Original Article

Enhancing Antimycobacterial Activity of Isoniazid and Rifampicin Incorporated Norbornene Nanoparticles

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Abstract

Background: Tuberculosis (TB) has been identified in skeletons over 6000 years old and still remains as the most prevalent infectious disease in the world; thus, there is a need for development of new drugs or tuning of old drugs. Nanotechnology, an advanced technology, plays a vital role in research for the diagnosis and treatment of TB, thus preventing adverse effects and drug resistance. The objective of this study was to enhance the antimycobacterial activity of isoniazid- (INH) and rifampicin (RIF)-incorporated norbornene (NOR) nanoparticles in comparison with plain INH and RIF without nanoparticles. **Methods:** Norbornene-polyethylene glycol – Isoniazid copolymer (NOR-PEG-INH) and norbornene polyethylene rifampicin Co polymer (NOR-PEG-RIF) were used for this study. The percentage of INH and RIF in NOR nanoparticles was 35% and 74%, respectively. Mycobacterium growth indicator tube containing Middlebrook 7H9 broth, the liquid medium, was used to analyze *in vitro* activity of the NOR-based drug and the plain drug. Minimum inhibitory concentration (MIC) of the drugs was determined from H37Rv control strain of mycobacterium TB (MTB). **Results:** The dosage of INH and RIF is minimal in the combination form with the NOR nanoparticles compared to the plain INH and RIF. The results indicate that the minimum concentration of NOR-PEG-INH and NOR-PEG-RIF required inhibiting H37Rv strain of MTB was 0.05 μg/ml and 0.5 μg/ml, respectively. The results were similar to plain INH and RIF MIC. **Conclusion:** Low dosage of INH and RIF along with NOR nanocarrier has similar activity to that of INH and RIF; thus this is expected to reduce adverse effects and NOR did not alter the functional activity of INH and RIF, thus becoming eligible for the newer drug carrier in TB treatment.

Keywords: Isoniazid, norbornene, rifampicin, treatment, tuberculosis

INTRODUCTION

The tuberculosis (TB) scourge shows continuous increase each year, thus proving itself as a top infectious disease in the world.[1] According to the World Health Organization (WHO) 2016 data, there were estimated 10.4 million TB cases worldwide, including 1.4 million new cases within a year.[2] India is now represented as the TB capital of the world with the highest TB cases.[3] Directly observed therapy short course (DOTS) was implemented by the WHO for treatment of TB, with the objective of TB control; in spite of a dramatic progress in TB case detection and treatment under the DOTS strategy, the reduction in the incidence rate of TB is only to a small extent.[4-6]

One of the major reasons behind the TB prevalence is patient incompliance in anti-TB treatment which directly increases the case of multidrug-resistant TB, relapse, and failure in patients. The main factors associated with patient incompliance are the adverse effects caused by two major first-line drugs isoniazid (INH) and rifampicin (RIF), which include ototoxicity, hepatotoxicity, neuropsychiatric manifestations, and hyperuricemia.[7]

The definitive goals of anti-TB chemotherapy are to inhibit or kill the actively multiplying tubercle bacilli, preventing acquired drug resistance and sterilization of infected host

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tissues to thwart clinical relapse.[8] With the currently available TB drugs, the goals cannot be fulfilled; thus, development of newer therapeutic technologies in treatment of TB is need of an hour.[9]

Since 1960, extensive research is going on in search of newer TB drugs, but still, we could find a better alternative for the powerful drugs INH and RIF. This shows that there is an ultimate need of modification of the older ones to increase the effectiveness of the treatment and improve the efficacy and compliance of current TB chemotherapy by changing the formulations, adding adjuvant or drug carriers, or using novel drug delivery systems.[10]

Nanotechnology gives the best platform for pharmacology through the designing of drug delivery systems which are able to target phagocytic cells infected by intracellular pathogens, such as mycobacteria.[11] Nanocarrier-based anti-TB therapy includes developing a controlled and sustained drug delivery system, increasing bioavailability of the anti-TB drug, reducing side effects, and enhancing blood circulation time.[12] Norbornene (NOR), a bridged cyclic hydrocarbon, is used as a carrier for effective delivery of TB drugs, and it has a multidrug compartment to carry INH and RIF along with a strong hydrophobic core.[13]

The *in vitro* drug release profile of NOR nanocarriers with drugs was validated using dialysis method, which demonstrated the feasibility of potential delivery of the drug from carrier in macrophage compartments whose pH was in the range of 4.7–5.5. Cytotoxicity assay and renal clearance assay were also made from NOR-derived copolymers, indicating that these nanocarriers are biocompatible in nature.[14] On liver cells, INH-conjugated NOR nanocarrier exhibited a more protective effect compared to INH, and normal histological tissue pattern was observed in liver tissues exposed to the INH-conjugated nanocarrier.[15] The cellular internalization property of the NOR nanocarrier was carried out in 4 T-cell line, indicating that these nanocarriers were easily internalized by the living cell.[16,17]

The Food and Drug Administration in 2000 approved NOR as indirect food additives and recommended the safe use of ethylene NOR copolymers.[18] Thus, our study aimed to check the enhancing antimycobacterial activity of INH and RIF at low concentration incorporated with NOR nanoparticles.

METHODS

Synthesis of norbornene-polyethylene glycol-isoniazid/norbornene-rifampicin-polyethylene glycol polymer

In two separate Schlenk flasks, a solution of known amount of NOR INH monomer/NOR RIF monomer and polyethylene glycol (PEG) monomer was dissolved in 2 mL of anhydrous dichloromethane–methanol (9:1 v/v%) solvent and kept under a nitrogen atmosphere. Into another Schlenk flask, the second-generation Grubbs' catalyst 0.21 mg (0.0002 mmol) was added, flushed with nitrogen, and dissolved in anhydrous dichloromethane (1 mL). All these three flasks were degassed

three times by freeze-pump-thaw cycles. NOR INH monomer was transferred to the flask containing Grubbs' catalyst using a cannula. The reaction was stirred at room temperature until the complete polymerization of INH monomer, and the solution of PEG monomer in anhydrous dichloromethane was transferred to the reaction mixture through cannula. The polymerization was allowed to stir for 8 h at room temperature while being protected from light. After completion of reaction, the polymers NOR INH copolymer and NOR RIF copolymer were precipitated in pentane. The polymer (product) was dissolved in tetrahydrofuran and passed through a neutral alumina column to remove Grubbs' catalyst. The polymeric nanoparticles were prepared by dissolving 1 mg of polymer in 1 mL tetrahydrofuran and drop-wisely added it to water (2 mL) and left it for overnight for slow evaporation of tetrahydrofuran.

The concentration of INH in NOR carrier was about 35%, and the concentration of RIF in NOR carrier was about 74%. The synthesized polymer along with anti-TB drugs was shown in Figures 1 and 2.[19] The synthesized polymer was observed under atomic force microscope, and the particles analyzed are shown in Figure 3.

Testing on H37Rv strain of mycobacterium tuberculosis

Standard H37Rv strain of mycobacterium TB (MTB) was received from reference laboratory and subcultured on Lowenstein–Jensen medium for further use. 2/3 loop of H37Rv was inoculated in Bijou bottles containing beads and 0.5 ml of double-distilled water. The bottle was vortexed and added 3.5 ml of double-distilled water, left for 15 min without disturbance. 0.5 ml of was inoculated in 5 ml of Middlebrook 7H9 broth and incubated for 7 days. The positive culture was further processed.

Mycobacterium growth indicator tube

800 μl of polymyxin, amphotericin, nalidixic acid, trimethoprim, and amoxicillin supplement was added to 7 ml of mycobacterium growth indicator tubes (MGITs), and 500 μl of H37Rv from Middlebrook 7H9 broth was added to tubes and then loaded in BACTEC MGIT and left undisturbed until positive signal comes. A fluorescent compound is embedded in silicone on the bottom of the tubes. The fluorescent compound is sensitive to oxygen. Large amount of actively respiring organism consumes the oxygen and allows the fluorescence to detect. The positive tubes contain approximately 10^5–10^6 colony-forming unit/milliliter.
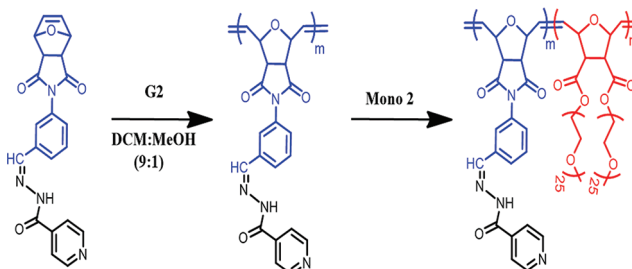

Figure 1: Norbornene + Isoniazid + polyethylene glycol copolymer

Figure 2: Norbornene + Rifampicin + polyethylene glycol copolymer

Figure 3: Atomic force microscopic pictures of norbornene-polyethylene glycol-isonizid copolymer/norbornene-rifampicin-polyethylene glycol copolymer
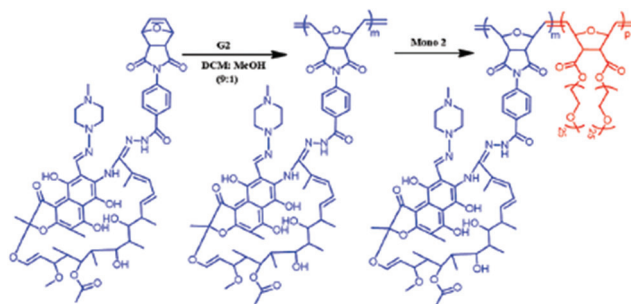
Drug concentration required for testing

Based on the results from drug sensitivity testing (DST) in solid Lowenstein–Jensen culture, seven different concentrations were chosen: they were 0.05–5 µg/ml for INH and NOR + INH + PEG and 0.5–32 µg/ml for RIF and NOR + RIF + PEG. NOR alone was used here as a control study, and three different concentrations were used for study which included 2.5–10 µg/ml. The concentration of the drug was calculated using the standard formula:

$$\text{Weight} = \frac{\text{Required volume} \times \text{Required concentration} \times 83}{\text{Drug potency}}$$

Drug susceptibility testing protocol

The DST was performed on the 4th day after the positive signal rose. The growth control (GC) was made by adding 0.1 ml of positive culture in 9.9 ml of saline (1:100 dilutions). The test culture was made by adding 1 ml of positive culture in 4 ml of saline (1:5 dilutions). 800 µl of SIRE supplement was added to tubes and 100 µl of drug was added to the respective tubes, and finally, 500 µl of culture was added to all the tubes and loaded in BACTEC MGIT. The tubes were recapped and mixed well. The tubes were set into the BACTEC MGIT 960 using the antimicrobial susceptibility testing set entry procedure. BACTEC MGIT 960 instrument will monitor susceptibility test sets until the growth unit of the GC reaches 400.

Quality control of pure NOR was made to three different concentrations and performed MGIT DST against H37Rv to check whether NOR has any antimycobacterial activity.

RESULTS

The results of INH and NOR + INH + PEG are shown in Table 1 and Table 2. Pure INH of 99.9% purity requires 0.05 µg/ml to inhibit the growth of H37RV strain of MTB, but NOR carrier along with 35% INH shows the same minimum inhibitory concentration (MIC) values.

The results of RIF and NOR + PEG + RIF are shown in Table 3 and Table 4. Pure RIF of 99.9% purity requires 0.5 µg/ml to inhibit the growth of H37RV strain of MTB, but NOR carrier along with 74% RIF shows the same MIC.
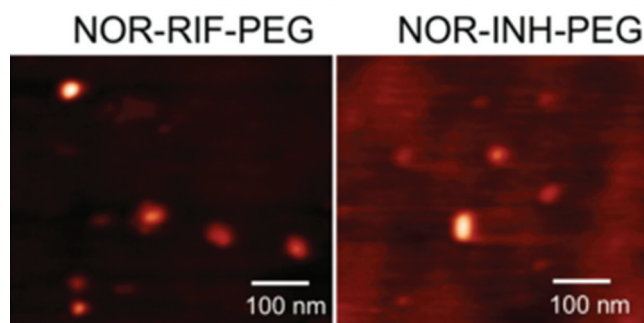
Table 1: Antimycobacterial activity of isoniazid on H37Rv

Drug name	Concentration in µg/ml	GU in BACTEC MGIT	Status
Growth control		400	Control (growth +)
INH (99.9% pure)	0.05	0	Sensitive (no growth)
	0.1	0	Sensitive (no growth)
	0.25	0	Sensitive (no growth)
	0.5	0	Sensitive (no growth)
	1	0	Sensitive (no growth)
	2.5	0	Sensitive (no growth)
	5	0	Sensitive (no growth)

MGIT: Mycobacterium growth indicator tube, GU: Growth unit, INH: Isoniazid

Table 2: Antimycobacterial activity of norbornene + polyethylene glycol + isoniazid on H37Rv

Drug	Concentration in µg/ml	GU in BACTEC MGIT	Status
Growth control		400	Control
NOR + INH + PEG (63% + 35% + 2%)	0.05	0	Sensitive (no growth)
	0.1	0	Sensitive (no growth)
	0.25	0	Sensitive (no growth)
	0.5	0	Sensitive (no growth)
	1	0	Sensitive (no growth)
	2.5	0	Sensitive (no growth)
	5	0	Sensitive (no growth)

MGIT: Mycobacterium growth indicator tube, NOR + INH + PEG: Norbornene + isoniazid + polyethylene glycol, GU: Growth unit

Norbornene as a control compound

NOR has a specific activity of cleaving in acidic pH and has multiple compartments to carry the different drugs. Some studies show that NOR itself has lipid disruption activity; thus to check whether NOR has antimycobacterial activity, NOR was tested with H37Rv.

DISCUSSION

The preparation of nanoparticles and analysis were similar to study done by Farnia *et al.*, 2016.[19] The results show that

Table 3: Antimycobacterial activity of rifampicin on H37Rv

Drug name	Concentration in µg/ml	GU in BACTEC MGIT	Status
Growth control		400	Control
RIF (99.9% pure)	0.5	0	Sensitive (no growth)
	1	0	Sensitive (no growth)
	2	0	Sensitive (no growth)
	4	0	Sensitive (no growth)
	8	0	Sensitive (no growth)
	16	0	Sensitive (no growth)
	32	0	Sensitive (no growth)

MGIT: Mycobacterium growth indicator tube, RIF: Rifampicin, GU: Growth unit

Table 4: Antimycobacterial activity of norbornene + polyethylene glycol + rifampicin on H37Rv

Drug name	Concentration in µg/ml	GU in BACTEC MGIT	Status
Growth control		400	Control
NOR + RIF + PEG (23% + 74% + 2%)	0.5	0	Sensitive (no growth)
	1	0	Sensitive (no growth)
	2	0	Sensitive (no growth)
	4	0	Sensitive (no growth)
	8	0	Sensitive (no growth)
	16	0	Sensitive (no growth)
	32	0	Sensitive (no growth)

MGIT: Mycobacterium growth indicator tube, NOR + RIF + PEG: Norbornene + rifampicin + polyethylene glycol, GU: Growth unit

low concentration of INH also has inhibitory effects, and this formulations enhanced 65% lesser concentration of the free drug in the presence of NOR nanocarrier. The results prove that nanoparticles conjugated with INH are active in *in vitro* condition, and thus, it has the ability to target the intracellular mycobacterium within macrophages.

One of our previous studies was on solid Lowenstein–Jensen medium by absolute concentration method. The same compounds were tested, but the results were varying that NOR + PEG + INH required high concentration for inhibition of MTB which was published by us earlier.[20] The result shows that low dosage of RIF could also have inhibitory effects in the presence of NOR nanocarrier.

RIF, the most powerful drug in TB therapy, has the maximum side effects compared to other anti-TB agents. Reduction of dosage without reducing the inhibitory effects is the need of an hour.[20] Thus, our study provides better results with low dosage of RIF. The results obtained from our study showed that NOR has antimycobacterial activity from 5 µg/ml; thus, it has a role in inhibition of MTB. NOR and its derivatives possess cell-penetrating peptides, thus expressing their antimicrobial activity.[21]

Macrophages infected with MTB produce an acidic Environment. Targeting the acidic condition and releasing the anti-TB drug is very important in drug delivery.[22,23] The nanoparticles used in this study also target the acidic macrophages to deliver the drugs.

Conclusion

According to the *in vitro* demonstration study results, even at lower dosage of two major drugs, INH and RIF show excellent results and revealed that NOR-based INH and NOR-based RIF act as good antimycobacterial agents which fulfill the need of drugs with low dosage. Reduced side effects were also proved from the different studies done on this polymer. As the INH and the RIF are the main drugs utilized for the treatment of TB infection and also responsible for MDR-TB. The nano-sized polymer with anti-TB drugs has an effective antimycobacterial activity, these nanodrugs can be included in TB treatment protocol. The *in vitro* activity shows excellent results, thus creating a platform of *in vivo* condition to check the targeted drug delivery.

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Conflicts of interest

There are no conflicts of interest.

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