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Mycolic acids, a promising mycobacterial ligand for targeting of nanoencapsulated drugs in tuberculosis

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ABSTRACT

The appearance of drug-resistant strains of *Mycobacterium tuberculosis* (*Mtb*) poses a great challenge to the development of novel treatment programmes to combat tuberculosis. Since innovative nanotechnologies might alleviate the limitations of current therapies, we have designed a new nanoformulation for use as an anti-TB drug delivery system. It consists of incorporating mycobacterial cell wall mycolic acids (MA) as targeting ligands into a drug-encapsulating Poly DL-lactic-co-glycolic acid polymer (PLGA), via a double emulsion solvent evaporation technique. Bone marrow-derived mouse macrophages, either uninfected or infected with different mycobacterial strains (*Mycobacterium avium*, *Mycobacterium bovis* BCG or *Mtb*), were exposed to encapsulated isoniazid-PLGA nanoparticles (NPs) using MA as a targeting ligand. The fate of the NPs was monitored by electron microscopy. Our study showed that i) the inclusion of MA in the nanoformulations resulted in their expression on the outer surface and a significant increase in phagocytic uptake of the NPs; ii) nanoparticle-containing phagosomes were rapidly processed into phagolysosomes, whether MA had been included or not; and iii) nanoparticle-containing phagolysosomes did not fuse with non-matured mycobacterium-containing phagosomes, but fusion events with mycobacterium-containing phagolysosomes were clearly observed.

1. Introduction

Tuberculosis (TB) is mainly a burden of the developing world, but has also a great impact globally with an estimated annual 8.6 million incident cases. It also ranks as the second leading cause of death from a communicable disease (second to Acquired Immunodeficiency Syndrome (AIDS)) with 1.3 million deaths reported in 2012 [1]. TB-AIDS can be classified as a new combination disease, since Human Immunodeficiency Virus/*Mycobacterium tuberculosis* (HIV/*Mtb*) co-infection has become such a frequent occurrence in third world countries. In comparison to TB alone, TB/AIDS patients have severely reduced survival times, are more challenging to diagnose [2] and may exhibit sometimes fatal immune reconstitution inflammatory syndrome (IRIS) when subjected to anti-retroviral therapy [3]. Standard first line chemotherapy is not effective for individuals that are infected with multi-drug resistant (MDR) *Mtb* strains and there is practically no cure for extensively-drug resistant (XDR) ones. Failure to complete the lengthy drug regimens allows the pathogens to become resistant especially to the two first line drugs, isoniazid (INH) and rifampicin (RIF). This is increasingly evident in persons who have been previously treated for TB [4]. It is now imperative to discover new chemotherapies against this mycobacterium as well as to enhance the efficacy of existing drugs to combat MDR and XDR forms [5]. In addition, it is important to find new technologies for targeting chemotherapeutic agents and toxic host cell effector molecules through the lipid-rich wax-like mycobacterial cell wall [6].

Innovative nanotechnologies have been designed and tested to meet the challenges posed by the pathogen and poor drug efficacy. Their potential to improve compliance, efficacy and affordability of therapy makes them especially suited for addressing the treatment of TB and AIDS. Nanomedicine (the medical application of nanotechnology) has the potential to improve bioavailability, reduce toxic side effects, reduce drug–drug interactions and overcome drug resistance. Furthermore, nanoformulations can improve drug solubility and facilitate

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intracellular drug delivery as well as target drugs to the site of infection. Nano drug delivery systems are generally prepared with natural or synthetic compounds (i.e. polymers and lipids) using various techniques to yield particles ranging in size between 10 and 1000 nm in diameter [7]. Many groups have used poly DL, lactic–co-glycolic acid (PLGA) as the polymeric encapsulating material because it is non-immunogenic, biodegradable and has the capacity to encapsulate hydrophobic and hydrophilic agents [8]. PLGA was also shown to be selectively taken up into macrophages and dendritic cells [9], the main targets for infection with *Mtb* [10]. In addition, the cytotoxicity of PLGA was evaluated in vitro and in vivo and no significant adverse effects were noted [11].

Nanoencapsulation of drugs for delivery provides an opportunity to incorporate targeting strategies to further enhance drug efficiency and limit systemic toxic side-effects to the patient. By functionalizing the surface of the particles with a targeting ligand, higher bioavailability could be generated at the site of infection and therefore the dose and side effects of the drug are minimized [12]. An effective targeting strategy will impact positively on drug delivery to the site of infection, may direct the trafficking of the drugs into organelles in which pathogens are harboured and may improve the release and longevity of the active drug. A comprehensive review of the recent advances in targeting with drug carrier designs for TB was done by Dube and colleagues [13].

The mycobacterial cell wall is composed of many compounds that could serve as targeting ligands. Among these, mycolic acids (MA) are promising candidates because they are the dominant lipids found in the outer cell wall envelope of *Mycobacterium* species [14]. MA are long 2-alkyl 3-hydroxyl fatty acids and in *Mtb*, MA consist mainly of alpha-, keto- and methoxy classes, typically 70–90 carbon atoms in length, each containing mixtures of homologs of varying chain lengths and, in some cases different stereochemistry around functional groups in the main (mero-) chain [15]. The various subtypes of MA have been shown to play a major role in the virulence of the pathogen [6,16]. MA from *Mtb* have interesting biological activities, including foam cell formation and immune steering towards Th1 cellular responses [17–19], as well as cholesteroid like properties [20,21]. MA was also shown to be immunogenic. When presented by CD1b molecules on dendritic cells, proliferation of human $CD4^-$, $CD8^-$ (double negative) T-lymphocytes occurs [22,23]. In addition, anti-MA antibodies are found in human TB-positive patient serum [24]. Mycobacterial MA has never been tested as a ligand for improving nanoencapsulated antituberculosis (anti-TB) drug targeting. An attractive hypothesis would be that the MA present on the external surface of the NPs may interact with anti-MA antibodies in the vicinity of the sites of infection to cause a localised immune complex that may enhance uptake of the NPs in the infected and surrounding uninfected macrophages. MA could also target cholesterol present in the plasma membrane of uninfected or infected macrophages [25,26] and, more interestingly, in the membrane of phagosomes in which pathogenic mycobacteria are harboured [27], by means of its attraction to cholesterol [20,21,28].

To understand whether or how this happens demands an intricate knowledge of the cellular and molecular mechanisms of survival of the pathogen of interest and more precisely how the pathogen uses or diverts the cellular machinery to its advantage. It is well known that the preferred site of residence of pathogenic mycobacteria in the host macrophage is the immature phagosome [29] which is unable to fuse with lysosomes [30,31]. However, mycobacteria internalized in clumps, a frequent event in the case of *Mtb*, are unable to block phagosome maturation [32–35]. In this case, phagosomes mature and fuse with lysosomes to become phagolysosomes. Interestingly, mycobacteria do not die in this cytolytic environment from which they are eventually rescued to reside in phagosomes that no longer fuse with lysosomes [29]. Several more recent studies also indicate that *Mtb*, but not *Mycobacterim bovis* BCG or *Mycobacterium avium*, may evade phagosomes to survive in the host cell cytosol immediately prior to host cell lysis [36–39].

The subcellular localisation of PLGA particles in cells is a field that has hitherto yielded conflicting results over the past decade. On the one hand, PLGA particles were suggested to be able to escape the endomembrane compartments into the cytosol of a smooth muscle cell line within minutes after phagocytic uptake [12,40]. More groups supported this interpretation for different cell types, including macrophages [41–43]. On the other hand, the PLGA particles were suggested to remain within the phagolysosomal compartment for several days [44–46]. Another issue is the ability for PLGA particles to reach the compartment in which mycobacteria reside. One group suggested this possibility [47], but others could not demonstrate it [10,45].

Previous efforts to demonstrate a potential benefit of MA as a drug-targeting ligand utilized fluorescently labelled MA in a human myelomonocytic cell line by means of confocal laser-scanning fluorescence microscopy (CLSM) [48]. The fluorescently labelled MA in NPs were shown to be actively taken up by the cell lines. In the present work, we assess the use of mycobacterial MA as a ligand for nanoencapsulated anti-*Mtb* drug targeting on the uptake and intracellular fate of the NPs by electron microscopy (EM) methods. It is important to recall that EM is the only technique that can combine sensitive protein detection methods with detailed information on the ultrastructure and interaction of mycobacteria and cellular compartments. A nanoformulation of incorporated MA in a PLGA polymer was achieved via the double emulsion solvent evaporation technique. INH was incorporated as the anti-TB drug model in our PLGA particles due to its known cytotoxicity as a first line drug against actively replicating *Mtb*. We chose to infect mouse bone marrow-derived macrophages (BMDM) with either *Mtb*, *M. bovis* BCG or *M. avium*. Six days later, cells were exposed to the synthesized NPs, with or without the potential targeting ligand MA. We show that MA enhanced the uptake of NPs into mycobacterium-infected macrophages. Newly formed nanoparticle-containing phagosomes were processed into phagolysosomes within hours. Such phagolysosomes were able to fuse with and deliver their contents to mycobacterium-containing phagolysosomes but not to immature phagosomes. We now clearly demonstrate that NPs do co-localise with mycobacteria in the phagolysosomal compartments.

2. Materials and methods

2.1. Chemicals and reagents

Dimethyl sulfoxide, Isoniazid (99%), mycobacteria-derived MA, PLGA 50:50 (Mw: 45,000–75,000), Polyvinyl alcohol MW: 13,000–2,3000 partially hydrolysed (87–89%), Sodium dodecyl sulphate, Triton X-100, Trypan blue, Basic fuchsin, Dulbecco's modified Eagle's medium (DMEM), Tyloxapol and Glutaraldehyde grade I (EM grade) were purchased from Sigma-Aldrich Chemical Co., (St Louis, MO, USA). Foetal bovine serum (FBS) was from Biowest (Nuaillé, France), phosphate-buffered saline (PBS) was from GIBCO (distributed by Invitrogen, Villebonsur Yvette, France), and osmium tetroxide and Spurr resin were from Electron Microscopy Sciences (distributed by Euromedex, Mundolsheim, France). Middlebrook 7H9, 7H10 agar and Oleic acid-albumin-dextrose-catalase (OADC) were from Difco Inc. (Detroit, MI, USA). Bovine serum albumin conjugated to gold particles (BSA-Au) was purchased from the Utrecht University School of Medicine (Utrecht, The Netherlands).

2.2. Preparation of NPs

The double emulsion solvent evaporation freeze drying technique was used as previously described with modifications [49]. PLGA 50:50 (MW: 45,000–75,000) was dissolved in 6 ml of dichloromethane (DCM) at a concentration of 1.25% weight/volume (w/v). For particles containing MA, isolated MA from *Mtb* (2 mg, 1.7 μmol) was dissolved in 2 ml DCM and added while stirring. For the first water-in-oil (w/o) emulsion, 2 ml of aqueous PBS, pH 7.4, was added to the mixture and homogenized at 5000 rpm for 3 min in a bench-top homogenizer at 4 °C. For NPs containing INH, INH (100 mg, 729 μmol) was dissolved