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EXPERT REVIEW

Nanotechnology in Tuberculosis: State of the Art and the Challenges Ahead

Estefania Grotz [1,2] • Nancy Tateosian [2,3] • Nicolas Amiano [2,3] • Maximiliano Cagel [1,2] • Ezequiel Bernabeu [1,2] • Diego A. Chiappetta [1,2] • Marcela A. Moretton [1,2]

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ABSTRACT Tuberculosis (TB) remains as the second most-deadly infection right behind the HIV/AIDS. Actually, in 2016, TB incidence was estimated in 10.4 million cases. Although an efficient and low-cost TB pharmacotherapy has been available for the last 50 years, the development of multi- and extra-drug-resistant *Mycobacterium tuberculosis* (Mtb) strains has put on the spot the necessity of improved TB regimens. In this framework, this review article presents the main relevant research outcomes of nanotechnology in TB. The novel delivery systems for antituberculosis drugs have been discussed. Moreover, the active-targeted nanomedicines to the Mtb reservoirs enlighten the possibility to eradicate low-replicant mycobacteria and diminish latent TB. Finally, we present an overview of the TB socio-economic impact and the cost-related features of TB regimens associated with the use of nanoformulations.

KEY WORDS active targeting · nanotechnology · respirable nanocarriers · tuberculosis

✉ Marcela A. Moretton
marcelamoretton@gmail.com

[1] Facultad de Farmacia y Bioquímica, Cátedra de Tecnología Farmacéutica I, Universidad de Buenos Aires, Buenos Aires, Argentina

[2] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

[3] Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Consejo Nacional de Investigaciones Científicas y Técnicas, Instituto de Química Biológica de la Facultad de Ciencias Exactas y Naturales (IQUIBICEN),, Ciudad Universitaria, Buenos Aires, Argentina

ABBREVIATIONS

AMs	Alveolar macrophages
FDCs	Fixed dose combinations
ETB	Ethambutol
INH	Isoniazid
LPs	Liposomes
MDR-TB	Multi-drug resistant tuberculosis
Mtb	*Mycobacterium tuberculosis*
NMs	Niosomes
NPs	Nanoparticles
PMs	Polymeric micelles
PYR	Pyrazinamide
RIF	Rifampicin
TB	Tuberculosis
XDR-TB	Extra-drug resistant tuberculosis

INTRODUCTION

Tuberculosis (TB) represents a major global health problem and it ranks alongside the human immunodeficiency virus (HIV) as the primary cause of death worldwide (1). According to the last World Health Organization (WHO) statistics, there was an estimated of 10.4 million new cases in 2016 (1). TB is a highly infectious chronic disease caused by *Mycobacterium tuberculosis* (Mtb) where an estimated one third of the world population is latently infected with Mtb and at risk of disease reactivation (2).

Due to its high incidence, morbidity, and mortality in the last decades, TB represents a challenge for public health, where the sanitary emergency stands since 1993 (3). Although TB mortality has fallen 47% since 1990, with nearly all of that improvement taking place since 2000, TB remains one of the world's biggest public health threats (1). It is a chronic disabling disease that can last for years in a latent stage, which impedes its follow up and eradication. Worldwide TB cases distribution is not homogenous and

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its highest incidence lies in developing countries. TB is also known as an "opportunistic" disease in immunosuppressed patients (4).

From 2016, the goal is to end the global TB epidemic by implementing the End TB Strategy. Adopted by the World Health Assembly in May 2014 and with targets linked to the newly adopted "*Sustainable Development Goals*", the strategy serves as a blueprint for countries to reduce the number of TB deaths by 90% by 2030 (compared with 2015 levels) (1). Nowadays, standard short-term treatment (6 months) comprises the combined oral administration of different antituberculosis (anti-TB) drugs as: rifampicin (RIF), isoniazid (INH), pyrazinamide (PYR), ethambutol (ETB) and streptomycin (STM). These are known as "first-line drugs" (1). Given the complexity of the therapeutics, one of the strategies proposed by the WHO and the International Union against TB and Pulmonary diseases, is the combination of at least two "first line" drugs in one dosage form. These are commonly known as *Fixed Dose Combinations* (FDC) (5,6). Thus, prescription mistakes can be minimized and the adherence to the treatment can be improved (5) However, in 2016, an estimated 490.000 people all over the globe developed multidrug resistant TB (MDR-TB) (1). The two reasons why multidrug resistance continues to emerge and spread are mismanagement of TB treatment and person-to-person transmission. Particularly, drug resistance could develop due to the inappropriate use of anti-TB drugs (single drug regimen and poor quality medicines) and premature treatment interruption.

In this framework, although the anti-TB pharmacotherapy has been available for a long time, TB still remains to be one of the main preventable causes of death by an infectious disease. Hence, it is important to develop new drug delivery systems that ensure high treatment adherence, low adverse effects and that are adequate for both, adults and children.

Thereafter, the development of novel drug-loaded nanotechnological platforms provides promising avenues to improve TB pharmacotherapy. In fact, many efforts have been directed to the assessment of "first line" anti-TB drug delivery systems, since these are the most effective and low-cost anti-TB drugs. Then, these nano-sized carriers could be extremely useful to overcome the main (bio)limitations of the anti-TB drugs such as low-aqueous solubility, chemical stability, and oral bioavailability. Also, alternative administration routes to the oral pharmacotherapy were explored as an attempt to optimize TB regimens, avoiding treatment failure and the development of drug-resistant Mtb strains.

In this context, the present review overviews the background of TB pathogenesis and pharmacology. Besides, the nanotechnological platforms evaluated for the development of novel anti-TB drug delivery systems are discussed. Finally, the socio-economic impact of the disease, the cost-related features of TB regimens and the potential benefits associated with the use of nanocarriers are explored.

TUBERCULOSIS PATHOGENESIS

Mtb is one of the most successful human pathogens, due to its ability to carry a primary infection to a state of dormancy, persisting in the body even in immune-competent people. In this regard, it is important to mention that there are two billion people infected worldwide and only nine million develop the disease annually (7). The presence of hereditary or acquired deficiencies of the immune system markedly increases the risk of progression to active TB (8). TB is usually a lung disease, due to the fact that these organs are the gateway and provide optimal conditions for the outbreak of the disease (9). The primary infection begins with the inhalation of the particles of Mtb, as it is schematized in Fig. 1. Although most of the inoculum is eliminated in the upper respiratory tract (10), approximately 10% of this invades the alveoli and bronchioles, where the bacteria is recognized and phagocytosed by alveolar macrophages (AMs) or dendritic cells (DCs) (11). Once inside the cells, the intracellular growth of Mtb will depend on the evasion capacity of the bacteria and the microbicidal mechanisms of the macrophages. Macrophages exposed to Mtb secrete pro-inflammatory cytokines (IL-1, TNF-α and IL-6) that will contribute to the subsequent formation of focal granulomatous lesions, a process that takes 2–3 weeks, and which generally leads to containment of the pathogen (11,12). Then, the clinical progression will be ruled by a combination of factors, including the competence of the immune system, the vaccinating state, the nutritional state and the patient's age. At a later stage, immuno-competent individuals will have the ability to form granulomas within the lung, where infected macrophages will remain contained, while fibrosis occurs at the peripheral level of the granuloma (9,13) (Fig. 1). Thus, these individuals will remain infected and act as carriers of the bacillus, without showing signs of infection (latent tuberculosis) (14). After 4–5 weeks of progressive infection, the microscopic granulomas increase in size and fuse with other granulomas, causing large necrotic areas surrounded by layers of epitheloid histiocytes, giant multinucleated cells, fibroblasts, lymphocytes and monocytes. Despite acidic pH, low oxygen concentration and the presence of toxic fatty acids, Mtb can remain viable for decades. The infection can be maintained at this stage or progress, according to cell-mediated immunity. In some cases, the granuloma will resolve, leaving small calcified fibrous lesions (11). It is important to point out that this bacillus is able to survive inside macrophages, as its cellular components inhibit the fusion of the phagocytic vacuoles, formed after its endocytosis with the lysosomes. Hence, the lysosome capacity for bacterial destruction is diminished (15).

A secondary acute infection is the last stage of this disease, which may be reached by two different ways: the additional inhalation of Mtb or the reactivation of the latent bacillus (11). The mechanism responsible for the reactivation is unknown, but it is clearly associated with factors of the patient's immune

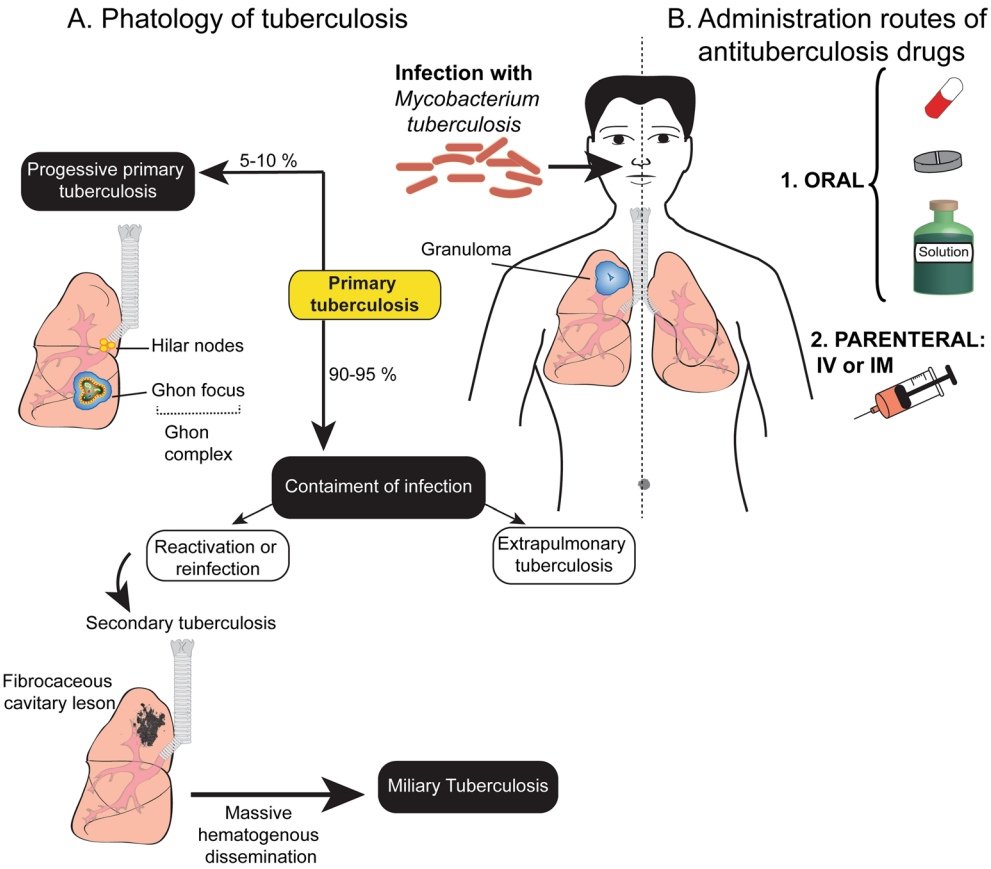

Fig. 1 (**a**) Pathology of TB infection in humans. (**b**) Administration routes for anti-TB drugs.

system. In these cases, infected macrophages escape the granuloma causing dissemination to the regional lymph node. If the cell-mediated immunity is inadequate, the delayed hypersensitivity response will try to eliminate the bacilli that multiply. However, at the same time, it will cause the destruction of the lung tissue, leading to the formation of cavities. The reactivation that progresses to the formation of cavities favors the spread of resistant and virulent Mtb strains (11). Phagocytic cells and DCs have a fundamental role in the initiation and targeting of T cell-mediated adaptive immunity. This is achieved through the presentation of antigens from mycobacteria and the expression of co-stimulatory signals and cytokines. The adaptive immune response in TB can be defined as the activation of T helper lymphocytes type1 (LTh1) response. The production of IFNγ by $TCD4^{+}$ cells is critical for the control of the disease (16,17). Although there is a strong humoral response during TB, the role of B cells is not well defined.

The process of presenting antigens is an important step in the transition from the innate immune response to the adaptive immune response, which is based on the specific recognition of antigens by different cell types. These cells are then activated and produce soluble factors, such as cytokines and chemokines. Between 6 to 8 weeks after the primo infection, it reaches the regional lymph nodes, where a recognition and ulterior activation of LTh occurs. The subsequent cytokine secretion, such as interleukin 12 (IL-12), IFN-γ and tumor necrosis factor α (TNF-α), induces the activation of macrophages and LT (18). Hence, these cytokines do not only stimulate the phagocytosis and the intracellular bacilli degradation, but also the localized inflammation and the ulterior cytotoxic LT activation, which will produce the death of infected cells. (19).

TUBERCULOSIS "FIRST LINE" PHARMACOTHERAPY

RIF, INH, PYR and ETB are combined to avoid the development resistant strains, both in adults and children (5,20). INH (class I drug, MW: 137.14 g/mol) is a bactericide antibiotic and it is highly effective against the active replicating forms of the bacillus, being recommended along the complete "short-term" regimen for pulmonary susceptible TB (Figs. 2 and 3) (21,22).

Secondly, RIF (borderline Class II drug, 822.95 g/mol) is a semi-synthetic antibiotic and one of the most powerful and effective drugs used in the treatment of TB since 1968 (23) (Figs. 2 and 3). RIF exhibits low pH-dependent aqueous solubility (2.56 mg/mL 25°C, pH: 5.0) and, in acidic media (gastric conditions), it undergoes degradation (24). What is

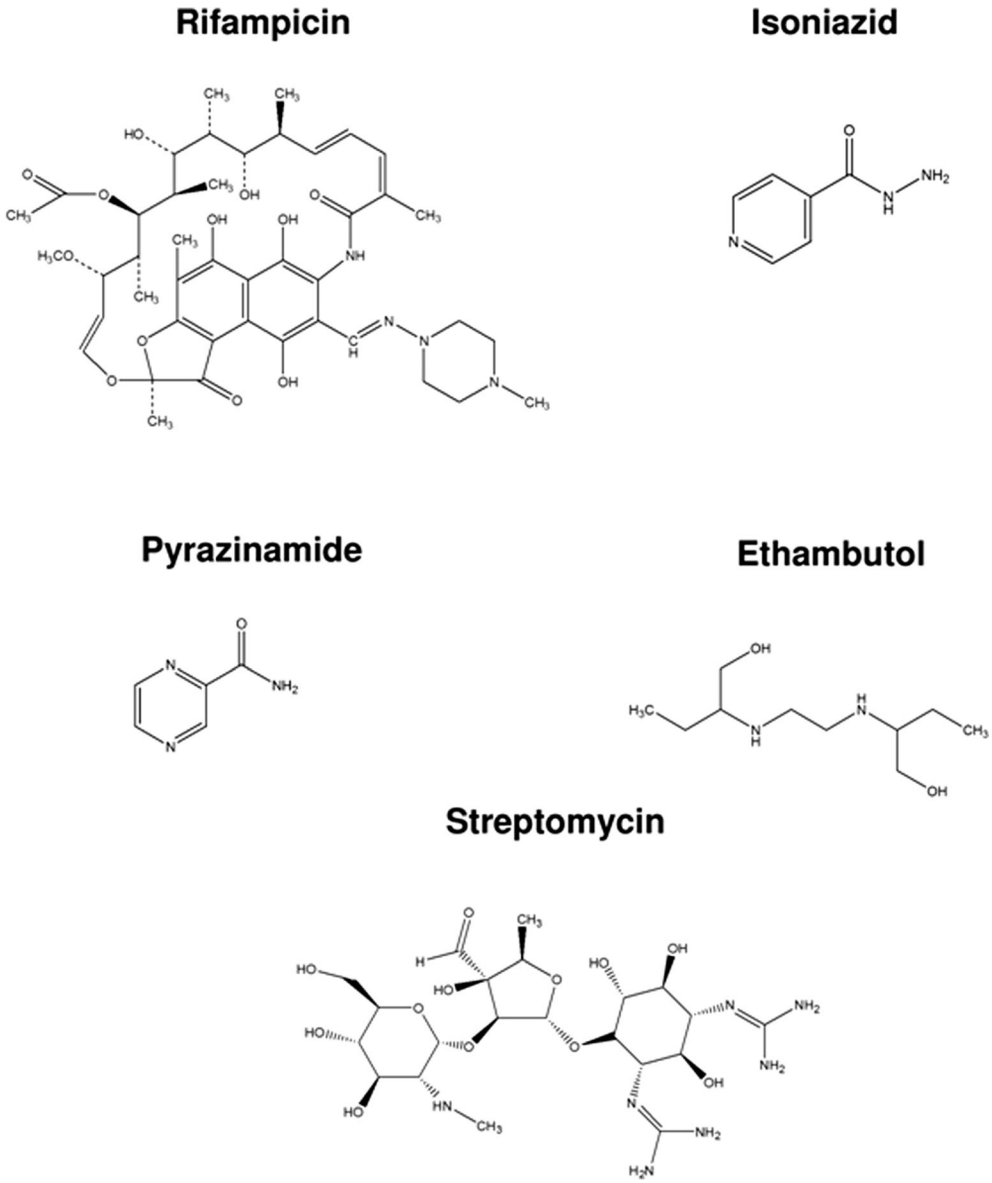

Fig. 2 Chemical structures of the "first-line" anti-TB drugs.

more, the presence of INH increases RIF degradation in acid media. In this context, the WHO has already expressed its concern about the lowered oral bioavailability of RIF in the fixed dose combination (FDC) of RIF/INH that is currently being used (25).

On the other hand, PYR (Class I drug, MW: 123.11 g/mol) is a synthetic pyrazinoic acid amide derivative with anti TB properties (Figs. 2 and 3). Interestingly, PYR plays a unique role in shortening the therapy from a period of 9–12 months to 6 months (26). RIF and PYR are crucial for the eradication of the slow replicating forms or the latent forms of the bacillus (27,28).

In the case of ETB (Class III drug, MW: 204.3 g/mol), it is used in combination with the other first line drugs in order to avoid the development of bacterial resistance (5) (Figs. 2 and 3).

Although nowadays the "first line" anti-TB drugs are the four mentioned before, streptomycin (STR, MW: 581.6 g/mol) was formerly considered to be one of them as well and, under particular circumstances, it is still used during the initial treatment. STR is a bactericidal aminoglycoside natural antibiotic obtained from *Streptomyces griseus* (Figs. 2 and 3).

It is worth mentioning that in the case of "new" (naïve) patients, the therapeutic regimen recommended by the WHO for susceptible pulmonary TB consists of a short-term treatment of 6 months (divided in two stages) (5). Drug combinations are orally administered daily or three times a week. Given the complexity of the treatment, FDCs with "first-line" drugs were developed as an attempt to simplify anti-TB therapy. Although there is no evidence that FDCs are superior to individual drugs, expert opinion suggests that these may decrease the frequency of acquired drug resistance (5). In

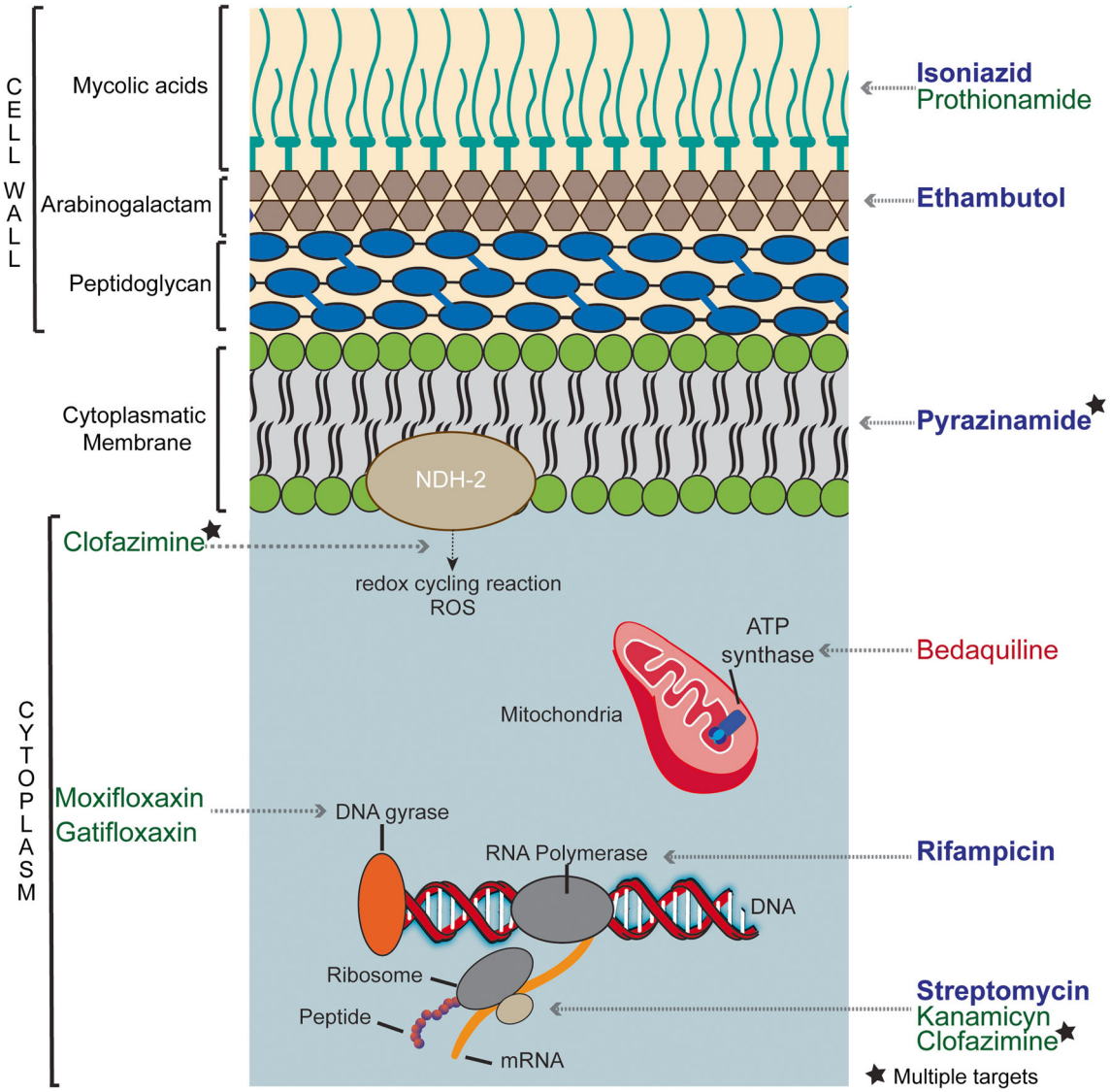

Fig. 3 Action mechanisms of anti-TB drugs. Blue font: "first-line" drugs; Green font: "second-line" drugs for MDR-TB short-therapy; Red font: Recently approved anti-TB drug for MDR-TB therapy.

addition, the most recent WHO update of the TB guidelines, recommends drug-susceptibility test for those patients who require re-treatment for TB, in order to verify the most suitable drug regimen for TB therapy and efficiently identify drug-resistant Mtb strains (1).

TUBERCULOSIS "SECOND LINE" PHARMACOTHERAPY

About 3.7% of new TB patients in the world become ill with MDR Mtb strains. Levels are much higher (~20%) in those patients previously treated with anti-TB drugs. Organisms that are resistant to the most effective "first-line" anti-TB drugs (INH and RIF) cause MDR-TB. On the other hand, extensively drug-resistant TB (XDR-TB) is a form of TB caused by organisms that are resistant to INH and RIF, as well as to any fluoroquinolone or "second-line" anti-TB injectable drug. The development of resistant Mtb strains gave rise to the need for the implementation of protocols with the so-called "second-line" drugs: amikacin (AMK), kanamycin (KNM) or capreomycin (CPM), rifabutin (RFB), rifapentine, aminosalicylic acid, ethionamide (ETM), protionamide (PTM), cycloserine, terizidone, ciprofloxacin (CPX), ofloxacin, levofloxacin (LVX) and moxifloxacin (MOX) (29).

Drug resistance emerges, because of inadequate treatment and, once TB organisms acquire resistance, they can spread from person to person in the same way as drug-suceptible TB. Besides, it is important to point out that MDR-TB results from either infection with organisms which are already drug-resistant or may develop in the course of a patient's treatment. MDR-TB does not respond to the standard 6-month

treatment with "first-line" anti-TB drugs and can take 2 years or more to treat with drugs that are less potent, more toxic and much more expensive (Table I) (30–32).

The recommended regimen by WHO in MDR-TB cases is based in the STREAM trial. It is a clinical study that compares the outcomes of a 9-month regimen *versus* a longer treatment in MDR-TB patients. The first stage of the enrolment was completed in mid-2015 (424 patients in 4 countries) and the results are expected to be reported in 2018. The regimen contains an initial 4-month phase of KNM, MOX, PTM, clofazimine (CFM), INH, PYR and ETH given together, followed by 5 months of treatment with MOX, CFM, PYR and ETH (32).

In spite of the proposed regimen, treating MDR-TB and XDR-TB is extremely difficult, as the treatment is long and expensive and the outcomes remain suboptimal, due to the frequent observed adverse effects and high rates of treatment failure.

The WHO has recently issued new recommendations on how to design MDR-TB regimens, moving from the previous stepwise approach based on five groups of drugs in priority order (33,34) to a new approach and a new drug classification (32,35). The new drugs delamanid (DLM) and bedaquiline (BDQ) are currently classified in group D2 (32). Therein, the WHO has approved the use of DLM for children over 6 years of age, based on recent pharmacokinetic data (36,37). Evidence in adults shows that DLM and BDQ increased sputum smear and culture conversions, as well as success rates at the end of treatment, although further studies are needed about potential drug-drug interactions related cardiotoxicity. (32,35,38). Despite the fact that some repurposed drugs are still useful as part of these regimens (e.g., linezolid and carbapenems), there is urgent need to know whether the new anti-TB drugs are better than the former (39,40). In pediatric population, the problem is even more pressing, as less information is available and recruitment of pediatric patients in clinical trials is usually more challenging.

NANOTECHNOLOGICAL APPROACHES FOR TB THERAPY EMPLOYING "FIRST-LINE" ANTI-TB DRUGS

In general terms, nanotechnology can thoroughly investigate and exploit novel factors and characteristics of materials in order to develop new strategies and delivery systems for diagnosis and treatment of diseases, as well as to enhance the properties of previously manufactured systems. Interestingly, by combining and mixing the most recent knowledge of nanomaterials with the understanding of different biological processes, nanotechnology could ameliorate and trigger the usage of brand new drug/antigen delivery systems.

Table I First-Line and Second-Line Drugs used for the Treatment of Drug-Resistant TB (WHO Classification) (31, 32, 119)

Group	Class	Drugs	Mechanism of action
A	Fluoroquinolones	Levofloxacin Moxifloxacin	Inhibition of DNA gyrase
		Gatifloxacin	
B (second-line injectable anti-TB drugs)	Aminoglycosides	Kanamycin Amikacin	Inhibition of protein synthesis
		Capreomycin	
		Streptomycin‡	
C (core second-line agents)	Thioamides	Ethionamide Prothionamide	Inhibition of cell wall synthesis
	Oxazolidinones	Cycloserine Terizidone	Inhibition of protein synthesis
		Linezolid	
		Clofazimine	
D (add-on agents)+	D1	High-dose isoniazide	Inhibition of mycolic acid synthesis
		Pyrazinamide	Disruption of plasma membranes
		Ethambutol	Inhibition of cell wall synthesis
	D2	Bedaquiline	Inhibition of mitochondrial ATP synthase
		Delamanid	Inhibition of mycolic acid synthesis
	D3	Para-aminosalicylic acid	Inhibition of DNA precursor synthesis
		Imipenem plus cilastatin or meropenem plus clavulanate	Inhibition of peptidoglycan synthesis
		Thiocetazone	Inhibition of mycolic acid synthesis

+ Various classes. ‡ Streptomycin can be used when the isolate is susceptible and none of the other injectable drugs are available

Specifically, nanotechnology represents a versatile alternative, in which different approaches have been studied to assess different types of nanocarriers, based on biomaterials with different administration routes (Table II; Fig. 4).

Liposomes

Liposomes (LPs) represent well-investigated platforms for drug delivery. They are spherical lipid carriers with an aqueous core surrounded by one or more bi-layered membrane structures, composed by natural or synthetic amphiphilic lipid compounds (41). Depending on their size and number of bilayers, LPs can be classified into three categories: multilamellar vesicles, large unilamellar vesicles, and small unilamellar vesicles (42). Interestingly, LPs can be effective against intracellular pathogens, like Mtb, as the macrophage uptake may result in the release of the load inside the cell (41). Nevertheless, they also present some drawbacks mainly related with their physical stability and loading capacity, in comparison with other nanocarriers, as the nanoparticles (NPs) (43).

In the past few years, many efforts have been directed to the development of LPs-based nanomedicines for TB therapy. Particularly, the respiratory administration route of these nanocarriers has been explored. This is the case of the freeze-dried powders for inhalation, studied by Changsan *et al.* RIF-loaded LPs were freeze-dried employing different cryoprotectant agents, such as lactose, trehalose and mannitol. However, the dried LPs with mannitol showed the most adequate aerodynamic properties, represented by the mass median aerodynamic diameter (3.4 μm) and fine particle fraction (66.8%) (Andersen Cascade Impactor). Therefore, the inhalation powder with RIF could reach the distal respiratory tract, representing a feasible platform for RIF respiratory delivery (44).

In a similar way, Manca *et al.* also aimed to develop inhalable LPs loaded with RIF. In contrast with Changsan *et al.*, the authors explored the lipid composition and its concentration, in order to improve the physicochemical properties and the aerosol performance of the nanocarriers. Data revealed that the addition of cholesterol to the phospholipid mixtures facilitated the development of LPs with better *in vitro* cell drug internalization properties and low drug toxicity in human A549 alveolar cells (45).

Furthermore, INH can also be entrapped into inhalable LPs. Particularly, Chimote *et al.* developed inhalable INH-entrapped multilamellar LPs, employing the natural lung surfactant known as dipalmitoylphosphatidylcholine with a mean particle diameter of 755 nm. A sustained release of INH was observed over 24 h, together with enhanced *in vitro* alveolar deposition efficiency *versus* free INH. These results combined with the LPs adequate *in vitro* cytocompatibility and haemocompatibility, make these nanocarriers a promising platform for the development of INH-respirable delivery systems (46).

Other approaches were based on PYR delivery for subcutaneous administration instead of respiratory drug delivery. This is the case of the neutral and negatively charged PYR-loaded LPs developed by El-Ridy *et al.* (26). It is worth stressing that the authors evaluated the biological efficacy of the drug-loaded LPs in an infection model in mice, employing Mtb. Data revealed that only 7 doses (25 mg/kg, twice a week) had the same biological effect as 18 doses of free PYR (25 mg/kg, 6 days per week). These results demonstrated the potential of LPs as PYR delivery system for the management of pulmonary TB.

Overall, developments based in LPs for TB therapy have been focused in inhalable nanocarriers. Without any doubt, respiratory administration on anti-TB drugs has been on the spot in the last decade as an alternative administration route. Further *in vivo* assays of inhalable powders or liquid dispersions in infected Mtb models are still required, in order to assess the therapeutic potential of these novel respirable nanocarriers.

Nanoparticles

Nanoparticles (NPs) were developed after LPs, in order to improve their physical stability and drug pay-load (43). Specifically, NPs are known as solid particles with a size in the range of 10–1000 nm. Their physico-chemical properties, like size, surface charge, hydrophobicity and composition contribute to their drug-load capacity, biodistribution, cellular uptake and immunogenicity (47). Particularly, a drug can be dissolved, entrapped, encapsulated or attached to a NP matrix (48). NPs can be classified in different groups according to their composition. For instance, polymeric NPs can be made from natural or synthetic polymers, where the latter can also be biodegradable and biocompatible. Further, solid lipid NPs (SLNPs) are sometimes considered as 'hybrid' nano-carriers between polymeric NPs and LPs. They consist of a solid lipid core stabilized by surrounding surfactants. This system presents better stability and drug loading capacity than LPs (43). Nowadays, different nanoformulations, like lipid-based and (branched) polymeric ones, are being explored to deliver different types of drugs.

In recent years, many efforts have been directed to the encapsulation of anti-TB drugs within NPs. In the first place, RIF oral delivery within SLNPs was studied by D. Pooja *et al.* Surface decorated SLNPs with wheat germ exhibited high *in vitro* binding efficiency (93.06%) to the intestine mucus glycoproteins and a sustained RIF release (76.9% over 120 h) (49). Apart from this, other investigations were focused on the prevention of RIF degradation in acid medium (pH = 1.2) for an oral administration. Besides, the authors evaluated the drug chemical stability in the presence of INH, taking into account the multi-drug regimen for TB. The *in vitro*

Table II Nanotechnological Approaches Employing First-Line Anti-TB Drugs

Nanocarrier	First-line anti-TB drug	Biomaterials	Administration route	*In vitro* data	Predinical studies	References
Liposomes (LPs)	Rifampicin (RIF)	Cholesterol/ soybean L-1-phosphatidylcholine/mannitol	Respiratory	LPs (cryoprotected with mannitol) demonstrated deep lung deposition.	–	(44)
		Soy phosphatidylcholine/ hydrogenated soy phosphatidylcholine/cholesterol/ oleic acid	Respiratory	LPs with cholesterol demonstrated the best nebulization properties and highest cellular uptake (A549 cells).	–	(45)
	Isoniazide (INH)	1,2-dipalmitoylphosphatidylcholine	Respiratory	LPs showed antimycobacterial activity, cytocompatibility and haemocompatibility.	–	(46)
	Pyrazinamide (PYR)	Dipalmitoyl phosphatidyl choline/ colesterol and dicetyl phosphate	Subcutaneous	–	High therapeutic efficacy (reduction in bacterial counts) against Mtb (H37Rv) in albino mice.	(26)
Solid lipid nanoparticles (SLNPs)	Rifampicin (RIF)	Stearic acid/glyceryl monostearate conjugated with wheat germ agglutinin	Oral	High binding efficiency to porcine mucin.	–	(49)
		Tween® 80/soya lecithin/ Compritol® 888 ATO	Oral	The drug release profile from the lipid matrix denoted a triphasic behavior at pH 6.8	Enhanced RIF oral bioavailability after a single dose (50 mg/kg) in Wistar rats. Plasma RIF concentration over the MIC90 was observed during 5 days.	(51)
		Cetyl palmitate/ Tween® 80/Chitosan	Respiratory	High binding efficiency of mucin to the nanocarriers was observed. RIF permeation through A549 cell monolayers was enhanced.	–	(52)
		Compritol® ATO 888/ polysorbate 80/	Oral	RIF degradation in acid medium (pH 1.2) was reduced in presence of free INH.	–	(50)
Polymeric nanoparticles (NPs)	Isoniazide (INH)	Chitosan	Oral	Swelling properties of the NPs were observed in acid medium (pH 1.2). The ex-vivo mucoadhesive tests showed higher adherence forces as the NPs size decreased.	–	(53)
	Rifampicin (RIF)	Poly(ethyleneoxide) monomethyl ether-block-poly(ε-caprolactone)	–	RIF uptake by macrophages was enhanced after the drug incorporation within NPs. Drug-loaded NPs demonstrated adequate antimicrobial efficacy against *Mycobacterium fortuitum*.	–	(54)
	Isoniazide (INH)	Poly (lactic-co-glycolic acid)-poly(ethylene glycol) derivatives	Oral	Sustained INH release (PBS pH 7.4) from the polymeric matrix up to 124 h, depending on the composition of the nanocarrier.	Enhanced INH oral relative bioavailability *versus* free drug in Wistar rats.	(55)
	Streptomycin (STR)	Poly (lactic-co-glycolic acid)	Oral	–	Improved oral STR relative bioavailability *versus* the free drug (intramuscular). A dose reduction (from 24 intramuscular injections to 8 oral doses) of STR was achieved in an infected (Mtb $H_{37}Rv$) mice model.	(56)
	Rifampicin+ Isoniazide+ Pyrazinamide (RIF + INH + PYR)	Poly (lactic-co-glycolic acid)	Respiratory	Deep lung deposition of the NPs.	Therapeutic concentrations of the 3 drugs were found in lungs up to 11 days post-nebulization (single	(57)

Table II (continued)

Nanocarrier	First-line anti-TB drug	Biomaterials	Administration route	*In vitro* data	Predinical studies	References
					administration) in infected (Mtb $H_{37}Rv$) Dunkin Hartley guinea pigs.	
		Sodium alginate	Respiratory	Deep lung deposition of the NPs.	3 nebulized NPs doses showed the same efficacy (undetectable colony forming units in lungs and spleen) as daily administered 45 oral doses of the free drugs in infected (Mtb $H_{37}Rv$) guinea pigs.	(58)
Polymeric micelles (PMs)	Rifampicin (RIF)	Poly(ε-caprolactone)-poly(ethylene glycol) tri-block copolymers	Oral	Drug-loaded PMs were freeze-dried employing hydroxypropyl-β-cyclodextrin as cryo/lyo-protectant additive.	Improved RIF relative oral bioavailability (3.3-fold) for the drug-loaded PMs *versus* a drug syrup, in presence of INH in Wistar rats.	(70)
		Kolliphor® HS-15	Oral	The RIF self-aggregation in aqueous medium was minimized by its encapsulation within PMs.	–	(63)
		Chitosan-graft-poly(ε-caprolactone)/(ferulic acid) multi-copolymers	–	The drug release from the polymeric matrix was higher at pH 5.3 (lysosomal microenvironment) than in physiological pH values (7.4). PMs were susceptible to lysozyme biodegradation. RIF-loaded PMs were internalized by A549 cells in a time-dependent manner.	–	(64)
	Rifampicin+ Isoniazide (RIF + INH)	N(2-hydroxypropyl) methacrylamide and poly lactic acid	–	RIF-loaded/INH-conjugated PMs showed lower hemolytic toxicity than the free drugs. PMs containing both drugs were more effective against sensitive and resistant Mtb strains than the free drugs.	–	(65)
Polymersomes (PS_s)	Rifampicin (RIF)	Poly(ε-caprolactone)-poly(ethylene glycol) di- and tri-block copolymers	Respiratory	RIF-loaded PS improved the *in vitro* drug intracellular concentration, in comparison to an aqueous RIF solution in murine macrophages (RAW 264.7).	–	(68)
Niosomes (NMs)	Pyrazinamide (PYR)	Span® 60/cholesterol with the addition of dicetyl phosphate	Subcutaneous	Biphasic release behavior of PYR from the nanocarriers up to 96 h.	Lower bacterial count in lungs for the drug-loaded NMs (administered twice a week) *versus* free drug (administered 2 and 6 times per week) in infected (Mtb H37Rv) guinea pigs.	(71)
	Ethambutol (ETB)	Span® 60/cholesterol with the addition of dicetyl phosphate	Subcutaneous	Biphasic release behavior of ETB from the nanocarriers in PBS pH 7.4.	Higher ETB lung deposition in Swiss Albino mice for the drug-loaded NMs (single dose). Lower bacterial count in lungs for the ETB-loaded nanocarriers than the	(72)

Table II (continued)

Nanocarrier	First-line anti-TB drug	Biomaterials	Administration route	*In vitro* data	Predinical studies	References
					free drug in an infected (Mtb H37Rv) model.	
	Rifampicin+ Isoniazide+ Pyrazinamide (RIF + INH + PYR)	Triton® X-100/ poly(ethylene glycol) 2000/Span® 80	–	The INH and PYR release profiles from the NMs demonstrated a biphasic behavior in PBS pH 7.4. In contrast, RIF showed a sustained release over 5 h (4%).	–	(73)
		Tyloxapol®/ poly(ethylene glycol) 2000	–	INH and PYR showed a sustained release form the NMs in PBS pH 7.4.	–	(74)

degradation studies demonstrated that the drug degradation was decreased *versus* free INH (19.5%) and INH-loaded SLNPs (12%) (50).

Secondly, regarding RIF pharmacokinetic, Singh *et al.* aimed to improve RIF erratic oral bioavailability and biodistribution. For this purpose, authors developed RIF-loaded SLNPs based on Tween® 80, soya lecithin and Compritol® 888 ATO (~130 nm). This lipid matrix promoted a sustained *in vitro* RIF release (70% over 9 days, pH 6.8). Remarkably, the RIF oral bioavailability was increased (8.14 times) after the administration of the drug-loaded SLNPs *versus* free drug, where significant RIF plasma levels were observed over 5 days (single RIF dose, 50 mg/kg) (51).

In addition to lipids, polymers such as chitosan have been assayed as SLNP-former biomaterial. Chitosan exhibits unique properties, such as biocompatibility, mucoadhesiveness and chemical versatility. To study this aspect, Vieira *et al.* developed RIF-loaded chitosan-coated SLNPs based on cetyl palmitate. Results showed greater *in vitro* mucoadhesive properties with higher *in vitro* RIF permeability in the alveolar epithelial cells, when compared to uncoated SLNPs. These results indicated that developing this system may be a promising strategy to improve respiratory TB therapy (52). A similar strategy was followed by Banik *et al.* to enhance the mucoadhesion properties of INH-loaded chitosan-based NPs (53).

Another interesting approach of RIF-loaded polymeric NPs was displayed by Trousil *et al*. Specifically, the poly(ethyleneoxide) monomethyl ether-block-poly(ε-caprolactone) NPs contained a sensor (Förster resonance energy transfer system) that enables real-time assessment of drug release. Surprisingly, RIF release from the polymeric matrix was more rapid for those systems retained by cells than in the dialysis tube. Moreover, *in vitro* assays demonstrated that not only the RIF-loaded but also the blank NPs, exhibited antimicrobial efficacy against *Mycobacterium fortuitum*. The latter was probably associated with the capacity of the block-copolymer to increase the permeability of the bacteria cell wall (54). Regarding PEG-based block copolymers, other investigations explored different anti-TB drugs, apart from RIF. For instance, M. Gajendiran *et al.* confirmed an enhancement of the oral INH bioavailability *versus* a drug solution, employing low-molecular weight poly (lactic-co-glycolic acid)-PEG derivatives (55). Moreover, Pandey *et al.* prepared NPs loaded with STR by a multiple emulsion technique to administer orally this injectable antibiotic. The authors demonstrated a feasible reduction of the administration frequency of this drug, upon its encapsulation within NPs. These results could be relevant to simplify the TB pharmacotherapy, especially for HIV-positive patients (56).

Finally, NPs have also been explored for the co-encapsulation of anti-TB drugs. Nanocarriers loaded with RIF, INH and PYR were developed for respiratory delivery.

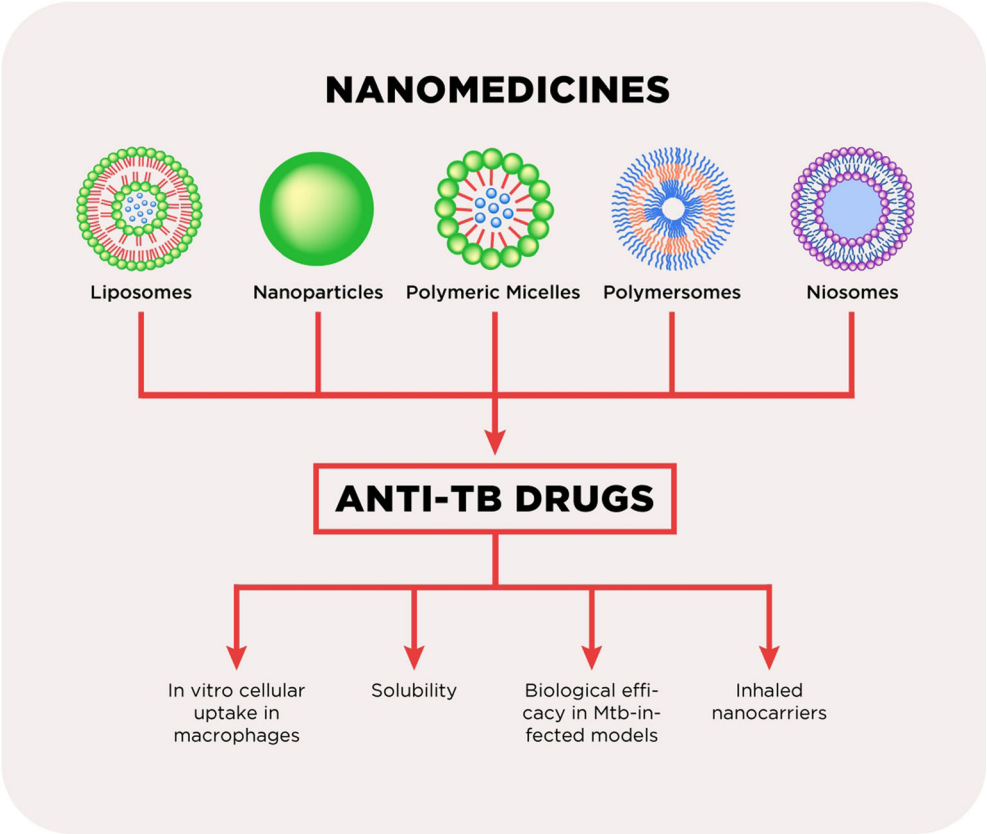

Fig. 4 Diagram of the different nanocarriers explored for anti-TB therapy.

Data demonstrated that the inhalable nanotechnological platform allowed improving the chemotherapeutic regimen in Mtb-infected guinea pig models (57). Similarly, alginate-based NPs were investigated to encapsulate these anti-TB drugs. For instance, 3 nebulized doses of the NP formulation (every 15 days) have the same efficacy to achieve undetectable colony forming units as daily administered 45 oral doses of the free drugs (58).

In contrast to LPs, NPs have been mainly explored for their oral administration as an attempt to improve the conventional TB therapy. Further studies should be focused in alternative administration routes for this nanotechnological platform, as inhalable nanocarriers.

Polymeric Micelles

Recently, polymeric micelles (PMs) have become one of the most attractive and well-investigated nano-vehicles (59,60). They are composed by amphiphilic biocompatible polymers that can self-assemble into nanostructures when the polymers concentration is above their critical micellar concentration (CMC). In general, micelle-forming biopolymers exhibit low CMC values, which confer excellent colloidal stability to the PMs. Specifically, PMs are formed by an outer hydrophilic corona and an inner hydrophobic core capable of hosting lipophilic drugs. On the one hand, the hydrophilic blocks are in contact with the aqueous medium, creating the micellar shell, which ameliorates its stability (60).

Particularly, for TB therapy, this strategy has not been as explored as LPs and NPs. The selection of the biomaterials and the pharmaceutical additives allowed developing different dosage forms.

For instance, RIF-loaded flower-like PMs were investigated to enhance the drug oral bioavailability. In this case, tri-block copolymers based in PEG and poly(epsilon-caprolactone) were employed as micelle-former biomaterials. Promising results were assessed, as a significant ($p < 0.05$) increment (3.3-fold) in the RIF oral bioavailability for the drug-loaded PMs was achieved *versus* a INH syrup, in Wistar rats (61). Furthermore, micellar powders could be obtained after the addition of hydroxipropyl-β-cyclodextrin as cryo/lyo-protectant additive (62). Other investigation focused on the development of a liquid RIF dosage form. In this case, a commercially available polymer as Kolliphor® HS-15 was used to minimize the drug self-aggregation in aqueous medium (63).

Other approaches employing PMs were based in different biopolymers, as the RIF-loaded chitosan-graft-poly(epsilon-caprolactone)/(ferulic acid) PMs (64). These micelles presented a size of approximately 200 nm, which is known to be favorable for intracellular uptake. Indeed, in an *in vitro* uptake study with A549 cells, RIF-loaded micelles were more efficient

and successfully internalized than the free drug, in a time-dependant manner.

Recently, a promising approach for multi-drug delivery was assessed by Upadhyay *et al.* A combination of two loading methods (conjugation and physical entrapment) was assessed. Its effectiveness *versus* susceptible and resistant Mtb strains was confirmed *in vitro*, denoting the potential of these polymeric micelles for co-delivery of two "first-line" anti-TB drugs (65).

Polymersomes

Nanopolymersomes (nPS) are nano-sized vesicles surrounded by a bilayer membrane, made of self-assembled amphiphilic copolymers. The inner represents the aqueous core and the membrane is composed by hydrophobic polymer domains, surrounded by hydrophilic polymers blocks. Due to their composition, nPS can load hydrophobic, hydrophilic and amphiphilic drugs (66,67). Due to their polymeric nature, nPS have demonstrated high colloidal stability in comparison with other nano-structured vehicles, as PMs and LPs.

This novel strategy, based on polymeric vesicles, employed di- and tri-block copolymers of PEG and poly(epsilon-caprolactone) and was designed for the respiratory administration of the drug. Results showed their adequate physical stability in aqueous solution over time (14 days, 72.2–97.5 nm). Further, these nanocarriers increased the *in vitro* drug intracellular concentration, in comparison to an aqueous RIF solution in murine macrophages (RAW 264.7). Additionally, only small amounts of biomaterial was administered, since the RIF dose for respiratory administration has been proved to be almost ~113-fold lower than the required amount of drug for oral administration (68).

Niosomes

Niosomes (NMs) are neutral surfactant vesicles with a bilayer structure formed by hydrated self-assembled surfactant monomers (69). They are biocompatible, biodegradable and non-immunogenic with proper plasticity in their structured characterization. Moreover, NMs are thermodynamically stable LP-like vesicles and their physical stability can be improved by the addition of cholesterol (70).

This nanotecnological strategy was explored for the delivery of PYR and ETB. The first drug was encapsulated within NMs composed by a mixture of Span® 60, cholesterol and dicetyl phosphate. The biological evaluation (subcutaneous administration) of the NMs in a guinea pig model infected with Mtb demonstrated lower bacterial counts in lungs after the niosomal administration twice a week, in comparison with free PYR administered either twice or 6 days per week. These results were in good agreement with the decrement of lung organomegaly observed for the niosomal formulation *versus* free drug and the control group (71). Further, similar results were observed after the biological evaluation of the ETB-loaded NMs (72).

An interesting approach of co-loading strategy was evaluated by Mehta *et al.* employing NMs based on Triton® X 100, poly(ethylene glycol) 2000 and Span® 80. Nanocarriers were co-loaded with RIF, INH and PYR, with an average diameter between 200 and 300 nm. The potential use of these NMs is focused on the possibility of co-administered anti-TB drugs with the possibility of RIF protection against acid degradation, due to its *in vitro* sustained release (73). Further studies by the same group were focused on the use of another biocompatible non-ionic surfactant known as Tyloxapol®, in replacement of Triton® X 100 and Span® 80, in search of a biomaterial with increased physical stability (74).

"SECOND" LINE PHARMACOTHERAPY: NANOTECHNOLOGICAL ADVANCES

The lack of adherence to the "first-line" anti-TB pharmacotherapy has frequently led to treatment failure. This represents one of the main causes of TB bacilli resistance. Unfortunately, those regimens based only in the combination of "first-line" anti-TB drugs are not an alternative for all the patients infected with MDR- and XDR- Mtb strains.

Furthermore, the ongoing nano-developments employing "first-line" drugs are at an early stage, thus more preclinical and clinical data is still required. Bearing this in mind, there is a strong urge to improve the anti-TB regimens for MDR- and XDR-TB. In this context, nanotechnology appears as an interesting strategy to achieve this aim (Table III; Fig. 4). Specifically, fluoroquinolones are the spine of MDR-TB therapy (Table I). Particularly, MOX is recommended by the WHO for the "shorter MDR-TB regimen" (75). However, its administration is associated with hepatotoxicity (76), a side effect that negatively affects patient adherence. The encapsulation of MOX within poly(lactic-co-glycolic acid) NPs may be one of the nanotechnological approaches attempted to minimize this side effect. Particularly, NPs were coated with hydrophilic polymers as chitosan and poly(ethylene glycol). Results suggested that the surface modification of the nanocarrier produced a sharp decrement of MOX liver accumulation. Authors associated this effect with a "brush-like" configuration of the hydrophilic polymers located on the NPs surface (77).

Following a similar strategy, Sarfraz *et al.* encapsulated MOX within NPs employing biomaterials as polyisobutylcyanoacrylate and gelatin. In this case, the authors aimed to boost the immune response mediated by the increment of the intracellular concentration of pro-inflammatory cytoquines. The *in vitro* results were assessed in murine macrophagues (78).

The objective of other investigations was to study the *in vivo* fate of nanocarriers loaded with a different fluoroquinolone,

Table III Nanotechnological Approaches Employing Second-Line and Novel anti-TB Drugs

Nanocarrier	Anti-TB drug	Biomaterials	Administration route	*In vitro* data	Preclinical studies	References
Polymeric nanoparticles (NPs)	Moxifloxacin (MOX)	Poly(lactic-co-glycolic acid) coated with chitosan and poly(ethylene glycol) 5000	Oral	Coated NPs showed low plasma protein binding, suggesting a "brush-like" configuration.	Drug bioavailability was improved *versus* uncoated NPs. A sharp decrement of MOX liver accumulation was observed in Wistar rats.	(77)
	Rifampicin + Moxifloxacin (RIF + MOX)	Polyisobutyl-cyanoacrylate/gelatin	–	Drug-loaded NPs promoted a cellular immune response in murine alveolar macrophages (MH-S cells).	–	(78)
	Bedaquiline (BDQ)	Methoxy-poly(ethylene glycol)-block-poly(lactic-co-glycolic acid), methoxy-poly(ethylene glycol)-block-poly(lactic-co-hydroxymethyl glycolic acid) and methoxy-poly(ethylene glycol)-block-poly(lactic-co-benzyloxymethyl glycolic acid)	–	Sustained BDQ release (3 days, PBS pH 7.4, 37°C) from the NPs.	–	(93)
Lipid nanoparticles (LNPs)	Bedaquiline (BDQ)	Soya lecithin, soybean Oil, Myrj S40® with the addition of 1,2-dioleoyl-3-trimethylammoniumpropane (chloride salt) (DOTAP)	Intravenous	MIC values of BDQ (against Mtb $H_{37}Rv$) were not modified after drug encapsulation.	–	(94)
Nanocapsules (NCs)		Chitosan-graft- poly(ethylene glycol) 5000	Respiratory	No cytotoxic effect on A549, HepG2 and THP-1 cell lines was observed for the BDQ-loaded nanocarriers.		
Lipid-polymer hybrid NPs (LPNPs)	Ciprofloxacin (CPX)	Soy Lecithin/1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy (polyethylene-glycol) 2000/ poly(lactic-co-glycolic acid)	Respiratory	Deep lung deposition of the spray-dried drug-loaded nanocarriers. The drug uptake from the LPNPs was improved *versus* the free drug in J774A.1 cells.	More than a half of the administered dose was found in lungs after 30 min. High drug accumulation in lungs over 24 h *versus* the free CPX.	(79)
Liposomes (LPs)	Clofazimine (CFM)	L-α-Dimyristoylphosphatidyl choline/ L-α-dimyristoylphosphatidyl glycerol	Intravenous	–	The therapeutic effect against Mtb was dose-dependant. Efficacy was higher in liver and spleen (bactericidal effect) than in lugs in an infected mice model.	(80)
	Cardiolipin + Levofloxacin (CDN + LVX)	Phosphatidylcholin/cholesterol/cardiolipin	–	The anti-TB efficacy of CDN was confirmed in an extensive drug resistant Mtb strain (CN-37). Further, the encapsulation of LVX to CDN-based LPs improved the antimicrobial effect of LVX *versus* free drug against Mtb resistant-strain.	–	(81)
	Rifabutin (RFB)	Dipalmitoyl phosphatidylcholine/ dipalmitoyl phosphatidylglycerol	Intravenous	–	High antimicrobial effect was observed in liver and spleen for the drug-loaded LPs. Lung inflammation was reduced after treatment with the liposomal formulation *versus* the free drug in a disseminated TB model.	(84)

such as CPX. This is the case of a promising nanosystem based in lipid-polymer hybrid NPs (LPNPs), developed by Bhardwaj *et al.* Once it was confirmed that the CPX *in vitro* cellular uptake was enhanced *versus* the free drug in J774A.1 cells, CPX plasma profiles were investigated in mice after pulmonary administration. The drug accumulation of the nanocarrier in lungs over 24 h was significantly ($p < 0.0001$) higher than that of the free drug, denoting the potential of this nano-development for pulmonary delivery (79).

Other "second-line" drugs have been explored as potential candidates to be encapsulated within nanocarriers for MDR-TB therapy. This is the case of CFM, an anti-TB agent, intended to be part of the "short MDR-TB regimen" (Table I). Due to its low aqueous solubility, Adams *et al.* developed an aqueous formulation composed of drug-loaded multilamellar LPs for intravenous administration. Remarkably, this investigation evaluated the therapeutic efficacy of CFM against Mtb in infected mice. Results were promising since the drug efficacy was improved in both acute and chronic TB infection models. Data revealed that this novel nano-platform showed a great potential to improve the "short MDR-TB regimen" (80).

An interesting approach was assessed by Gaidukevich *et al.*, towards the improvement of XDR-TB therapy. The authors demonstrated that cardiolipin (CDN) based-LPs were able to suppress the *in vitro* growth of a XDR-Mtb strain (CN-37). Considering these results, they developed an optimized liposomal formulation employing CDN and LFX. This attempt was focused on decreasing the amount of CDN employed in the liposomal formulation (81).

Finally, it is of utter importance to analyze the TB therapy in those patients who are co-infected with HIV. In this case, TB pharmacotherapy needs to be optimized according to the drug interactions of each regimen. Particularly, subtherapeutic doses of the antiretroviral drugs have been reported in presence of RIF (82). Hence, RFB has been recommended for the treatment of mycobacterial infections in HIV-positive patients. It is a member of the rifamycin family that has been hardly employed in TB therapy, due to its high cost. However, this drug is now off-patent and it is arriving to different developing-countries (83,84). Considering this, Gaspar *et al.* assayed multilamellar LPs (mean size of 100 nm) loaded with RFB in Mtb infection for the treatment of extra-pulmonary TB. The results in infected (Mtb, strain H37Rv) mice model showed that the bacilli concentration of the liposomal formulation in liver and spleen was significantly lower than free RFB, after the intravenous administration. However, this effect was not observed in the lungs. In these organs, the administration of RFB-loaded LPs minimized the inflammatory response, in comparison with the free drug. Overall, these results denote the potential of these nanocarriers for extra-pulmonary TB therapy, especially for HIV-TB co-infected patients (84).

NOVEL ANTI-TB DRUG CANDIDATES. WHAT HAS NANOTECHNOLOGY DONE?

Despite the availability of a combined effective anti-TB therapy, the morbidity and mortality associated with TB infection remain as a public health concern worldwide. In the past few years, the development of MDR and XDR strains of Mtb has raised special attention. Alarmingly, about half of the patients with MDR-TB are cured when treated with the current anti-TB regimens (85).

Therefore, in order to achieve the targets set in the "End TB Strategy and Sustainable Development Goals", a considerable increase in funding for TB research and development, e.g. improved anti-TB regimens and novel anti-TB drug candidates, is needed.

In this framework, only a few anti-TB drug candidates have been developed over the past decades. Particularly, BDQ (a diarylquinoline) has received accelerated FDA approval as a complement of the second-line regimen for MDR-TB in 2012. Hence, it has become the first approved anti-TB drug over the last 50 years (86,87). Also, BDQ and DLM (a nitroimidazo-oxazole derivative) have been approved in 2014 by the European Comission for MDR-TB in adults (31).

Furthermore, BDQ is actually being investigated in different ongoing randomized controlled trials known as NEXT-TB (NCT02454205) (88), STREAM Stage 2 (NCT02409290) (89), Nix-TB (NCT03086486) (90) and NC-005 (NCT02193776) (91).

A major drawback of BDQ is the fact that it presents a side effect associated with the risk of QT prolongation. This is the main reason why it is only recommended for adult patients with pulmonary MDR-TB that cannot be eligible for the short treatment (92). In this context, nanotechnology raises as an attractive platform to minimize BDQ systemic side effects and improve its potential as anti-TB drug. Unfortunately, only a few studies have explored BDQ as a candidate to be loaded within nanocarriers **(**Table III**)**. One of these investigations studied the BDQ encapsulation within polymeric NPs. The authors employed di-block copolymers based in poly(ethylene glycol) as biomaterials. In this case, they observed a sustained *in vitro* BDQ release (3 days, PBS pH 7.4, 37°C) from the polymeric NPs, associated with the drug diffusion through the polymeric matrix (93). For their part, De Matteis *et al.* observed similar sustained *in vitro* release profiles. In this case, two nanotechnological platforms were assayed: lipid NPs and chitosan nanocapsules. These nanocarriers released approximately 10 and 40% of the drug in Middlebrook 7H9 medium over 1 week. Interestingly, the authors demonstrated that these nanomedicines did not exhibit *in vitro* cytotoxic effect against lung epithelial human (A549), liver epithelial human (HepG2) and THP-1 cells (94).

These investigations constitute the basis for a deeper understanding of how novel anti-TB formulations can be

optimized to enhance TB therapy. However, further studies are needed in terms of nanotechnological development. A wide variety of nano-sized platforms can be assayed to overcome the main drug biopharmaceutical limitations, biodistribution issues and side effects associated with these novel anti-TB drug candidates. These recent researches are the first steps in the development of nanomedicines based on novel anti-TB drugs. Overall, these approaches constitute a strong and attractive alternative to optimize MDR-TB therapy in the near future.

Future investigations may be focused on the preclinical data observed after the administration of BDQ-loaded nanomedicines. This can result useful to gain further insight in the fate of these systems after their administration and the possibility to enhance the efficacy of a novel TB pharmacotherapy.

NANOTECHNOLOGY APPLIED FOR ACTIVE TARGETING TO *M. TUBERCULOSIS* RESERVOIRS

Active targeting of nanomedicines implies the specific ligand–receptor interaction between nanocarriers and cells or tissues. Particularly, ligands (e.g. monosaccharides, antibodies and proteins) are usually located on the surface of the nanocarrier. These specific moieties can be conjugated to the surface of drug delivery systems or they can be used to coat or modify the delivery system, in order to achieve an active targeting (95).

Consequently, the development of actively targeted nanomedicines to the AMs, remains of great significance for TB infections, since AMs constitute a cellular reservoir of latent Mtb forms (Table IV). In this context, pulmonary drug delivery remains of clinical relevance to minimize systemic side effects and enhance the bioavailability of anti-TB drugs in the lungs (96).

Among the variety of explored ligands for TB active targeting, mannose has been on the spot in the last years since macrophages present lectin-like receptors, which specifically recognize this monosaccharide (97).

In the first place, NPs have been investigated as nano-drug delivery systems for TB active targeting. The observed results obtained with mannose as the active targeting moiety have been more than promising. For instance, cationic mannose-lipid NPs demonstrated a significantly ($p < 0.01$) higher *in vitro and in vivo* uptake than their mannose-free counterparts in AMs. The researchers denoted that the cationic nature of the nanocarriers allowed their lung deposition due to the formation of NPs-plasma protein aggregates in the micro-scale range (98). Similar results were assessed by Vieiria *et al.* and Maretti *et al.* employing RIF-loaded mannosylated SLNPs. Once again, the effect of the monosaccharide located on the nanocarrier surface resulted in an enhancement of the *in vitro* cellular uptake in human THP1 differentiated macrophages and J774 cells, respectively. The former study revealed that the uptake mechanism of the mannosylated SLNPs was probably mediated by clathrin in THP1 differentiated macrophages (99). The latter highlighted a disadvantage related with the mannosylation of the carriers. The presence of the monosaccharide reduced its *in vitro* respirability properties, probably due to adhesion forces (100).

Furthermore, Costa *et al.* investigated a similar strategy for INH encapsulation, employing Witepsol® E85 as a lipid component of the SLNPs. As previously observed, the surface decoration with mannose promoted the *in vitro* uptake of the fluorescent-labeled SLNPs in dTHP-1 macrophages (101).

Since nanotechnology offers a wide variety of strategies, other studies have been focused on different platforms. This is the case of chitosan NPs and poly(epsilon-caprolactone)-poly(ethylene glycol)-poly(epsilon-caprolactone) flower-like PMs. The presence of hydrolyzed galactomannan on their surface clearly enhanced the *in vitro* RIF intracellular concentration in RAW 264.7 cells. In addition, PMs demonstrated greater RIF content, in comparison with the NPs, which could be useful to optimize the anti-TB therapy, by minimizing the amount of administered biomaterial (102).

Following the mannosylation strategy, dendrimers (DNs) have also been studied for RIF active targeting to AMs. Briefly, DNs are three-dimensional branched nanoscopic macromolecules that can be classified in different generations with variable molecular size (103).

The results revealed that mannosylated polypropylene imine 5G DNs exhibited higher RIF content within the AMs mediated by phagocytosis *versus* a free drug solution. Moreover, the authors observed a differential RIF release profile, due to pH variations. A higher drug *in vitro* release at acidic conditions could improve the RIF release in the phogolysosome environment (104). This could be useful since Mtb survives inside these vesicles.

Surprisingly, mannosylation demonstrated other advantage, as the decrement of the *in vitro* cytotoxicity (~ 2.2%) in red blood cells. This effect could be associated to the reduction of the cationic nature of the nanocarrier, due to the presence of mannose moieties on its surface (104).

In addition, macrophages express other receptors that could be studied for active targeting, such as the hyaluronic acid (HA) receptor (105). This strategy has been explored by Gao *et al.*, where the incorporation of HA into tocopherol/succinate micelles enhanced the RIF *in vitro* uptake in murine AMs (MH-S cells), in comparison with the free drug. The uptake mechanism probably occurred via phagocytosis and receptor-mediated endocytosis, in a time and dose-dependent manner. Interestingly, RIF uptake was decreased after the addition of HA to the cell culture medium, confirming the relation between the increment in the drug

Table IV Active-Targeted Anti-TB Drug Delivery Systems Employing Nanotechnological Strategies

Drug (abbreviation)	Ligand	Nanocarrier	Main features	References
Rifampicin (RIF)	Mannose	Cationic lipid nanoparticles	Improved FITC-labeled mannosylated nanocarrier uptake in alveolar macrophages *versus* their mannose-free, *in vitro* and *in vivo*.	(98)
		Solid lipid nanoparticles	Higher *in vitro* uptake efficiency of fluorescent mannosylated SLNs than the mannose-free nanocarriers in human THP1 differentiated macrophages.	(99)
		Solid lipid nanoparticles	Enhanced *in vitro* uptake of the modified nanocarrier by murine macrophages (J774 cells) *versus* un-modified counterparts, assessed by intracellular fluorescence	(100)
		5G dendrimers	Low *in vitro* citotoxicity in red blood cells. Higher *in vitro* RIF content within AMs mediated by phagocytosis *versus* a free RIF solution	(104)
	Hydrolyzed galactomannan	Polymeric "flower-like" micelles and polymeric nanoparticles	*In vitro* increment on the RIF intracellular concentration in macrophages (RAW 254.7)	(102)
	Hialuronic acid	Tocopherol succinate-based self-assembling micelles	Higher RIF intracellular content in MH-S cells employing the ligand-decorated nanocarrier *versus* free RIF. Probably uptake by phagocytosis and receptor-mediated endocytosis.	(106)
Isoniazid (INH)	Mannose	Solid lipid nanoparticles	Improved *in vitro* uptake of the fluorescent INH delivery system in dTHP-1 cells.	(101)

intracellular content and the presence of HA as a surface-located ligand (106).

Overall, the modification of the surface of drug delivery systems represents a promising platform for the development of novel anti-TB respirable nanocarriers. However, further clinical information needs to be obtained. In this way, future investigations should be focused on the *in vivo* results after the administration of these active-targeted nanocarriers. This data results crucial to fully characterize their potential as targeting agents, thus offering an alternative route for the administration of anti-TB drugs.

In addition, an interesting approach can be directed to the investigation of active-targeted nanocarriers loaded with more than one anti-TB drug. This goes in line with the current combined oral TB pharmacotherapy. In this way, different "first-line" anti-TB drugs may be co-delivered to AMs, representing a feasible strategy to explore a pulmonary adjuvant therapy to the conventional oral treatment. This may represent an improvement in the TB pharmacotherapy, aiming for better therapeutic outcomes.

INCIDENCE, PREVALENCE AND SOCIO-ECONOMICAL IMPACT

The consequences of TB for the patient and the environment are extremely harmful and can generate a high socio-economic impact. TB mostly affects productive year's population. However, all age groups are at risk. The sanitary emergency stands since 1993 (3) where over 95% of TB deaths occur in low- and middle-income countries (1). It is important to note that seven countries account for 64% of the total, with India leading the count, followed by Indonesia, China, Philippines, Pakistan, Nigeria, and South Africa. Further, TB is also known as an "opportunistic" disease in immunosuppressed patients (4). Hence, an adequate pharmacological regimen is vital for decreasing TB lethal index, since unfortunately; TB now ranks alongside HIV as a leading cause of death worldwide (1).

If we take further insight, MDR-TB still remains a public health crisis and a threat to health safety. WHO estimates that there were 600,000 new cases with RIF resistance, the most effective "first-line drug", of which 490,000 were infected with MDR-TB. Interestingly, the "End TB" program developed by the WHO aims is to adapt the strategy and targets at country level, with global collaboration. Furthermore, its pillars and components are to achieve an integrated, patient-centered care and prevention (early diagnosis of TB, drug susceptibility testings, correct treatment of infected patients, between others), to promote bold policies and supportive systems and to develop deeper research and innovation tools (107).

From a financial point of view, it is estimated that US$ 52 billion are required over 5 years (2016–2020) in low and middle income countries to implement the currently available interventions of the *Stop TB Partnership's Global Plan to End TB*. Around US$ 9.2 billion are required for 2017 and this number increases to US$ 12.3 billion for 2020. The majority of this funding is destined for diagnosis and treatment of drug-susceptible TB; however, the amount intended for diagnosis and treatment of MDR-TB rises from US$ 2.0 billion in 2017 to US$ 3.6 billion in 2020. Regarding high-income countries,

around US$ 6.3 billion are needed for the period 2016–2020, while an additional US$ 9.0 billion are required for TB research and development (1). Among the latter, few diagnostic technologies have emerged in 2017 and many of them have not shown an acceptable performance in field evaluation studies. On the other hand, much effort has been given to the study of new or repurposed drugs for the treatment of drug-susceptible, MDR or latent TB infection (1). Unfortunately, the cost of some of these novel drugs, such as the cases of BDQ and DLM, does not match the economic situation of the patients from low and middle income countries. They are more expensive than the standard regimens for MDR-TB and with equal effectiveness (108). It was estimated that the median cost per patient treated for drug-susceptible TB was US$ 1253, while that of MDR-TB was US$ 9529. Nonetheless, research and development of novel drugs remains of utter importance. In 2017, eight new compounds were included in clinical trials (delpazolid, GSK-3036656, OPC-167832, PBTZ169, pretomanid, Q203, SQ109 and sutezolid) and two of the most expensive ones have already been granted accelerated or conditional approval and are currently in phase III studies (BDQ and DLM) (1).

In the past few years, nanotechnology has emerged as an interesting alternative to be exploited in the medicine field. The application of nano-sized systems to different pathologies may enhance the efficacy of the treatments, increase detection sensitiveness and reduce the side effects of commonly used drugs, either by employing active targeting or by modifying the pharmacokinetics of the active compound (109). In the particular case of TB, the development of anti-TB drug-loaded nanocarriers may be useful for targeting the drug to specific action sites in a controlled manner with the aim of reducing the dose and dose frequency. Considering that one of the main causes of treatment failure is the non-adherence of the patient, the fact of lowering both doses and dose frequencies would help to improve the patient compliance to the therapy (110). Besides, depending on the chosen nanosystem, the development of a nanoformulation loaded with a conventional and well-studied anti-TB drug would be less expensive and more accessible to the patients from low and middle income countries than the lengthy and costly process of discovery and development of a new chemical entity to be clinically studied.

Finally, nanotechnology also offers the possibility to overcome or minimize drug-resistance since the encapsulation within a nano-sized carriers could decreased drug elimination from the intracellular medium mediated by efflux pumps, a common resistant-mediated mechanism observed in different pathologies as cancer, HIV and hepatitis (111). Particularly, for TB therapy, macrophage and mycobacterial membrane efflux pumps should be considered as they may play a key role in resistance-development. For instance, it has been studied that monocytes from TB patients mostly express multidrug resistance–associated proteins (MRPs). Interestingly, MRPs are involved in the efflux of the reverse transcriptase inhibitors employed for anti-HIV therapy (112). Moreover, there have been described multidrug efflux pumps belonging to the Mtb Major Facilitator Superfamily Transporters (MFS), some of them being upregulated in RIF-resistant insolates (113). Since Mtb is an intracellular pathogen, the possibility to enhance the anti-TB intracellular/cell concentrations remains of clinical relevance to maximize drug effectiveness. In this framework, nanotechnology offers a wide variety of platforms to deliver (by passive or active targeting) anti-TB drugs to infected macrophages, minimizing the development of MDR-TB strains.

FUTURE PERPECTIVES

A wide variety of nanocarriers and biomaterials have been explored for mono- and multi-drug delivery in TB. In this way, they have not only proved their potential to overcome the physicochemical limitations, as poor aqueous-solubility and chemical instability, but they have also improved drug bioavailability in Mtb infected animal models.

The oral administration of these nanomedicines has been studied to a great extent, since it represents the conventional administration route for TB therapy. Besides, many efforts have been in order to avoid the parenteral administration of anti-TB drugs (56). On the other hand, the inhaled TB therapy employing nano drug delivery systems could be an interesting alternative to the oral route, taking into account that pulmonary TB represents the most common TB form.

In the past few years, the pulmonary administration of drugs has gained great attention, due to its benefits, as it is not an invasive route, its large surface area and the possibility of minimizing systemic side effects and first hepatic metabolism. A clear example is represented by the different commercially available products for the respiratory therapy of cystic fibrosis. Different antibiotics, such as tobramycin (TOBI® and TOBI® Podhaler™), levofloxacin (QUINSAIR®) and aztreonam (Cayston®) have been approved as inhaled formulations (solutions and powders) by regulatory agencies (114).

Regarding TB therapy, nanotechnology offers a clear alternative of developing an adjuvant respiratory regimen to the oral pharmacotherapy. Their size in the nano-range can be useful to improve the apparent solubility of drugs and further exhibit a sustained release profile. Besides, nanocarriers can promote drug permeation though membranes and cellular uptake (68).

The possibility to reach the lung cellular reservoirs of the Mtb represents a key advantage to increase the intracellular drug concentration in infected macrophages. Nonetheless, the uniform administration of the nanocarriers into the lungs is a key parameter to be optimized. Moreover, another feature to be considered is the aerodynamic behavior of the nano-sized particles. It represents a main goal to assess deep lung

deposition and avoid rapid clearance from the respiratory system. In this sense, some biomaterials may improve membrane mucoadhesion (e.g. chitosan) and facilitate mucus penetration (e.g. PEG-based copolymers) (115,116).

However, the design of inhalable powders, suspensions or solutions requires the consideration of different factors, such as particle aggregation, the presence of preservatives, antioxidants, buffers and tonicity agents, mainly due to the lack of buffer capacity of the lungs. Furthermore, there is a limited list of pharmaceutical additives that are currently approved for pulmonary administration (117).

Furthermore, the lyophilization process to develop dry powders based on nanocarriers is not an easy task. A wide variety of cryo/lyoprotectors might be employed to ensure an easy re-dispersion of the freeze-dry powder, avoiding nanoparticle aggregation and drug precipitation (62,118). It is worth stressing that some nano-systems will demand high amount of lyoprotectors, which could enrich the cost-related features of the product. This is crucial, taking into account that TB is a poverty-related disease. Besides, the total amount of biomaterial and lyoprotector to be inhaled over time should be evaluated in terms of chronic lung toxicity. In this framework, many of the described nanomedicines for TB respiratory therapies should be optimized to reduce the amount of daily-administered excipients to the patients.

Interestingly, the development of novel drug formulations was investigated hand-in-hand with active-drug targeting, employing macrophage ligands, as mannose and hyaluronic acid. This strategy represented an attempt to develop "smart" nanoformulations that could be preferentially internalized by macrophages, thus optimizing the respiratory pharmacotherapy.

Despite of the recent advances in TB field employing nanotechnological platforms, more *in vivo* data is still required. Only a few investigations have shown that their nanoformulations were effective in infected preclinical models of TB. This represents a critical drawback, as future clinical studies will depend on the available preclinical data. Thereafter, the possibility of obtaining a commercially available nanoformulation is still at its early stages. The hypothesis of a respiratory adjuvant therapy for TB, employing nanotechnology, demands further preclinical and clinical data to become a feasible reality. Besides, the financial support to face these studies is limited, representing a major drawback for the improvement of this kind of poverty-related diseases. Nanotechnology in TB is just around the corner. In order to promote such innovation, a multisectoral and multidisciplinary approach at both country and global levels is required. This may be achieved through advocacy, education, knowledge sharing and support to international institutes for research and building-capacity.

Conclusion

Nowadays, TB represents an infectious disease that strikes public health with more than 10 million new cases worldwide. Although efforts have been directed to diminish its incidence and mortality, the development of multi-drug resistance Mtb strains threats the possibility to successfully control this illness. Nanotechnology has opened a window through the development of novel drug delivery systems to optimize TB pharmacotherapy.

All the "future medicines" elucidated for TB regimens are still waiting for financial support and further preclinical studies to move on to the next step and reach to the patients. Another important issue to consider is the approval status of the biomaterials employed as nanovehicles. Those materials which are not approved by regulatory agencies have fewer chances to reach clinical studies. In this context, the required toxicity studies would increase the final value of a novel nanoformulation.

Undoubtedly, nanomedicines stand out as an attractive tool with multiple advantages to face this mycobacterial infection. Future preclinical and clinical data are still required to gain a greater understanding of their potential to become commercially available nanotechnological formulations.

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