An effective Nano Drug Delivery and combination therapy for the treatment of Tuberculosis

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Abstract

Background

Drug-resistance in tuberculosis is exacerbating the threat this disease is posing for human beings. Antibiotics that were once effective against the causative agent, *Mycobacterium tuberculosis* (Mtb), are now no longer usable against multi- and extensively drug-resistant strains of this pathogen. To address this issue, new drug combinations and novel methods for targeted drug delivery could be of considerable value. In addition, studies have shown that the use of the antidepressant drug fluoxetine, a serotonin reuptake inhibitor, can be useful in the treatment of infectious diseases, including bacterial infections.

Method

In this study, an isoniazid and fluoxetine-conjugated multi-walled carbon nanotube nanofluid was designed to increase drug delivery efficiency alongside eliminating drug-resistance *in vitro*. The prepared nanofluid was tested against Mtb. Expression levels of *inhA* and *katG* mRNAs were detected by Real-time PCR. ELISA was applied to measure levels of cytokine secretion (TNF- α , and IL-6) from infected macrophages treated with the nano delivery system.

Result

The results showed that these nano-drug delivery systems are effective for fluoxetine at far lower doses than for free drug. Fluoxetine also has an additive effect on the effect of isoniazid, and their concomitant use in the delivery system can have significant effects in treating infection of all clinical strains of Mtb. In addition, it was found that the expression of isoniazid resistance genes, including *inhA*, *katG*, and the secretion of cytokines TNF α and IL6 under the influence of this drug delivery system is well regulated.

Conclusion

This nano-drug delivery method combined with host targeted molecules could be a game-changer in the development of a new generation of antibiotics that have high therapeutic efficiencies, low side effects and have the potential to overcome the problem of drug-resistance.

Introduction

According to the WHO reports, tuberculosis (TB) is among the top ten annual major causes of fatality and morbidity worldwide ¹. The disease is mainly caused by *Mycobacterium tuberculosis* (Mtb), a human pathogen that has been evolving to acquire resistance to drugs, leading to the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) phenotypes ^{2,3}. To prevent further spread of such strains, novel approaches in diagnosis and treatment are intensely needed.

Isonicotinic acid hydrazide or isoniazid (INH) has a strong antimycobacterial activity and is being used in clinic as the main first line agent since decades. The treatment however suffers from its length and presence of side effects. Emergence of bacterial resistance is also a detrimental issue that should be addressed to maintain the usability of this life-saving drug ^{4–7}. Two genes that are highly involved in INH resistance are *katG* and *inhA*, with the former coding for KatG, responsible for INH bio-activation ⁸ and the latter coding the target of the INH metabolite ⁹. Therefore, resistance to INH in *Mtb* is associated with mutations within these two genes ^{10,11}. Rapid sequencing of INH resistant strains showed significant differences in mutation rates from country to country. Differences in *katG* and *inhA* gene mutation rates between Beijing strains and non-Beijing strains could explain such discrepancies. For example, reverse hybridization studies successfully detected over 80% of INH-resistant strains among Korean isolates. Similar studies indicated that mutations in these two genes (*katG* and *inhA*) were found in 89.3% of the MDR and XDR isolates in Shanghai (China), 64% in Africa and about 70–90% in Iran ^{12–16}.

Assessment of different antidepressant drugs including fluoxetine (FLX) revealed the antibacterial feature of CNS drugs, when used alone or in combination with different antibiotics, mainly through inhibition of efflux pumps ¹⁷. Likewise, the reported synergic effects of FLX with gentamycin and erythromycin in treating MDR strains of *Pseudomonas aeruginosa* and *Escherichia coli* illustrate not only its antimicrobial activity but also its antibiotic modulatory role ¹⁸. In a recent review article, the collective impact of FLX with other antiviral drugs has been proposed as a promising treatment and preventative approach for coronavirus disease ¹⁹.

FLX is an approved serotonin reuptake inhibitor upregulating serotonin level through agonistic activity toward 5-HT_{2B} ²⁰, as well as 5-HT_{2C} receptors ²¹, both belonging to the G- protein coupled receptor family. When used in macrophages infected with *Mtb*, FLX was found to inhibit mycobacterial growth by enhancing TNF- α secretion and inducing autophagy in infected macrophages ²².

In terms of targeted drug delivery, conjugation of drugs with carbon nanotubes (CNTs) has drawn much attention with regards to their potential use in diagnosis and therapy of different diseases ^{23–25}. To circumvent multidrug-resistance in bacteria and reduce the dosage of drugs, nano-drug delivery via functionalized CNTs can be utilized. Studies have shown that both single-walled CNT (SWCNT) and multi-walled CNT (MWCNT) were able to hinder multidrug-resistance, exerting their antibacterial effect through cell wall destruction, induction of oxidative stress, and fracturing of the bacterial DNA or macromolecules ²⁶. This CNT system can also be formulated in the form of nanofluids or nanoparticle suspension as they have high dispersion stability and bioavailability, and, therefore, are a viable method for drug delivery ^{27,28}.

In our previous study, we developed a nano-drug delivery system for INH that displayed a higher antimicrobial effect *in vitro* against the reference laboratory strain of *Mtb* (H37Rv) compared to the pure drug ²⁹. The primary goal of this study was to investigate the possible antibacterial effect of the FLX and INH combination when used as a synthesized nano-drug delivery system, as compared to the pure drugs used in combination or alone on three different strains of *Mtb*: the reference laboratory strain H37Rv and two clinical isolates classified as MDR or XDR. In addition, our study aimed at exploiting MWCNTs, which have lower toxicity compared to SWCNTs ³⁰. Further, the gene expression profile of *inhA* and *katG* in all

treated groups was captured in an effort to understand the influence of these drug formulations on the bacterial resistance. Finally, variations in the level of TNF- α and IL-6, both pivotal markers of TB-induced inflammation, were analyzed in response to the treatment.

Materials And Methods

Preparation of INH, FLX and MWCNTs

4-pyridinecarboxylic acid hydrazide (INH) was purchased from Darou Pakhsh Pharmaceutical Co. (Tehran, Iran). Carboxylated (-COOH) MWCNTs were purchased from Neutrino Co. (Tehran, Iran) with specifications as presented in Table 1. Fluoxetine (FLX) was purchased from Abidi Pharmaceutical Co. (Tehran, Iran).

Chlorination of functionalized MWCNTs

A suspension of 200 mg MWCNT in 20 ml thionyl chloride (Merck, Germany) was refluxed for 14 hours at 60°C in a chemical hood. Then the chlorinated (COCl) MWCNT were dried at 60°C under the hood.

Drug conjugation

For loading the drug on CNT, a suspension of 100 mg chlorinated MWCNT and 300 mg drug (ratio 1:3 w/w) in 20 ml dimethylformamide (DMF) (Merck, Germany) was stirred for 20 minutes before sonication at 200 W in an ultrasonic device. The obtained fluid was incubated at room temperature for 6 hours, then refluxed at 70° C for 16 hours. This was followed by several stages of centrifugation (at 8,000 rpm, 15 min each time) and washing with tetrahydrofuran, methanol, and ethanol (96%). Finally, the precipitate was dried at room temperature.

Nano-drug characterization

To ensure binding of the drug on the CNT, elemental analysis (C-H-N-S) and Fourier transform infrared (FT-IR) analysis tests were carried out. Conjugated nanotubes and nanotubes alone were also observed and analyzed under scanning electron microscopic observation (SEM).

Nanofluid preparation

To a suspension of 200 mg nano-drug powder in 6 mL of ethanol (96%) was added 60 mg of Arabic gum dissolved in 100 mL of deionized water and the resulting mixture was stirred for 20 min. The mixture was then placed on ice and sonicated for 20 minutes at 200 W.

Stocks preparation and microbial testing

Mtb strains including the reference laboratory strain H37Rv, together with a MDR, and a XDR clinical isolates were obtained from the microbial bank of the Mycobacterium and Pulmonary Research Department, Pasteur Institute, Iran. For each strain, microbial suspension of 0.5 McFarland turbidity (equal to 1.5×10^8 bacteria) was prepared and bacterial strains were cultured in Lowenstein-Jensen culture medium.

Initial stocks of INH, FLX, MWCNT-INH and MWCNT-FLX were prepared with the first concentration of 1 mg/mL, 1 mg/mL, 2 mg/mL, and 2 mg/mL respectively. Then different dilutions of INH and FLX were prepared with the first concentration of 143, and 40 $\mu\text{g/mL}$ respectively. MWCNT-INH and MWCNT-FLX were prepared, starting from an initial concentration of 100 and 111 $\mu\text{g/mL}$ respectively. The summary of all dilutions and the corresponding concentrations is presented in Table 2.

Evaluation of the drug effects and their dose-dependencies were conducted on the bacterial strains using both the pure and conjugated forms of the drugs. At first, for determination of growth, a microplate-based assay which uses Alamar blue reagent was evaluated for all studied concentrations. Then each obtained results of MICs against Mtb H37Rv, MDR, and XDR strains were validated by tube assay test. Checkerboard assay was performed for the combination of MWCNT-INH and MWCNT-FLX. Observations were made based on the growth of bacteria after two to three weeks incubation.

Evaluation of synergy

The fractional inhibitory concentration (FIC) index was calculated to characterize quantitatively the activity of the antimicrobial combinations. The FIC represents the sum of the FICs of each drug tested. The calculation was done based on the following equation: $A / \text{MIC}_A + B / \text{MIC}_B = \text{FIC}_A + \text{FIC}_B = \text{FIC}$ Index, where A and B are the MIC of each antibiotic in combination (in a single well), and MIC_A and MIC_B are the MIC of each drug individually^{31,32}. The FIC Index value is then used to categorize the interaction of the INH and FLX, and also MWCNT-INH and MWCNT-FLX as follow:

FIC value < 0.5= synergy, > 4= antagonism, and 0.5-4= Additive or indifference.

Cell culture

Experiments were carried out with A549 and THP-1 human cell lines, which were provided by the National Cell Bank of Iran (NCBI) (Pasteur Institute, Iran). A549 and THP-1 cells were cultured at 37° C in DMEM and RPMI, respectively, supplemented with 10% of fetal bovine serum (FBS). The cells were seeded in 6 well plates and THP-1 cells differentiated in macrophages with PMA at a concentration of 50 ng/ μL for one day.

In vitro Mtb infection and cellular treatments

The supernatant was removed from each well and the cells washed with PBS, followed by addition of fresh culture medium. Cells were infected for 4 hours at 37°C, at a multiplicity of infection (MOI) of 10, with a

suspension of the MDR, XDR or the H37Rv bacterial strain, adjusted to a turbidity of 0.5 McFarland with RPMI medium.

Based on the results obtained from the tube-tests studies, further evaluations such as gene expression and cytokine quantification were performed. The cells were treated with INH, FLX, MWCNT-INH, MWCNT-FLX and combination of drugs and nano-drugs. In each 6 well plate, the different doses (1/512, 1/1024, 1/2048) of the INH, (1/32, 1/64, 1/128) of MWCNT-INH, (40, 60) of FLX, and (2, 4, 8) of MWCNT-FLX were repeated twice. At the end of the incubation period, the pellets and supernatants of treated cells were collected for molecular and cytokines studies.

Analysis of gene expression

RNA extraction was performed for cells treated with doses less than the obtained MIC value of each group. Total RNAs were extracted from tubes in which bacteria had less growth than the control using the Gene all kit (Cat. No. 301-001) according to manufacturer's protocol. Total RNA was transcribed into single stranded cDNA using the Easy™ cDNA Synthesis Kit (Pars Tous, Iran). Finally, Real-Time PCR was carried out using specific primer pairs as shown in table 3.

Cytokine quantification and statistical analysis

ELISA were performed on supernatant collected 24 hours after treatment to quantify the secretion of IL6 and TNF- α cytokines using specific kits (IBL international, Germany). The data were organized in Excel and analyzed by means of student T-test and ANOVA using Prism software (GraphPad). Results were considered significant when P-value was lower than 0.05.

Results

Characterization of the nano systems

3.1.1. Elemental analysis

The results of the elemental analysis conducted to ensure binding of drugs to the carbon nanotubes can be seen in Table 4. Naked carbon nanotubes does not contain any nitrogen, but detectable amounts were seen in drug-functionalized nanotubes, indicating effective binding of the drugs to the carbon nanotubes. Considering the mass content of azote (N) in 100 g of MWCNT, a loading of 0.6 mmol INH/g can be inferred for MWCNT-INH, and 1.4 mmol FLX/g for MWCNT-FLX. Because of minor CNT mass reduction due to centrifugation and washing, the carbon balance could not be used to estimate the loading.

Comparison of infrared spectroscopy of non-functionalized nanotubes with drug-activated nanotubes

The Fourier Transform Infrared (FTIR) spectrums of carboxylated and drug-functionalized nanotubes are shown in Figures 1-3. The peak at $\sim 1525\text{ cm}^{-1}$ corresponds to the C=C double bonds from the nanotube

wall. Also, the peaks at 3304 cm^{-1} (Figure 1) 3437 cm^{-1} (Figure 2) and 3433 cm^{-1} (Figure 3) correspond to the carboxylic acid group. The appearance of new peaks with lower wave number at $\sim 1678\text{ cm}^{-1}$ in figure 2 and $\sim 1630\text{ cm}^{-1}$ in figure 3 were assigned to amide bond. So The FTIR spectrum of MWCNTs-INH (Figure 2) and MWCNTs-FLX (Figure 3) confirmed the formation of amide groups on the MWCNTs surface.

Scanning electron microscopic results

Evidence for the functionalization of multi-walled carbon nanotubes can be obtained by SEM electron microscopy images. Figure 4 shows the SEM images of MWCNTs, MWCNTs-INH and MWCNTs-FLX. As visible on the figures, the drug-functionalized nanotubes have a different morphology and appear rougher compared to the naked nanotubes, which confirms that MWCNTs were functionalized with drugs.

Results of different types of treatment with INH, FLX, MWCNTs-INH and MWCNTs-FLX

The effective concentration which could be considered as MIC value for each treatment are summarized in table 5. The results obtained by bacterial growth observation for all treatment groups for H37Rv, MDR, and XDR strains is summarized below.

H37Rv treatments with the different doses of INH, FLX, INH+ FLX, MWCNTs-INH, MWCNTs-FLX and MWCNTs- INH+ MWCNTs- FLX

After incubation with INH for two weeks, a significant decrease in bacterial growth was observed from a dose of $1/1024$ ($0.14\text{ }\mu\text{g/mL}$) and no growth was observed from dilutions of $1/512$ ($0.28\text{ }\mu\text{g/mL}$) (Figure S1). Also, a significant reduction in growth was observed at a dilution of $1/64$ from MWCNTs-INH ($1.56\text{ }\mu\text{g/mL}$ containing $0.13\text{ }\mu\text{g/mL}$ INH) and no growth was observed at a dilution of $1/32$ ($3.125\text{ }\mu\text{g/mL}$ containing $0.26\text{ }\mu\text{g/mL}$ INH) (Figure S2). The efficacy results for FLX indicate that bacterial growth is stopped at dilutions of 40, (1.6 mg/mL) (Figure S3). The treatment results of MWCNTs-FLX showed that no growth was observed from dilution of 2 ($222\text{ }\mu\text{g/mL}$, containing $96\text{ }\mu\text{g/mL}$ FLX) (Figure S4). For Checkerboard treatments on H37Rv, the different concentrations of both drugs from dilution of 2 to $1/4$ for MWCNT-FLX and $1/64$ to $1/512$ of MWCNT-INH were tested. The bacterial growth was significantly reduced when the two nano drug systems were combined, with dilutions of $1/128$ ($0.78\text{ }\mu\text{g/mL}$ containing $0.067\text{ }\mu\text{g/mL}$ of INH) of MWCNTs-INH sufficient to prevent bacterial growth when used in combination with dilution of $1/2$ ($55.5\text{ }\mu\text{g/mL}$ containing $24\text{ }\mu\text{g/mL}$ of FLX) of MWCNTs- FLX (Figure S5). Checkerboard treatment on H37Rvwith FLX and INH was carried out in a previous study and results showed that dilutions of $1/256$ of INH ($0.56\text{ }\mu\text{g/mL}$) combined with dilution 20 of fluoxetine (0.8 mg/mL) inhibited bacterial growth. The antibacterial effect of MWCNT alone on H37Rv was observed at the concentration $228\text{ }\mu\text{g / mL}$ (Figure S6).

MDR & XDR treatments with the different doses of INH, FLX, INH+ FLX, MWCNTs-INH, MWCNTs-FLX and MWCNTs-INH + MWCNTs-FLX

For the MDR strain, INH at dilution of $1/128$ ($1.12\text{ }\mu\text{g/mL}$) was required to observe a significant effect on bacterial growth (Figure S7). Similarly for the XDR strain, INH at dilution of $1/128$ ($1.12\text{ }\mu\text{g/mL}$) had

significant effect on bacterial growth (Figure S8). Besides, growth of the MDR strain was observed at the dose of 3.2 mg/mL and only has few growth reduction at 1.6 mg/mL for FLX (Figure S9), while for the XDR strain only a slight growth reduction was observed at this dose of FLX (Figure S10). Results showed that bacterial growth of MDR and XDR strains were been inhibited at the dilution of 1/16 (6.25 µg/mL, containing 0.52 µg/mL of INH) and 1/8 (12.5 µg/mL, containing 1.04 µg/mL of INH) of the MWCNTs-INH respectively (Figures S11, S12).

MWCNTs-FLX has few antibacterial effect on MDR at dilution of 1/2 (55.5 µg/mL, containing 24 µg/mL of FLX) (Figure S13) and also on XDR at the dilution of 1/16 (6.9 µg/mL, containing 3.5 µg/mL of FLX) (Figure S14).

Checkerboard treatment results of MDR and XDR strains with INH and FLX showed that, in the treatment with dilution of FLX 20 (800 µg/mL) in combination with isoniazid in the dilution of 1/256 (0.56 µg/mL), the bacterial growth were stopped (Figure S15). Besides, in XDR treatment group at the dose of FLX 40 (1.6 mg/mL) in combination with INH at a dose of 1/128 (1.12 µg/mL) and also at doses of FLX 20 (800 µg/mL) in combination with INH at a dose of 1/256 (0.56 µg/mL), a significant reduction in growth was observed (Figure S16).

After the effect of MWCNTs-INH and MWCNTs-FLX, bacterial growth observation indicated that in the MDR strain, at the dose of 1/2 of MWCNTs-FLX (55.5 µg/mL, containing 24 µg/mL of FLX) in combination with the dose of 1/32 of MWCNTs-INH (3.125 µg/mL, containing 0.26 µg/mL of INH), no growth was observed. In the XDR strain group, the results showed that at doses of 1/16 of MWCNTs-FLX (6.93 µg/mL, containing 3.5 µg/mL of FLX) with a dose of 1/8 of MWCNTs-INH (12.5 µg/mL, containing 1.04 µg/mL of INH), bacterial growth was inhibited (Figure S17).

Quantitative Analyses by Fractional Inhibitory Concentration Index (FICI) for FLX and INH

According to the calculations of FIC index value for all states of action of isoniazid and fluoxetine in both free and conjugated form with carbon nanotubes, it was shown that in all strains, these two drugs have an additive effect on each other both in free and conjugated forms (Table 6).

Gene expression analysis and IL6 and TNFα secretion studies

The data analysis of gene expression in various treatment groups was carried out by the One Way ANOVA method and showed that the expression of both *inhA* and *katG* genes in all strains increased significantly in the presence of INH (Figure S18-S20). In the presence of FLX however, both gene expression was unchanged. For all strains tested, every treatment group involving INH saw a sharp increase in *inhA* expression, irrespective of the presence of FLX or the formulation in MWCNT. In contrast, the expression of *katG* was significantly reduced by combination therapy with free FLX, and the conjugated form of INH led to weaker levels of expression (Figure S18-S20). Results of the cytokine secretion studies showed that the levels of IL6 and TNFα secretion were significantly increased in all the treated groups, indicating that the drug treatment was able to induce pro-inflammatory pathways in TB-infected macrophages (Figure S21-S23), presumably due to efficient killing of the bacteria. The secretion of IL6 and TNF-α cytokines from TB-

infected macrophages were similar between free and MWCNT-conjugated drugs, indicating that the CNT vectorization strategy could maintain an efficient targeting of the intracellular bacteria.

Discussion

In this study, the main focus was on comparing the efficacy of nano-type drugs with that of the pure, free drugs and to evaluate the combination effects with host targeting agent like FLX, belonging to the group of serotonin reuptake inhibitors (SSRIs). INH is still one of the most active anti-tuberculosis agents, used for both the treatment and prevention of TB. Although INH is one of the safest and most cost-effective drugs for the treatment of TB, it causes many side effects in patients with high and long-term doses ⁷.

Selective serotonin reuptake inhibitors are antidepressants and have shown significant activity against bacterial resistance, but the antibacterial effects, as well as the antibiotic modulatory properties of FLX, are not yet clear. The results of previous studies showed that SSRIs drugs have significant antimicrobial activity against gram-positive bacteria, but are ineffective against Enterobacteria other than *Citrobacter* and *Pseudomonas aeruginosa* ³³. In this study, the mentioned effects of FLX were confirmed against different strains of Mtb ³⁴, including a MDR and XDR strain. Additional studies are needed to describe the antimicrobial properties of this drug as well as the clinical implications of its use in the treatment of infections by resistant microorganisms. On the other hand, the molecular mechanisms of INH resistance include several genes that are involved in several biosynthetic networks and pathways. Mutations in the *katG* and *inhA* genes are major factors in INH resistance, followed by *ahpC*, *kasA*, *ndh*, *iniABC*, and *fadE* ^{35,36}. Therefore, in this research, the expression of these two main genes (*inhA*, *katG*) was studied before and after drug treatments by Real-Time PCR. The results of this study showed that the expression of *katG* was significantly reduced by combination therapy with both free and conjugated drugs.

As a result of a study in 2018 on a large number of CNS drugs, a synthetic analogue of FLX was introduced as a selective and specific anti-tuberculosis agent and the antibacterial effects of this drug on the standard strain of tuberculosis (H37RV) were tested ³⁷. The results of some researches showed that the use of a series of small molecules called Host-targeted inhibitors could target TB-infected macrophages and stop the growth of Mtb. The use of drugs such as FLX, a selective serotonin reuptake inhibitor, can increase the secretion of pro inflammatory cytokines, induce autophagy in TB-infected macrophages and stop the growth of Mtb ^{38,39}.

On the other hand, nanotubes can be activated with groups that give them new properties so that they can be easily used in the environment. With the help of nanotubes and their attachment to drug compounds such as INH and FLX by chemical reactions, a new nano product was prepared. We showed here that these new compounds have equivalent biological activity and could be classified in the family of nano-drugs ^{23,24,40}.

Conclusion

In this study, using MWCNTs, a new form of the antibiotic INH and the drug FLX were made. This study aimed to increase the level of activity of the antibiotic INH against resistant bacteria and thus increase tolerance and reduce the spread of infection. Because the process of approving a drug is time-consuming and costly, these methods can be obtained sooner through combination therapies because these drugs are known for their pharmacokinetic profile, safety, are cheaper, more accessible, and can enter Phase 2 of the clinical trial faster. Also, with the increase in the number of people affected with MDR-TB, XDR-TB, and so-called totally drug-resistant tuberculosis (TDR-TB), new treatment strategies are needed that go beyond the conventional antibiotics. Targeting the host is one strategy considered in this study. However, extensive studies on the toxicity of these nanostructures need to be conducted.

Declarations

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Author Contributions

MSH: designed and supervised the study, performed experiments, interpreted data, wrote, read and approved manuscript; VD: interpreted data, read and approved final manuscript; ABK: interpreted data; VA: performed experiments; MM: performed the experiments; MS: interpreted data; NE, MM and SHP: performed the experiments.

Additional Information

Competing Interests

The authors declare no competing interests.

Availability of data and materials

All data generated or analysed during this study are included in this published article (and its supplementary information file).

References

1. Who. Global Tuberculosis Report. 2020. (2020).
2. Mabhula, A. & Singh, V. Drug-resistance in Mycobacterium tuberculosis: where we stand. *MedChemComm* **10**, 1342-1360 (2019).
3. Saifullah, B., Hussein, M. Z. B. & Al Ali, S. H. H. Controlled-release approaches towards the chemotherapy of tuberculosis. *International journal of nanomedicine* **7**, 5451 (2012).
4. Tafazoli, S., Mashregi, M. & O'Brien, P. J. Role of hydrazine in isoniazid-induced hepatotoxicity in a hepatocyte inflammation model. *Toxicology and applied pharmacology* **229**, 94-101 (2008).

5. Saifullah, B. *et al.* Development of a biocompatible nanodelivery system for tuberculosis drugs based on isoniazid-Mg/Al layered double hydroxide. *International journal of nanomedicine* **9**, 4749 (2014).
6. Erwin, E. R., Addison, A. P., John, S. F., Olaleye, O. A. & Rosell, R. C. Pharmacokinetics of isoniazid: The good, the bad, and the alternatives. *Tuberculosis (Edinburgh, Scotland)* **116s**, S66-s70, doi:10.1016/j.tube.2019.04.012 (2019).
7. Schaberg, T., Rebhan, K. & Lode, H. Risk factors for side-effects of isoniazid, rifampin and pyrazinamide in patients hospitalized for pulmonary tuberculosis. *European Respiratory Journal* **9**, 2026-2030 (1996).
8. Zhang, Y., Heym, B., Allen, B., Young, D. & Cole, S. The catalase—peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature* **358**, 591-593 (1992).
9. Banerjee, A. *et al.* inhA, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science* **263**, 227-230 (1994).
10. Musser, J. M. *et al.* Characterization of the catalase-peroxidase gene (katG) and inhA locus in isoniazid-resistant and-susceptible strains of *Mycobacterium tuberculosis* by automated DNA sequencing: restricted array of mutations associated with drug resistance. *The Journal of infectious diseases* **173**, 196-202 (1996).
11. Basso, L. A., Zheng, R., Musser, J. M., Jacobs, W. R., Jr. & Blanchard, J. S. Mechanisms of Isoniazid Resistance in *Mycobacterium tuberculosis*: Enzymatic Characterization of Enoyl Reductase Mutants Identified in Isoniazid-Resistant Clinical Isolates. *The Journal of Infectious Diseases* **178**, 769-775, doi:10.1086/515362 %J The Journal of Infectious Diseases (1998).
12. Park, Y. K. *et al.* Comparison of drug resistance genotypes between Beijing and non-Beijing family strains of *Mycobacterium tuberculosis* in Korea. *Journal of microbiological methods* **63**, 165-172 (2005).
13. Luo, T. *et al.* Selection of mutations to detect multidrug-resistant *Mycobacterium tuberculosis* strains in Shanghai, China. *Antimicrobial agents and chemotherapy* **54**, 1075-1081 (2010).
14. Haas, W. H. *et al.* Molecular analysis of katG gene mutations in strains of *Mycobacterium tuberculosis* complex from Africa. *Antimicrobial agents and chemotherapy* **41**, 1601-1603 (1997).
15. Doustdar, F., Khosravi, A. D., Farnia, P., Masjedi, M. R. & Velayati, A. A. Molecular analysis of isoniazid resistance in different genotypes of *Mycobacterium tuberculosis* isolates from Iran. *Microbial Drug Resistance* **14**, 273-279 (2008).
16. Khosravi, A. D., Goodarzi, H. & Alavi, S. M. Detection of genomic mutations in katG, inhA and rpoB genes of *Mycobacterium tuberculosis* isolates using polymerase chain reaction and multiplex allele-specific polymerase chain reaction. *Brazilian Journal of Infectious Diseases* **16**, 57-62 (2012).
17. Munoz-Bellido, J. L., Munoz-Criado, S. & Garcia-Rodriguez, J. A. Antimicrobial activity of psychotropic drugs: selective serotonin reuptake inhibitors. *International journal of antimicrobial agents* **14**, 177-180 (2000).
18. de Sousa, A. K. *et al.* New roles of fluoxetine in pharmacology: Antibacterial effect and modulation of antibiotic activity. *Microbial pathogenesis* **123**, 368-371 (2018).
19. Sheikhpour, M. The Current Recommended Drugs and Strategies for the Treatment of Coronavirus Disease (COVID-19). *Therapeutics and Clinical Risk Management* **16**, 933 (2020).

20. Peng, L., Gu, L., Li, B. & Hertz, L. Fluoxetine and all other SSRIs are 5-HT_{2B} agonists-importance for their therapeutic effects. *Current neuropharmacology* **12**, 365-379 (2014).
21. Ni, Y. G. & Miledi, R. Blockage of 5HT_{2C} serotonin receptors by fluoxetine (Prozac). *Proceedings of the National Academy of Sciences* **94**, 2036-2040 (1997).
22. Stanley, S. A. *et al.* Identification of host-targeted small molecules that restrict intracellular Mycobacterium tuberculosis growth. *PLoS Pathog* **10**, e1003946 (2014).
23. Sheikhpour, M., Golbabaie, A. & Kasaeian, A. Carbon nanotubes: a review of novel strategies for cancer diagnosis and treatment. *Materials Science and Engineering: C* **76**, 1289-1304 (2017).
24. Yang, W., Thordarson, P., Gooding, J. J., Ringer, S. P. & Braet, F. Carbon nanotubes for biological and biomedical applications. *Nanotechnology* **18**, 412001 (2007).
25. Kamazani, F. M., Siadat, S. D., Pornour, M. & Sheikhpour, M. A success targeted nano delivery to lung cancer cells with multi-walled carbon nanotubes conjugated to bromocriptine. *Scientific Reports* **11**, 1-15 (2021).
26. Mocan, T. *et al.* Carbon nanotubes as anti-bacterial agents. *Cellular and molecular life sciences* **74**, 3467-3479 (2017).
27. Sheikhpour, M., Arabi, M., Kasaeian, A., Rabei, A. R. & Taherian, Z. Role of nanofluids in drug delivery and biomedical technology: Methods and applications. *Nanotechnology, Science and Applications* **13**, 47 (2020).
28. Kamazani, F. M., Sotoodehnejad nematalahi, F., Siadat, S. D., Pornour, M. & Sheikhpour, M. A success targeted nano delivery to lung cancer cells with multi-walled carbon nanotubes conjugated to bromocriptine. *Scientific Reports* **11**, 24419, doi:10.1038/s41598-021-03031-2 (2021).
29. Zomorodbakhsh, S., Abbasian, Y., Naghinejad, M. & Sheikhpour, M. The Effects Study of Isoniazid Conjugated Multi-Wall Carbon Nanotubes Nanofluid on Mycobacterium tuberculosis. *International Journal of Nanomedicine* **15**, 5901 (2020).
30. Sheikhpour, M. *et al.* The Applications of Carbon Nanotubes in the Diagnosis and Treatment of Lung Cancer: A Critical Review. *International Journal of Nanomedicine* **15**, 7063 (2020).
31. Jain, S. N. *et al.* Antibiotic synergy test: Checkerboard method on multidrug resistant Pseudomonas aeruginosa. *Int. Res. J. Pharm* **2**, 196-198 (2011).
32. Lorian, V. *Antibiotics in laboratory medicine*. (Lippincott Williams & Wilkins, 2005).
33. Munoz-Bellido, J., Munoz-Criado, S. & Garcia-Rodriguez, J. Antimicrobial activity of psychotropic drugs: selective serotonin reuptake inhibitors. *International journal of antimicrobial agents* **14**, 177-180 (2000).
34. Ni, Y. & Miledi, R. Blockage of 5HT_{2C} serotonin receptors by fluoxetine (Prozac). *Proceedings of the National Academy of Sciences* **94**, 2036-2040 (1997).
35. Rawat, R., Whitty, A. & Tonge, P. J. The isoniazid-NAD adduct is a slow, tight-binding inhibitor of InhA, the Mycobacterium tuberculosis enoyl reductase: adduct affinity and drug resistance. *Proceedings of the National Academy of Sciences* **100**, 13881-13886 (2003).
36. Vögeli, B. *et al.* InhA, the enoyl-thioester reductase from Mycobacterium tuberculosis forms a covalent adduct during catalysis. *Journal of Biological Chemistry* **293**, 17200-17207 (2018).

37. Kumar, M. M. K., Madhavi, K., Mohan, T., Nagasree, K. P. & Sangeeta, G. Novel synthetic analogues of Fluoxetine as potent and selective anti-TB agents. *Journal of Applied Pharmaceutical Science* **8**, 107-115 (2018).
38. Schrlau, M. G., Falls, E. M., Ziober, B. L. & Bau, H. H. Carbon nanopipettes for cell probes and intracellular injection. *Nanotechnology* **19**, 015101 (2007).
39. Ladel, C. H. *et al.* Lethal tuberculosis in interleukin-6-deficient mutant mice. *Infection and immunity* **65**, 4843-4849 (1997).
40. Sheikhpour, M., Barani, L. & Kasaeian, A. Biomimetics in drug delivery systems: a critical review. *Journal of Controlled Release* **253**, 97-109 (2017).

Tables

Table 1. Specifications of carboxyl multi-walled carbon nanotubes (MWCNT-COOH).

Purity	>95%
-COOH content	3.86 wt%
The rate of surface carbon atom	8-10 mol%
Outer diameter	< 8 nm
Inner diameter	2-5 nm
Length	10-30 μm
Special surface area	> 500 m^2/g
Color	Black
Tap density	0.27 g/cm^3
True density	$\sim 2.1 \text{ g}/\text{cm}^3$
Electric conductivity	> 100 s/cm
Manufacturing method	CVD

Table 2. Dilutions and concentrations of pure and conjugated drugs used in this study

Dilution	Concentration ($\mu\text{g/mL}$)			
	INH	FLX	MWCNT-INH	MWCNT-FLX
40	-	1600	-	-
20	-	800	-	-
10	-	400	-	-
5	-	200	-	-
2	-	80	-	222
1	143	40	100	111
1/2	71.5	20	50	55.5
1/4	35.75	10	25	27.75
1/8	17.87	5	12.5	13.87
1/16	8.93	2.5	6.25	6.93
1/32	4.47	1.25	3.125	3.47
1/64	2.23	-	1.56	1.73
1/128	1.12	-	0.78	0.87
1/256	0.56	-	0.39	0.43
1/512	0.28	-	0.19	0.22
1/1024	0.14	-	-	-
1/2048	0.07	-	-	-

Table 3. Primer sequences used for RT-PCR

Target gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Product (bp)
<i>katG</i>	AGAAGAAGTACGGCAAGAAG	TACCGCTGTAACGCTCAT	183
<i>inhA</i>	CATCTCGGCGTATTCGTA	CGTCATCCAGTTGTAGGC	124
<i>16s rRNA</i>	AAGTCGGAGTCGCTAGTA	TACGGCTACCTTGTTACG	183

Table 4. CHNS elemental analysis results of isoniazid and fluoxetine nano systems

Sample Name	%C	%H	%N	%S
MWCNT	96.40	0.04	0.00	0.00
INH	60.00	6.02	34.70	0.00
MWCNT-INH	90.71	0.62	2.66	0.00
FLX	61.00	5.70	4.26	0.00
MWCNT-FLX	71.28	0.51	1.29	0.00

Table 5. Summary table of effective concentrations

Treatment	Strain		
	H37Rv	MDR	XDR
	<i>Concentration that inhibited bacterial growth($\mu\text{g/mL}$)</i>		
INH	0.28	1.12	1.12
FLX	1600	1600	1600
INH + FLX	0.56 + 800	0.56 + 800	0.56 + 800
MWCNT-INH	3.125 (INH 0.26)	6.25 (INH 0.52)	12.5 (INH 1.04)
MWCNT-FLX	222 (FLX 96)	55.5 (FLX 24)	6.93 (FLX 3.50)
MWCNT-INH + MWCNT-FLX	0.78 + 55.5 (INH 0.067 + FLX 24)	3.125 + 55.5 (INH 0.26 + FLX 24)	12.5 + 6.93 (INH 1.04 + FLX 3.50)

Table 6. FIC index calculation of treated groups

<i>Treatment</i> & <i>MIC/$\mu\text{g/mL}$</i>	<i>INH</i>	<i>FLX</i>	<i>INH+FLX</i>	<i>FICIndex</i>	<i>MWCNT-INH</i>	<i>MWCNT-FLX</i>	<i>MWCNT-INH + MWCNT-FLX</i>	<i>FIC Index</i>
<i>H37Rv</i>	0.28	1600	0.56+800	2.5	3.125	222	0.78+55.5	0.5
<i>MDR</i>	1.12	1600	0.56+800	1	6.25	55.5	3.125+55.5	1.5
<i>XDR</i>	1.12	1600	0.56+800	1	12.5	6.93	12.5+6.93	2

Figures

Figure 1

The infrared spectrum of carboxylated nanotubes.

Figure 2

The infrared spectrum of carboxylated nanotubes.

Figure 3

The infrared spectrum of nanotubes functionalized with FLX.

Figure 4

SEM images of the raw MWCNTs (Left), MWCNTs – INH (Right) and MWCNTs – FLX (Below).

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