The toxicity of rifampicin polylactic acid nanoparticles against *Mycobacterium bovis BCG* and human macrophage THP-1 cell line

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**Abstract.** Tuberculosis is rapidly becoming a major health problem. The rise in tuberculosis incidence stimulates efforts to develop more effective delivery systems for the existing antituberculous drugs while decreasing the side effects. The nanotechnology may provide novel drug delivery tools allowing controlled drug release. Rifampicin is one of the main antituberculous drugs, characterized by high toxicity, and Poly (L-lactic acid) (PLLA) is a biodegradable polymer used for the preparation of encapsulated drugs. The aim of our work was to evaluate the toxicity of rifampicin-PLLA nanoparticles against *Mycobacterium bovis BCG* using human macrophage THP-1 cell line. Our data demonstrate that rifampicin-PLLA is effective against *M. bovis BCG* in the infected macrophages. The drug is inducing the dysfunction of mitochondria and apoptosis in the macrophages and is acting as a potential substrate of Pgp thereby modulating cell chemosensitivity. The severity of the toxic effects of the rifampicin-PLLA nanoparticles is increasing in a dose-dependent manner. We suggest that free rifampicin induces death of *M. bovis BCG* after PLLA degradation and diffusion from phago-lysosomes to cytoplasm causing mitochondria dysfunction and affecting the Pgp activity.

1. **Introduction**

Tuberculosis is caused by *Mycobacterium tuberculosis* that predominantly resides macrophages. In the past two decades tuberculosis has again developed into a major health problem, with 1.7 million deaths every year and 2 billion latently infected [1, 2]. Among the problems with the application of the existing antituberculous drugs are toxicity, rapid degradation and limited bioavailability [3]. The increase of tuberculosis incidences stimulates development of new antibacterial drugs as well as more effective delivery systems for the existing drugs while decreasing their side effects. The progress in nanotechnology and particular encapsulation of the drugs inside the slow biodegradable, polymer microparticles (>1000 nm, PMPs) and nanoparticles (PNPs) may advance the development of novel drug delivery systems and controlled drug release [2, 3, 4]. Highly porous polymeric materials are...
often used as drug carriers. Poly(L-lactic acid) (PLLA) and poly(lactide-co-glycolide acid) (PLGA) are biodegradable polymers used for the preparation of encapsulated drugs [5, 6, 7].

Rifampicin is one of the main antituberculotic drugs, characterized by low water solubility and high toxicity. In bacterial cells rifampicin binds with RNA polymerase and thus prevents the elongation of RNA molecules [8]. In mammalian cells rifampicin initiates a complex of pathological changes ([9, 10]. In epithelial cells mitochondria are a potential target for rifampicin: the damage to mitochondria causes their dysfunction leading to oxidative stress [11]. In macrophages PMPs and PNPs predominantly localize in phago-lysosomes, where the polymer degrades, releasing the drug [12]. Compared with the accumulation of free rifampicin, rifampicin-P MPs and -PNPs can give up to a 20-fold higher concentration in macrophages [13, 14]. Released rifampicin can affect the viability of intracellular mycobacteria as well as the functional activity and structure of cellular organelles in the infected and uninfected macrophages. Thus the aim of our work was to study the toxicity of rifampicin-PLLA NPs against Mycobacterium bovis BCG in infected macrophages, and to determine the effects of rifampicin-PLLA NPs on cell viability, type of the induced cell death, and the role of mitochondria in this process.

2. Material and methods
2.1 Nanoparticle preparation
Rifampicin (100.8 mg, Sigma-Aldrich) and PLLA (700.1 mg, Mw -60,000, Sigma-Aldrich) were dissolved in 10 ml chloroform with heating to 40 degrees and stirring. Then the solution was stirred 30 min at room temperature. For the water phase 180 mg of PVA (polyvinyl alhocol) was dissolved in 36 ml of water (pre-filtered using a 0.2 mm filter). The two solutions were mixed, stirring vigorously with a magnetic stirrer for 1h at room temperature, and homogenized (24,000 rpm, 3 times x 1 min) to obtain the primary emulsion. The resulting mixture of the solvent is removed by evaporation of the solution under vacuum for 1 h. Then the emulsion was filtered through a glass filter (pore size 40-110 μm). To the resulting colloidal solution was added 201.6 mg of D-mannitol, frozen in liquid nitrogen, freeze dried and stored at 4°C. The average particle size, measured using the method of autocorrelation spectroscopy of submicron laser spectrometer Coulter N4MD (USA), is 300 ± 71 nm. The loading of rifampicin in the PLLA-NPs is 8.5% (loading is given as % by weight (w/w)).

2.2 Cell lines
Both human cell lines, THP-1 (derived from human monocytic leukaemia) and KG1 (derived from human myelogenous leukaemia), were obtained from Russian Cell Culture Collection and were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum. The cultures were maintained at 37°C in a humidified atmosphere (95% air/5% CO2) as a stationary suspension culture. To differentiate suspension THP-1 cells in macrophage107 M phorbol ester PMA (phorbol 12-myristate 13-acetate, Sigma) was used. After treatment for 24 h, the cells were induced to adhere to the growth substratum and to differentiate for 24-72h.

2.3 MTT-assay
The cell survival in the presence of increasing rifampicin-PLLA and rifampicin (Sigma) concentrations (from 10 to 250 μg/ml) was determined by monitoring the mitochondrial reduction activity according to standard protocol [15].
2.4 Staining of mitochondria
Mitochondria in cells were stained with 300 nM Mitotracker Orange (Molecular Probes) for 15 min at 37°C, then cell were fixed 3.7% formaldehyde in phosphate-buffered saline solution (PBS). DAPI (3 µg/ml, Molecular Probes) were added to label cell nuclei. Images were analyzed with Leica TCS SPE confocal laser microscope (Germany). The population of the active mitochondria without cytotoxic effects was stained with 10 µg/ml rhodamine 123 (Rh123) for 20 min at 37°C. The living cells was immediately observed with an Axiovert 200M (Carl Zeiss, Germany).

2.5 Immunostaining of cytochrome c
For immunocytochemical staining of cytochrome c, the cells were fixed for 10 min in 3.7% formaldehyde, washed in PBS (pH 7.2-7.4), treated with 0.1% Triton X-100 for 3-5 min. Then the cells were incubated in 1% BSA solution in PBS for 30 min, washed 3x10 min in PBS. Mouse monoclonal antibodies against cytochrome c (Sigma) were used as the first antibodies. The secondary antibodies were conjugated with Alexa 488 (Sigma). DAPI (3 µg/ml, Molecular Probes) were added to label cell nuclei. Images were analyzed with Leica TCS SPE confocal laser microscope (Germany).

2.6 Efflux of rhodamine 123
P-glycoprotein activity was determined by efflux of Rhodamine 123 (Rh123, Sigma), a fluorescent dye that is a substrate for P-gp, as previously described [16]. Briefly, 100 µl of purified cell suspension was incubated with 5 µg Rh123 for 15 min. After washing, cells were incubated in Rh123-free medium in the presence or absence of 2 mM verapamil (Sigma), a P-gp inhibitor, for 30 min at 37°C. Finally, cells were washed and up to 10,000 events were detected and analyzed with a FACScan flow cytometer (Becton Dickinson) equipped with an argon-ion laser with a wavelength setting of 488 nm and with 530/30 nm band pass (FL1).

2.7 Bacterial culture
_Mycobacterium bovis BCG_ expressing green fluorescent protein GFP (M. bovis BCG-GFP) were grown on Middlebrook’s 7H9 broth medium (Difco) supplemented with 10% OADC (V/V), glycerol 0.02% and 0.05% of Tween 80 v/v until exponential phase at 37°C.

2.8 Staining of dead bacteria
THP-1 cells were seeded on glass cover slips in 24-well plates at a density of 500 000 cells per 1 ml per well and incubated with 100 nM PMA for 48 hours in a 5% CO2 incubator. M. bovis BCG-GFP in the exponential grown phase washed twice in PBS (pH 7.4), and resuspended in serum-free RPMI medium. A 10-minute pulse in a water-bath sonicator was followed by passage through a 23-gauge needle to disrupt bacterial clumps. Before infection, residual bacterial aggregates were removed by low-speed centrifugation (120 g) for 2 minutes. Single-bacteria suspension was verified by fluorescence microscopy. Infection proceeded with BCG in the ratio of 1:10 bacteria per macrophage, to achieve about 1–10 bacteria inside macrophages after 4 hours uptake. The cells were washed twice with PBS to remove extracellular bacteria. After a 2-hour chase in medium without bacteria, rifampicine-PLLA NPs were added to the cells at 50 µg and 100 µg to each well – a total volume of 1 ml per well. After 72 hours of incubation, cells were washed with PBS, were permeabilized by 0.1% Triton for 1 min, stained by 2 µg/ml PI 5 min, washed PBS and fixed 3.7 % formaldehyde in PBS 10 min. Antibiotic was replaced freshly every day.

3. Results and Discussion
Due to the fact that the accumulation of rifampicine-PMPs and -PNPs in macrophages can be considerable, we first compared the rifampicin-PLLA NP effect on cell viability with the pure rifampicin toxicity (figure 1). Our data shows that rifampicin-PLLA NPs does not reduce the cell viability even at high concentrations (250 µg/ml) while free rifampicin induces cell death starting from
150 μg/ml. The macrophage THP-1 cells are more rifampicin-resistant compared to the suspension THP-1 cells in which 25 μg/ml of rifampicin induced apoptosis [17].

![Figure 1. Rifampicin-PLLA NP effect on the viability of macrophage THP-1 cells. Data were expressed as the percent survival of the control (100% the viable cells).](image)

To study rifampicin-PLLA effect on mitochondria, the cells were exposed to 50, 100 and 250 μg/ml of rifampicin-PLLA for 72 h. Rh123 staining showed that functional-active mitochondria formed reticular-like structure in the control cells. The similar reticular-like structures were formed upon treatment rifampicin-PLLA NPs (figure 2).

![Figure 2. Mitochondrial reticular-like structure in THP-1 cells exposed to rifampicin-PLLA NPs for 72 h. a) control; b) 50 μg/ml of rifampicin-PLLA NPs; c) 100 μg/ml of rifampicin-PLLA NPs.; Rh123-staining. Bar 10 μm.](image)

Increasing the concentrations of rifampicin-PLLA up to 250 μg/ml allowed identifying the small population of cells with perinuclear localization of mitochondria in the area of the cell centre. Analysis by Mitotracker Orange revealed the disappearance of the reticular-like the mitochondria and their redistribution to the cell centre (7.9 ± 0.3% versus 1.3 ± 0.6% cells in controls) (figure 3).
Figure 3. The disappearance of mitochondrial reticular-like structures under the rifampicin-PLLA NP exposure for 72 h in THP-1 cells. a) control; b) 250 μg/ml of rifampicin-PLLA NPs.; Staining by Mitotracker Orange. Bar 10 μm.

Induction of apoptotic death could be caused by the changes in the mitochondrial membrane permeability and by releasing of a proapoptotic factor cytochrome c from the mitochondria [18, 19]. Therefore, we studied localization of the cytochrome c in the untreated and treated macrophages. Under the influence of rifampicin-PLLA, a diffuse distribution of the cytochrome c which is specific for apoptotic cells is observed in cells characterized by perinuclear localization of mitochondria (figure 4).

Figure 4. Localization of cytochrome c under rifampicin-PLLA NP exposition for 72 h in THP-1 cells. a) control, cytochrome c localizes inside mitochondria; b) 250 μg/ml of rifampicin-PLLA NPs, diffuse distribution of the cytochrome c in perinuclear zone. Staining with first antibodies against cytochrome c and the secondary antibodies conjugated with Alexa 488. Bar 10 μm.

In control cells cytochrome c had a typical localization inside the mitochondria, including the mitochondria in the perinuclear zones around cell centre (figure 4a). Upon rifampicin-PLLA treatment, cells characterized by perinuclear localization of mitochondria displayed a diffuse
distribution of the cytochrome c specific for the apoptotic cells (figure 4). Thus, the treatment with rifampicin-PLLA induced the release of cytochrome c from the mitochondria to the cytosol, implicating mitochondria in activation of apoptosis. It has been reported that damage to mitochondria and their dysfunction lead to the changes in distribution of mitochondria, development of oxidative stress and trigger the mitochondrial pathway of apoptosis [20]. We suggest that the increased frequency of the cells with perinuclear localization of mitochondria indicates that rifampicin-PLLA NPs induce dysfunction of mitochondria. The release of the cytochrome c from the mitochondria to the cytosol is an early event of apoptotic death that leads to activation of effector caspases and degradation of cell structures. We observed the diffuse distribution of the cytochrome c in the mitochondria area under the influence of rifampicin-PLLA. It may indicate that membrane organelles such as Golgi apparatus, endoplasmic reticulum, lysosomes and cytoskeleton elements maintain their integrity and localization for a certain period after the release of the cytochrome c. We suggest that the treated macrophages reveal specific pathways of the caspase activation and the degradation of the cell structures.

P-glycoprotein (Pgp) is a multidrug transporter that belongs to the ATP-Binding Cassette (ABC) family, and is a product of the Multidrug Resistance-1 (MDR-1) gene. Pgp acts as a transporter molecule pumping a wide variety of endogenous substances and drugs from the cytoplasm to the extracellular environment [21].

**Figure 5.** Effects of different drugs on P-glycoprotein activity in KG1 cells. a) 50 μg/ml of rifampicin-PLLA NPs; b) 100 μg/ml of rifampicin-PLLA NPs; c) 100 μg/ml of pure PLLA NPs; d) 10 μg/ml of rifampicin; e) 2 mM of vinblastine (a typical blocker of Pgp). FACScan analysis of the fluorescence intensity; blue areas fluorescence of cells stained with Rh123; green curves - fluorescence of cells stained with Rh123 and incubated in dye-free medium; red curves - fluorescence of cells stained with Rh123 and incubated in the dye-free medium in the presence of drugs.
Rh123 and several other fluorescent dyes are substrates for the Pgp-mediated transport, and Rh123-efflux is the functional test for Pgp-activity. We tested the effect of rifampicin-PLLA NPs (50 and 100 µg/ml) on Rh123 efflux in human leukemic KG1 cell line characterized by high Pgp expression [22]. It has been shown that rifampicin-PLLA NPs affect the Rh123-efflux in a concentration-dependent manner: 50 µg/ml of rifampicin-PLLA has no impact on Rh123 efflux, whereas 100 µg/ml completely blocks the Rh123 efflux (figure 5a and 5b).

We have found that PLLA-NPs alone (100 µg/ml), don't have effect on Rh123 efflux (figure 5c). In contrast, 10 µg/ml of free rifampicin (comparable to rifampicin concentration in the NP composition) inhibits the efflux (figure 5d). Thus, the rifampicin PLLA-NPs have a stronger effect on the functional activity of Pgp as compared to both, rifampicin and PLLA NPs. We propose that effect is reached due to the higher intracellular concentration of free rifampicin after polymer degradation. These data indicate that rifampicin acts as a potential substrate of Pgp and therefore may modulate cell chemosensitivity.

Analysis of rifampicin-PLLA NP effect on viability of M. bovis BCG-GFP in the infected macrophage THP-1 cells showed the appearance of dead M. bovis BCG (PI-staining is effective only for bacteria with damaged permeability of bacterial cell wall) inside the macrophages (figure 6).

![Figure 6](image-url)

**Figure 6.** Influence of rifampicin-PLLA NPs on viability of *M. bovis BCG-GFP* in infected macrophage THP-1 cells. a)-c) – control; d)-f) - 50 µg/ml rifampicin-PLLA NPs; a) and d) intracellular *M. bovis BCG-GFP*; b) and e) PI-staining; c) and f) DIC, merge. Confocal laser microscopy. Bar 10 µm.
4. Conclusion
Our data demonstrate that rifampicin-PLLA NPs: (i) are non-toxic for a significant part of macrophage population; (ii) can induce dysfunction of mitochondria and lead to apoptosis only in a small part of the cell population; (iii) are effective against *M. bovis BCG* in the infected macrophages; (iv) act as potential substrate of Pgp and therefore may modulate cell chemosensitivity. We suggest that after degradation of polymer in phago-lysosomes, the free rifampicin induces death of *M. bovis BCG* and diffuses from phago-lysosomes to cytoplasm causing the dysfunction of mitochondria and modulating the Pgp activity. The increasing concentration of rifampicin-PLLA NPs increases the severity of the toxic effects on the macrophage cells.

Acknowledgments
The authors thank Dr. Valentina Factor for her very helpful suggestions in the preparation of this manuscript. This work was supported by the *Russian Science Foundation* (project №14-50-00029).

References

[1] Young D B, Perkins M D, Duncan K and Barry C E, 2008 *J. Clin. Invest.* 118 1255
[2] Dube D, Agrawal G P, Vyas S P 2012 *Drug Discov. Today* 17(13–14) 760
[3] Sosnik A, Carcaboso A M, Glisoni R J, Moretton M A and Chiappetta D A 2010 *Adv. Drug Deliv. Rev.* 62 547
[4] Shegokar R, Al Shaal L, Mitri K* J. Pharm. Pharmac. Sci.* 2011 14 (1) 100
[5] Mao S, Guo C, Shi Y and Li L C 2012 *Expert Opinion Drug Delivery* 9 1161
[6] Mao S, Guo C, Shi Y and Li L C 2012 *Expert Opinion Drug Delivery* 9 1209
[7] Sasaki T, Matsuura H and Tanaka K 2014 *ISRN Polymer Science* 2014 1
[8] Campbell E A, Korzheva N, Mustaev A, Murakami K, Nair S, Goldfarb A and Darst S A *Cell* 2001 104 901
[9] Bottcher T, Gerber J, Wellmer A, Smirnov A, Fakhrjanali F, Mix E, Pilz J, Zettl U K and Nau R 2000 *J. Infect. Dis.* 181 2095
[10] Bi W, Zhu L, Jing X, Liang Y and Tao E 2013 *Neurol. Sci.* 34 137
[11] Erokhina M, Kurynina A, Onishchenko G 2013 *Biochemistry (Mosc)* 78(10) 1473
[12] Kalluru R, Fenaroli F, Westmoreland D, Ulanova L, Maleki A, Roos N, Madsen PM, Koster G, Egge-Jacobsen W, Wilson S, Roberg-Larsen H, Khuller G K, Singh A, Nyström B and Griffiths G 2013 *J. Cell Sci.* 126 3043
[13] Anisimova Y, Gelperina S, Peloquin C and Heifets L B 2000 *J. Nanopart. Res.* 2 165
[14] Hirota K, Hasegawa T, Nakajima T, Inagawa H, Kohchi C, Soma G, Makino K and Terada H 2010 *J. Control. Release* 142 339
[15] Carmichael J, De Graff W, Gazdar A F, Minna J and Mitchell J 1987 *Cancer Res.* 47 936
[16] Stromskaya T, Rybalkina E, Shtil A A, Zabotina T N, Filippova N A and Stavrovskaya A A 1998 *Br. J. Cancer* 77 1718
[17] Erokhina M B, Aleksandrova E A, Prokopenko A V, Lepeka L N and Onishchenko G E 2009 *Tuberkulez Bolezni Legkikh* 11 49
[18] Bossy-Wetzel E, Goldberg M, Allen T, Barber M J, Green D R and Newmeyer D D 1999 *J. Cell Biol.* 147 809
[19] Scorrano L, Ashiya M, Buttke K, Weiler S, Oakes S A, Mannella C A and Korsmeyer S J 2002 *Developmental Cell*, 2, 55
[20] Izyumov D S, Domnina L V, Nepryakhina O K, Avetisyan A V, Golyshev S A, Ivanova O Y, Korotetskaya M V, Lyamzaev K G, Pletjushkina O Y, Popova E N, Chernyak B V 2010 *Biochemistry (Mosc)* 75 123
[21] Stavrovskaya A A 2000 *Biochemistry (Mosc)* 65(1) 95
[22] Bailly J, Muller C, Jaffrézou JP, Demur C, Gassar G, Border C and Laurent G 1995 *Leukemia* 9(5) 799