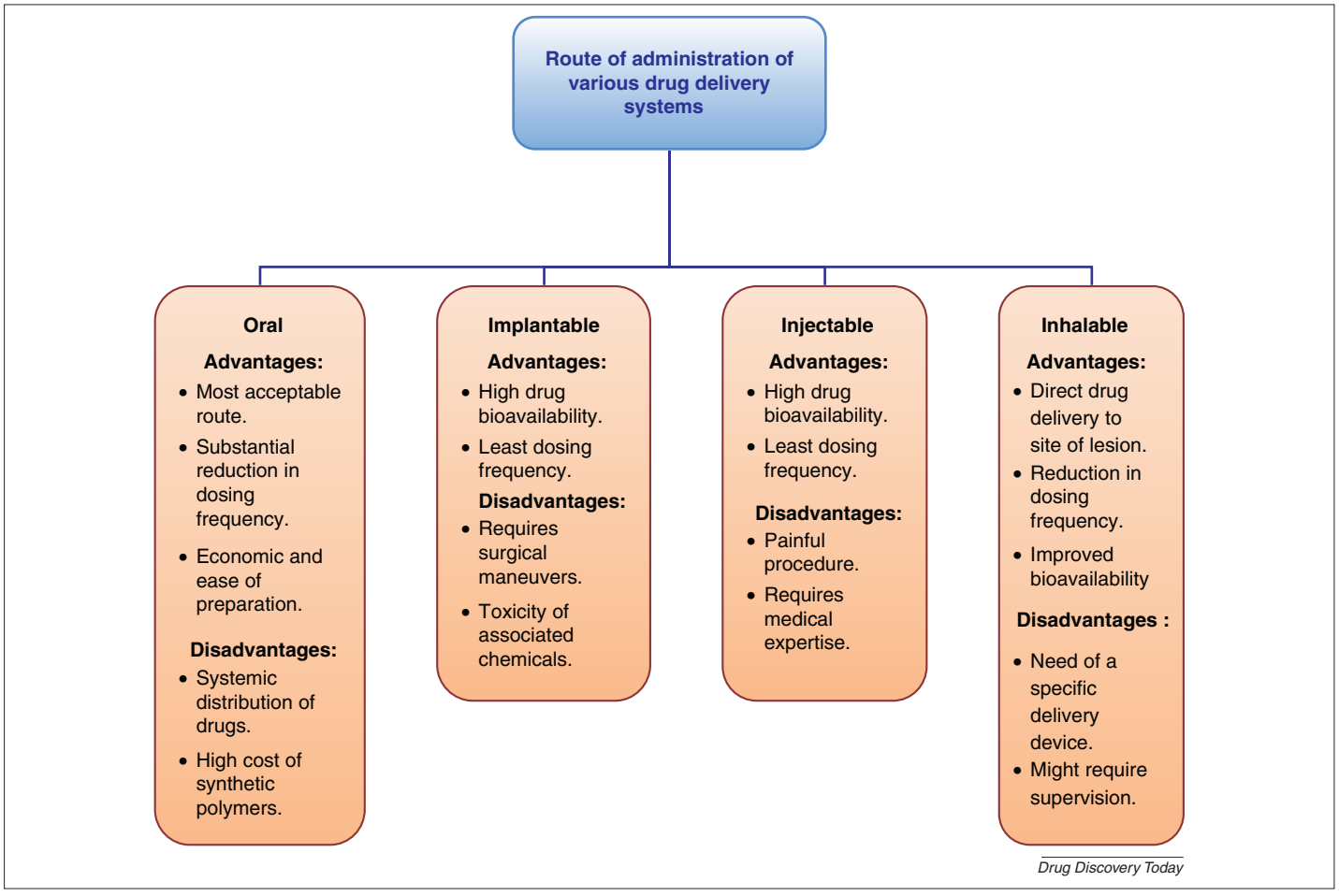

FIGURE 2

Various routes of administration for antimycobacterial drug delivery systems.

of the drug was localized in the lungs by adjusting the size of the carrier. The same group further extended the investigations and evaluated the biodistribution of niosomes with smaller sizes produced with different sorbitan esters (Span® 20, 40, 60, 80 and 85) and cholesterol [45]. The extent of drug entrapment increased gradually with the increase of the hydrophobicity of the surfactant. When administered through the intraperitoneal (i.p.) route niosomal formulations attained substantially higher RIF concentrations in thoracic lymph nodes (i.e. 46.2% of the administered dose as opposed to 13.1% for the free drug). By contrast, when administered intravenously, only 7.3% of the drug was found in the thorax. Thus, suggesting compartmentalization of the drug took place in the lymphous tissue when administered through i.p. route.

Nanoparticles and microparticles. Nanoparticles (NPs)-based drug delivery systems have considerable potential for treatment of TB. The important technological advantages of NPs used as drug carriers are high stability, high carrier capacity, feasibility of incorporation of both hydrophilic and hydrophobic substances, and feasibility of variable routes of administration, including oral application and inhalation. NPs can also be designed to enable controlled drug release from the matrix. Thus, this approach has been one of the most extensively investigated with respect to antitubercular drug delivery systems.

Anisimova *et al*. investigated the encapsulation of RIF, INH and streptomycin within poly(n-butylcyanoacrylate) (PBCA) and poly(isobutylcyanoacrylate) (PIBCA) NPs and tested the accumulation in human blood monocytes *in vitro* towards the development of a drug depot [46]. The intracellular concentration of the drugs increased with respect to the extracellular concentration. Also, these researchers showed that the activity of encapsulated RIF against intracellular bacteria was not higher than that of the free drug. Similarly ciprofloxacin [47] and moxifloxacin [48,49] have been encapsulated in PIBCA and PBCA nanoparticles.

Regardless of the potential of the nanoparticulate strategy, the non-biodegradability of the polymers employed constitutes a major limitation. Anti-TB drugs have been successfully entrapped and delivered in biodegradable polymers, such as poly (DL-lactide-co-glycolide) (PLG), which are biocompatible and releases the drug in a controlled manner at therapeutic levels [50]. When INH and RIF containing microparticles (MPs), having a diameter ranging from 11.75 μm to 71.95 μm, were injected subcutaneously as a single dose they provided sustained release of drugs over 6–7 weeks when tested in mice [51]. The particles with a size range ≥10 μm remained at the site of injection forming a depot and the entrapped contents of the MPs were gradually released by diffusion through the polymeric particles. Such depots can show release

TABLE 2

Novel drug delivery systems containing anti-TB drugs

Carrier	Polymer/lipid	Drugs	Ligand	Study	Refs.
Liposomes	Lecithin	Streptomycin		Intravenous injection of Streptomycin loaded liposomes to infected mice led to a significant decrease of the *Mycobacterium* count in the spleen, but not in the lungs. Prolonged mouse survival and reduced acute drug toxicity were also observed.	[42]
	ePC	Gentamycin		The encapsulated drug significantly reduced the bacterial count in spleen and liver. In addition, a dose-related reduction of the bacterial load, without sterilization was found.	[43]
	PC and phosphatidylglycerol	Sparfloxacin		Treatment with free or liposome-encapsulated sparfloxacin (6 mg/ml) for 24 hours resulted in the reduction of the growth index to 25 and 30% of that of untreated controls, respectively. When cultures were treated for 4 days, free sparfloxacin reduced the growth index to 6% of that of the untreated control, whereas liposome-encapsulated sparfloxacin reduced it to 8% of that of the control.	[67]
	Lecithin	Amikacin		Once-weekly and even once-monthly treatments with liposomal amikacin significantly reduced bacterial replication in infected tissues and extended the survival time of infected mice.	[68]
	PC	Clofazimine		Encapsulation resulted in significant reduction of the *in vitro* and *in vivo* toxicity of the drug. The anti-TB activity in both acute and chronic models was enhanced; particularly higher in the liver and lung. In addition, chronically infected mice treated with the encapsulated drug showed total clearance from the liver and spleen with no signs of recovery 2 months post-treatment.	[69,70]
	ePC, DSPE, PEG	INH, RIF		Once weekly-administered liposomal drugs for 6 weeks reduced the mycobacterial load significantly in lungs, liver and spleen of infected mice compared with untreated animals.	[71]
	DPPC	PZA		When injected twice weekly, high therapeutic efficacy of PZA liposomes was observed in the treatment of *M. tuberculosis* in mice.	[72]
	DPPC, DPPG	RFB		Compared with mice treated with free RFB, mice treated with the liposomal formulation exhibited lower bacterial loads in the spleen, liver and lung. The system is a promising approach for the treatment of extrapulmonary TB in human immunodeficiency virus co-infected patients.	[73]
	PC, dicetylphosphate	INH, RIF	O-SAP, Monosialogangliosides/ DSPE-PEG 2000	Significant decrease in the hepatoxic activity of the anti-TB agents was observed upon encapsulation in liposomes. Within 30 min the accumulation of nanocarriers in the lungs was 31% with PEGylated systems containing O-SAP as compared to 5.1% with conventional liposomes.	[58]
	PC, CH, dicetylphosphate	INH, RIF	O-SAP, Monosialogangliosides/ DSPE-PEG 2000	As compared with therapeutic doses (12 and 10 mg/kg for INH and RIF, respectively) administration of sub-therapeutic doses (4 and 3 mg/kg for INH and RIF, respectively) led to a higher decrease in CFU. Overall, a significant increase in the anti-TB activity was found.	[74]
	DSPC, DPPC, HPC	Capreomycin		The vesicles made of DSPC had a narrow size distribution, with a mean diameter lower than 200 nm. Thus demonstrated their suitability for use in inhaled formulations	[75]
	DSPC, DPPC, HPC	Capreomycin		Both freeze–thawing technique and a response surface methodology were used to improve the drug content.	[76]
	ePC	RIF	O-SAP and MBSA	Percent viability of *M. smegmatis* inside macrophages (*in vitro*) after administration of drug (*in vivo*) was 7–11% (ligand-anchored liposomal aerosols), 45.7 and 31.6% in case of plain drug and plain neutral liposomal aerosol-treated macrophages. Preferential accumulation of MBSA- and *O*-SAP-coated formulations in alveolar macrophages. O-SAP-coated liposomes were more effective than MBSA, likely due to better accumulation in alveolar macrophages.	[66]

TABLE 2 (*Continued*)

Carrier	Polymer/lipid	Drugs	Ligand	Study	Refs.
	HSPC, DOPE		4-Aminophenyl-α-D-mannopyranoside	A pronounced increase in the uptake was observed with the mannosylated-nanocarriers. In addition, greater accumulation of modified liposomes was found in the lungs after pulmonary administration to rats.	[77]
	HSPC, DOPE	Ciprofloxacin	4-Aminophenyl-α-D-mannopyranoside	The targeting efficiency towards rat AMs of mannosylated liposomes was significantly greater than that of unmodified liposomes. PK/PD analysis suggested that the mannosylated liposomes exhibited potent antibacterial effects against many bacteria although unmodified liposomes were ineffective against several types of bacteria, and the probability of microbial mutation by mannosylated liposomes was extremely low.	[78]
	DSPC		Man-C4-Chol	The higher the mannose concentration on the surface, the more pronounced the cellular uptake. Also, the uptake of modified liposomes ***in vitro*** was inhibited with an excess of solubilized mannan, confirming the specificity of the carrier–receptor interaction. Accumulation in alveolar cells after intratracheal administration to rats indicated the higher uptake of mannosylated-nanocarriers, preferably (15–17-fold) by alveolar macrophages over alveolar epithelial type II cells.	[79]
Niosomes	Span 85	RIF		Up to 65% of the drug was localized in the lungs by adjusting the size of the carrier.	[44]
	Span 20, 40, 60, 80, 85	RIF		Niosomal formulations attained substantially higher RIF concentrations in thoracic lymph nodes.	[45]
		RIF		A significant increase in the extent of drug accumulation in the lungs, liver, kidneys and blood serum was apparent for RIF-loaded niosomes. After i.v. administration, niosomes preferentially accumulated in the lung, liver and kidney. After intathoracic (i.t.) administration, the lung and/or plasma ratios for niosomes and free drug represented a 145-fold increase in the accumulation capacity of RIF-loaded niosomes in the lungs as compared to the free drug.	[80]
Nanoparticles and microparticles	PLG	INH		Porous and non-porous microparticles released INH in plasma for up to 2 days. Hardened PLG microparticles sustained release of INH for up to 7 weeks. Concentrations of INH obtained were higher than the MIC of INH.	[81]
	PLG	RIF, INH, PYZ, ETB		Entrapped drugs remained in circulation up to 72 h. Level of PLG encapsulated INH was found to be higher than its MIC value (0.1 μg/ml). Increased C_{max}; $AUC_{o-\infty}$; $t_{1/2(a)}$ and $t_{1/2(e)}$ when drug were given entrapped in PLG microparticles	[82]
	PLG	INH, RIF		Single dose of PLG microparticles – sustained release of INH and RIF for up to 7 and 6 weeks, respectively. Free drugs (in combination) injected in the same doses were detectable *in vivo* up to 24 h only. One dose of PLG microparticles cleared bacteria more effectively from lungs and liver in experimental murine model of TB (compared with a daily administration of the free drugs)	[51]
	PLG	RIF		Bioassay assessment of cell culture supernatants from monocyte cell lines showed release of RIF up to 7 days. The levels of RIF released within monocytes were more effective at reducing *M. tuberculosis* intracellular growth than equivalent doses of free drug.	[83]
	PLGA	RIF		Animals treated with single and double doses of microparticle entrapped RIF reduced numbers of viable bacteria, inflammation and lung damage compared with RIF-only treated animals 28 days post-infection. Two doses of RIF-PLGA-reduced splenic enlargement.	[84]
	PBCA, PIBCA	RIF, INH, Streptomycin		Encapsulated INH, Streptomycin and RIF showed 4–8-, 7- and 22–25-fold increases in the intracellular concentration with respect to the extracellular concentration. The activity of encapsulated RIF against intracellular bacteria was not higher than that of the free drug.	[46]

PIBCA	Ciprofloxacin		Loading in NPs led to increased AUC, $t_{1/2}$ and V_d as compared to free drug. Drug loaded PIBCA NPs were more effective against *M. avium* complex in human macrophages than free drug.	[47]
PBCA	Moxifloxacin		*In vitro* drug release for formulation containing encapsulated moxifloxacin showed initial burst release followed by sustained drug release of 65% at end of 48 h. The moxifloxacin loaded NPs were more toxic to the macrophages than the free drug. Cellular uptake showed pronounced increase (2–3 fold) in the intracellular drug concentration. After i.v. administration, anti-TB activity in infected mice showed a significant decrease in the total mycobacterial count in the lungs.	[48,49]
PLGA	RIF, INH, PZA		Encapsulated drugs were detected in plasma for up to 9 days and therapeutic concentrations in tissues were maintained for 9–11 days. It was shown in the preclinical studies that in infected mice 5 oral doses of drug-loaded PLGA NPs when administered over 50 days effectively eliminated the pathogen from the different organs.	[52]
PLGA	RIF, INH, PZA, ETB		It was observed that after oral administration therapeutic levels were maintained for 5–8 days in blood and 9 days in plasma; one administration every 10th day (5 doses) eliminated the bacteria in the meninges.	[85]
PLG	RIF, INH, PZA, ETB		Combination of four drugs has a significant potential to shorten the duration of chemotherapy besides reducing the dosing frequency.	[86]
PLG	RIF, INH, PZA		A single subcutaneous dose maintained drug plasma, lungs and spleen concentrations for more than 1 month and led to undetectable bacterial counts in the different organs. The particle preparation showed a better chemotherapeutic efficacy compared with a daily drug treatment.	[87]
Alginate	RIF, INH, PZA, ETB		Encapsulated drugs were observed in plasma up to 7, 9, 11 and 11 days after administration for ETB, RIF, INH and PZA, respectively, and in tissues until day 15.	[53]
Gelatin	RIF		Encapsulation in NPs not only sustained the plasma level but also enhanced the AUC and MRT of the drug. Significant reduction in bacterial counts in the lungs and spleen of TB-infected mice was also found.	[88]
PLG	Econazole, Moxifloxacin, RIF		Only eight doses of NPs individually were sufficient to suppress bacterial clearance in infected mice, in contrast to 56 daily doses of moxifloxacin and 112 doses twice a day of econazole. Addition of third drug RIF to this combination showed complete bacterial clearance within 8 weeks.	[89]
Stearic acid	RIF, INH, PZA		After a single oral administration, the therapeutic concentrations of the drugs were maintained in the plasma for 8 days and in the organs (lungs, liver and spleen) for 10 days, whereas the free drugs were cleared within 1–2 days. The initial CFU count 15 days after the infection with *M. tuberculosis* H37Rv was 4.20 and 4.34 log in lungs and spleen, respectively.	[54]
PLG	RIF, INH, PZA		The efficacy of NPs encapsulated drugs administered every 10 days *versus* that of daily nonencapsulated drugs against *M. tuberculosis* aerosol infection in guinea pigs was evaluated. Both treatments significantly reduced the bacterial count and lung histopathology.	[90]
PLG	RIF, INH, PZA	Wheat germ agglutinin	Three doses administered fortnightly for 45 d were sufficient to produce a sterilizing effect in lungs and spleen.	[59]
Gelatin	INH	Mannose	Study concluded that NPs are potential carrier for safer and efficient management of TB through targeted delivery.	[60]

TABLE 2 (*Continued*)

Carrier	Polymer/lipid	Drugs	Ligand	Study	Refs.
	PLG	RIF, INH, PZA		Nebulization of drug-loaded NPs resulted in plasma-detectable concentrations after 6 h and therapeutic drug concentrations were detected until day 6 for RIF and day 8 for both INH and PZA. In nebulization of NPs to ***M. tuberculosis***-infected guinea pigs at every 10th day, no tubercle bacilli could be detected in the lungs after only five doses of treatment, whereas 46 daily doses of orally administered drug were required to obtain an equivalent therapeutic benefit.	[64]
	Alginate	RIF, INH, PZA		Following aerosol administration, drug levels above MIC were detected in the lungs, liver and spleen up to 15 days, compared with just 1 day for the free drugs. However, the results are promising; a lack of a deep particle characterization (e.g. morphology, size and size distribution, *in vitro* release) makes the observed results difficult to explain.	[91]
	PLGA, mannitol	RIF		Encapsulation of the NPs in mannitol improved the *in vivo* uptake of the drug by alveolar macrophages in rat lungs as compared to RIF-containing PLGA and mannitol microspheres.	[63]
	PLGA	RIF		Particles were taken up very efficiently by macrophages inducing a potent bactericidal effect. Phagocytosis of RIF loaded PLGA MS does not generate the toxic humoral factors to AMs, such as TNF-α and NO, and the phagocytosis does not affect the viability of AMs, showing that, PLGA MS are not toxic to AMs.	[92]
	PLGA	RIF		Daily doses of RIF solutions over 10 or 20 days had a positive effect on pulmonary and splenic inflammation but not on the number of viable bacteria in the lungs, while a single administration of particles or 20 days of dosing with free RIF equally decreased the bacteria population in the spleen. Besides, PLGA MPs increased drug residence time in the lungs.	[65]
	PLA	RFB, INH		Intracellular concentrations of drug encapsulated respirable MPs was found to be four-fold higher than the drug solutions.	[93]
	PLA	INH		The particles were found to be suitable to target and provide sustained release of INH to AMs.	[94]
	PLG	INH, RIF		The intracellular drug concentrations resulting from particle inhalation were found to be higher than vascular delivery of soluble drugs.	[95]
	Hyaluronan	Ofloxacin		Intratracheal administration of Ofloxacin-loaded hyaluronan particles resulted in 50% lower serum bioavailability with respect to intravenous or oral ofloxacin. This observation supported the view that inhaled MPs may reduce systemic side effects, but also suggested that extrapulmonary TB might not be addressed by inhaled therapies alone.	[96]
Polymeric micelles	P(CL-GA)–PEG-P(CL-GA)	RIF	–	RIF sustained release was obtained over 32 days from 25% gel matrix.	[56]
	MPEG-PLLA and MPEG-PDLA	RIF	–	RIF loading capacity and encapsulation efficiency of the stereo-complexes were higher. There was a fast initial release (50% after 4–8 hours) and a more moderated one (100% after 48 hours) afterwards.	[97]
	PLA-modified chitosan oligomers	RIF	–	RIF-chitosan oligomer micelles showed initial burst drug release of 35% within 10 h followed by more sustained drug release till 5th day	[98]
	INH-PEG-PAA	INH	–	The micelle-forming prodrug showed a 5.6-fold increase in antituberculous activity against *M. tuberculosis in vitro* when compared to the free drug	[99]
	PYZ-PEG-PAA	PYZ		The size of the micelles prevented renal filtration, increased the residence time in the blood stream with improved antimicrobial activity.	[100]
	RIF-PEG-PAA	RIF		The size of the micelles prevented renal filtration, increased the residence time in the blood stream with improved antimicrobial activity.	[101]
	INH lipid derivatives	INH		Micelles showed increased penetration of the drug into the pathogen leading to promising antibacterial activity	[57]

Dendrimers	PPI	RIF	Mannose	Mannose on surface significantly reduced the hemolytic toxicity of the nanocarriers and drug and also sustained the drug release. Surface modification improved the selective uptake of the drug-loaded nanocarriers by cells of the immune system.	[61]
	PEGylated PPI	RIF		PEG grafted dendrimers showed significant increase in drug entrapment, sustained release of RIF and low hemolytic activity.	[102]

Abbreviations: AUC: area under the curve; CH: cholesterol; DOPE: Dioleoyl phosphatidylethanolamine; DPPC: Dipalmitoyl phosphatidylcholine; DSPC: Distearoyl phosphatidylcholine; ePC: egg phosphatidylcholine; HSPC: hydrogenated soy phosphatidylcholine; HPC: hydrogenated phosphatidylcholine; MBSA: maleylated bovine serum albumin; MPEG-PLLA: poly (ethylene glycol)–poly(L-lactide); MPEG-PDLA: poly(ethylene glycol)-poly(D-lactide); MRT: mean residence time; O-SAP: o-stereoylamylopectin; PAA: poly(aspartic acid); PC: phosphatidylcholine; PEG-PAA: poly(ethylene glycol)–poly(aspartic acid); P(CL-GA)–PEG-P(CL-GA): poly(ε-caprolactone-coglycolide)–poly(ethylene glycol)–poly(ε-caprolactone-co-glycolide); PD: pharmacodynamic; PLG: poly(lactide-co-glycolide); PK: pharmacokinetic; PPI: polypropyleneimine.

profiles extending over several months culminating in degradation of the entire polymeric device.

Pandey *et al.* investigated the encapsulation of different first-line anti-TB drugs (RIF, INH and PZA) within biodegradable PLGA NPs [52]. In contrast to free drugs which were cleared from circulation after 12–24 h, encapsulated drugs were detected in plasma for up to 9 days and therapeutic concentrations in tissues were maintained for 9–11 days. It was shown in the preclinical studies that in infected mice five oral doses of drug-loaded PLGA NPs when administered over 50 days effectively eliminated the pathogen from the different organs.

Zahoor *et al.* also produced anti-TB drug-loaded alginate NPs by means of ionotropic gelation [53]. The encapsulation efficiency ranged between 80 and 90% for RIF, 70 and 90% for INH and PZA and 88 and 95% for ETB. In contrast to the free drugs which were cleared from blood 12 to 24 hours after administration and were detectable in tissues (e.g. spleen, liver and lung) only until day 1, encapsulated drugs were observed in plasma for up to 7, 9, 11 and 11 days after administration of ETB, RIF, INH and PZA, respectively, and in tissues until day 15.

As opposed to polymeric NPs, the production process of solid lipid nanoparticles (SLN) involves minimal amounts of organic solvents and SLNs have good stability on nebulization. Thus, the chemotherapeutic potential of oral SLNs loaded with RIF, INH and PZA was evaluated against TB [54]. After a single oral administration, the therapeutic concentrations of the drugs were maintained in the plasma for 8 days and in the organs (lungs, liver and spleen) for 10 days, whereas the free drugs were cleared within 1–2 days. The initial colony forming units (CFU) count 15 days after the infection with *M. tuberculosis* H37Rv was 4.20 and 4.34 log in lungs and spleen, respectively. The study concluded that this therapy can reduce the dosing frequency and improve patient compliance for a better management of disease. Surprisingly, almost identical pharmacokinetic profiles after pulmonary administration of lipid MPs or SLN oral administration have been obtained by the same authors. It is a matter of concern as different kinds of particles and different routes of administration are generally not supposed to provide such similar pharmacokinetics behavior. Therefore, SLN pulmonary delivery remains an open concern as no significant and reliable data support their use for TB treatment. However, the usefulness of SLN is unaltered owing to their faster degradation, rapid cellular uptake and low cytotoxicity compared with polymeric particles.

Polymeric micelles. Micelles are submicroscopic aggregates (20–80 nm) of surfactant molecules resulting in liquid colloid. Solubilization of RIF within polymeric micelles of a variety of linear and branched poly(ethylene oxide)–poly(propylene oxide) (PEO–PPO) with a broad spectrum of compositions showed a minimal solubilization effect. The incorporation of the drug was strongly limited by the size of the micellar core. The solubilization extent was increased from 5- to 7-fold with other amphiphilic block copolymers synthesized by linking mono- and bi-functional poly(ethylene glycol) (PEG) precursors of different molecular weights with poly(ε-caprolactone) (PCL) enabled the fine tuning of the hydrophile-lipophile balance (HLB) and the enlargement of the micellar core [55].

Thermo-responsive poly(ε-caprolactone-coglycolide)–poly(ethylene glycol)-poly(ε-caprolactone-co-glycolide) (P(CL-GA)–PEG-P(CL-GA)) smart block copolymers were synthesized displaying micelle-forming and gelation properties [56]. They can be used

for development of drug depot system. RIF sustained release was obtained over 32 days from 25% gel matrix. To reduce the drug resistance, INH lipid derivatives were designed by Jin and co-workers [57]. Flexible medium-long tails formed self assembling nano-sized vesicles whereas short lipid tail-derivatives resulted in weak hydrophobic interactions. Lipid vesicles showed increased penetration of the drug into the pathogen leading to promising antibacterial activity.

Various carriers for active targeting for the treatment of TB

Liposomes. Lung-specific Stealth® liposomes containing o-stereoylamylopectin (O-SAP) and monosialogangliosides/distearoylphosphatidylethanolamine-poly(ethylene glycol) (DSPE-PEG) as targeting moiety, for the targeted delivery of INH and RIF exhibited controlled release and reduced toxicity *in vivo* in mice infected with *M. tuberculosis* [58]. The cytotoxicity of the drug-loaded nanocarriers in peritoneal macrophages showed a significant decrease in the toxic effects as compared with free drugs. Furthermore, statistically significant decrease in the hepatoxic activity of the anti-TB agents was observed upon encapsulation in liposomes. Within 30 min the accumulation of nanocarriers in the lungs was 31% with PEGylated systems containing O-SAP as compared with 5.1% with conventional liposomes. Moreover, when both healthy and infected animals were pretreated with conventional liposomes (1 hour before the administration of modified liposomes), RES was saturated and the uptake levels in the lungs rose to approximately 40% within 30 min. By contrast, modified liposomes showed reduced (30–50%) uptake and accumulation in the liver and spleen. The therapeutic activity of free and encapsulated INH and RIF was evaluated in both therapeutic and sub-therapeutic dose and significant decrease in CFU was found for liposome encapsulated drugs in both the cases.

Nanoparticles

Lectins have been shown to improve mucoadhesion of the drug due to the biorecognition of the lectin-grafted carriers by glycosylated structures in the intestine and lung mucosa. To improve the intestinal mucoadhesion of the nanocarriers and the drug absorption and bioavailability, PLG NPs were surface-grafted with lectins [59]. Oral administration of wheat germ agglutinin-coated PLG NPs loaded with RIF, INH, and PZA in mice produced considerably extended serum half-life: detectable RIF serum levels were observed for 6–7 days and INH and PZA for 13–14 days (versus 4–6 days and 8–9 days for plain NPs). In addition, total bacterial clearance was achieved in lungs, liver and spleen after only three doses (once every 14 days; versus 45 daily doses of free drugs). Lectins enhance prolonged adhesion of the particles to the intestinal surface to enable an increase in the time interval available for absorption and a localized increase in the concentration gradient between luminal and serosal sides of the membrane. Authors suggested that the above factor might be responsible for the prolonged circulation of drugs encapsulated in wheat germ agglutinin-grafted NPs.

Saraogi *et al*. prepared mannosylated gelatin NPs for the selective delivery of INH to alveolar macrophages and concluded that gelatin NPs can be explored as a potential carrier for safer and efficient management of TB through targeted delivery [60].

Dendrimers

Dendrimers are macromolecules displaying well defined, regularly hyperbranched and 3D architecture, relatively low molecular weight polydispersity and high and adjustable functionality. Owing to this unique structure, these molecules represent attractive candidates for the encapsulation and delivery of anti-TB agents for diverse administration routes, although only a few research works have been reported. Kumar *et al*. developed mannosylated fifth generation (5G) polypropyleneimine (PPI) dendrimeric nanocarriers for delivery of RIF to macrophages [61]. RIF encapsulation extents were approximately 37% with hydrophobic interactions and hydrogen bonding contributing to the physical binding of the drug to the core. The mannose on the surface significantly reduced the hemolytic toxicity of the nanocarriers. RIF-containing dendrimers reduced hemolytic effect of free RIF from 9.8 to 6.5%. As opposed to the fast delivery found with regular dendrimers, modified dendrimers sustained the release for approximately 120 hours. The phagocytic uptake of RIF and RIF-loaded dendrimers in alveolar macrophages harvested from rat lungs showed a clear increase in the intracellular concentration of the antibiotic.

Local delivery and targeting for the treatment of TB

Although systemically administered nanocarriers to target the lungs have been intended, this strategy appears to be less preferable owing to its invasiveness and the potentially harmful systemic exposure to the antibacterial agents. Because most of the TB manifestations are observed mainly in the lungs, direct delivery of the drugs has emerged as one of the most attractive administration routes to target the main site of infection (alveolar macrophages), in addition to reducing systemic adverse effects (Fig. 3). Thus, carriers that were previously investigated with the aim of optimizing different technological aspects of anti-TB drugs are currently being explored for the targeted delivery to the lungs.

Incorporation of drugs into particles offers improved stability and protection of the molecule of interest against, for example, enzymatic degradation, sustained drug action and release, enhancing bioavailability of poorly soluble therapeutics and drug

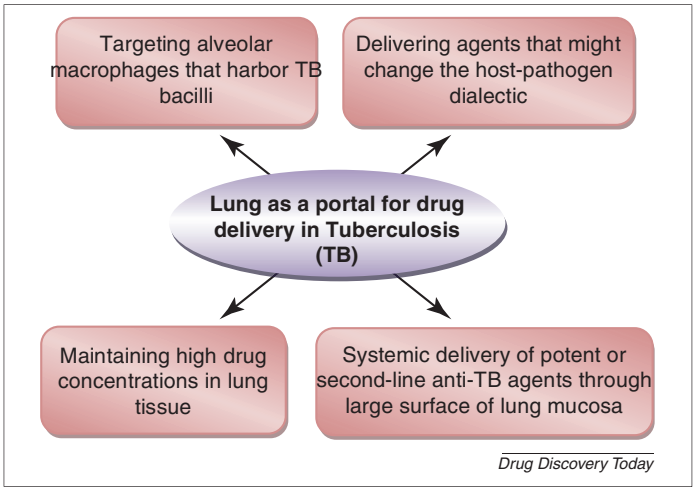

FIGURE 3

Various objectives behind selecting lungs as a portal for drug delivery in tuberculosis.

targeting. Also, the small size of NPs (≤200 nm) enables them to escape both phagocytic and mucociliary clearance mechanisms in the lung. The pharmacokinetics and antibacterial effect of the NPs bound anti-TB drugs administered through the respiratory route was investigated in guinea pigs [62]. Nebulization of drug-loaded NPs resulted in plasma-detectable concentrations after 6 hours and therapeutic drug concentrations were detected until day 6 for RIF and day 8 for both INH and PZA. Pharmacokinetic experiments indicated that the C_{max} of encapsulated RIF and PZA administered by nebulization were similar to those attained by the oral route with free drugs although the time to the peak concentration was longer for the NPs. Biodistribution data revealed that as opposed to the free drugs that were undetectable after 24 hours, encapsulated drugs were detected in the lungs until day 11. In nebulization of NPs to *M. tuberculosis*-infected guinea pigs at every 10th day, no tubercle bacilli could be detected in the lungs after only five doses of treatment, whereas 46 daily doses of orally administered drug were required to obtain an equivalent therapeutic benefit.

Ohashi *et al.* produced RIF loaded biodegradable PLGA that were incorporated into mannitol microspheres in one single step by means of a four-fluid nozzle spray drier [63]. The composite particles possess the advantages of NPs without their drawbacks (e.g. poor aerosolization). Encapsulation of the NPs in mannitol improved the *in vivo* uptake of the drug by alveolar macrophages in rat lungs as compared to RIF-containing PLGA and mannitol microspheres. Studies by O'Hara and Hickey demonstrated significantly higher efficacy of RIF in PLGA MPs [64]. When compared with free drug, the MPs led to a significant reduction of spleen inflammation when administered in infected guinea pig [65]. Daily doses of RIF solutions over 10 or 20 days had a positive effect on pulmonary and splenic inflammation but not on the number of viable bacteria in the lungs, whereas a single administration of particles or 20 days of dosing with free RIF equally decreased the bacteria population in the spleen. Besides, PLGA MPs increased drug residence time in the lungs. Results indicated that direct delivery to the lungs indeed results in high local concentrations and reduced bacterial burden compared with the same treatments delivered through other routes, offering the possibility of reduced doses and systemic side effects.

Nanocarriers bearing surface modifications to specifically target alveolar macrophages following pulmonary and other administration routes have been investigated (i.e. sugars such as mannose that are recognized by lectin receptors sited on the surface of macrophages). Vyas *et al.* developed RIF-containing aerosolized micrometric liposomes to target the alveolar macrophages [66]. MBSA and O-SAP were anchored on the surface of the nanocarriers with the intention to improve the selectivity for the lung; the former is recognized by macrophage scavenger receptors and the latter shows affinity for alveolar macrophages. A significant decrease in bacterial viability from 45.7 to 7–11% for the ligand-modified liposomes and the free drug was found. Unmodified liposomes showed intermediate activity with 31.6% viability, whereas controls showed 69.5% viability. O-SAP-coated liposomes were more effective than MBSA, likely owing to better accumulation in alveolar macrophages. Biodistribution showed that all the liposomes, independently of the modification, led to higher concentrations in the lungs and lower concentrations in the plasma when compared with the free drug. Therefore, aerosol administration of ligand-tagged liposomes showed preferential accumulation in the lungs with a high detectable drug concentration even after 24 hours.

As discussed above there are numerous papers available in the literature on formulating inhaled therapies for TB (Table 2), but no antitubercular inhalable formulation is yet available in clinics. The reason for this delay might reside in the complexity of engineering drugs in respirable formulations, using safe and accepted excipients and developing scalable processes.

Concluding remarks

It is well established that TB is a curable infection and most of the negative therapeutic outcomes are related to poor patient compliance. New drug delivery approaches offer a solution to this problem. As discussed throughout this article an increasing number of studies support the use of drug delivery vehicles to treat TB. Several carrier-based drug delivery systems incorporating anti-TB agents have been fabricated that either reduce the dosing frequency or target the site of infection. These developments in drug delivery represent attractive options with significant merit, such as improved drug bioavailability and reduction of the dosing frequency, feasibility of the versatile routes of drug administration, long term stability and thus, might serve as basis for better management of the disease, thereby making the treatment more practical and affordable.

Micrometric or nanometric carriers, together with alternative routes of administration have shown advantages over conventional formulations but there are also some issues to be considered. In fact, formulation and large-scale production of these systems are costly, and their use, other than oral, is not convenient for patients, especially in developing countries where economic and social dire straits have a determinant role in the failure of TB therapies. As pulmonary drug delivery is becoming more and more important, nanotoxicological aspects of inhaled drug delivery systems have to be considered and *in vitro* methods must be established to ensure safety. A serious shortage of data regarding the biological safety of the used excipients in healthy lungs and, more importantly, in diseased organs, does not afford a conclusive opinion on the realistic application of inhaled therapy against TB.

The success of any approach for drug delivery to this intracellular pathogen depends on the ability to construct a biocompatible carrier with a high loading capacity of therapeutic drugs with no or minimum premature release of the cargo before reaching the replicative niche of the bacteria. It can be expected that future research will concentrate on the development of the vectorized delivery systems combining advantages of the colloidal carriers, such as large payloads of a drug, with active targeting to the infection sites. Thus, by establishing clinical efficacy, affordability, accessibility, and acceptance for new technologies, these therapeutic systems can be better translated for use on a global scale.

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