

**LUCIFERASE REPORTER MYCOBACTERIOPHAGES FOR EVALUATING NORBORNENE-BASED ANTITUBERCULOSIS DRUG SUSCEPTIBILITY TESTING ON *MYCOBACTERIUM TUBERCULOSIS***KALAISELVI K<sup>1</sup>, MANGAYARKARASI V<sup>1\*</sup>, GOMATHI NS<sup>2</sup>, BALAJI S<sup>2</sup>, SHIVSHANKAR R MANE<sup>3</sup>, RAJA SHUNMUGAM<sup>3</sup><sup>1</sup>Department of Microbiology, SRM Medical College Hospital and Research Centre, Kattankulathur, Kanchipuram, Tamil Nadu, India.<sup>2</sup>Department of Bacteriology, National Institute for Research in Tuberculosis, Chetpet, Chennai, Tamil Nadu, India. <sup>3</sup>Department of Chemical Sciences, Polymer Research Centre, Indian Institute of Science Education and Research, Kolkata, West Bengal, India.

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**ABSTRACT**

**Objective:** In 2015, 9.6 million people around the world became sick with tuberculosis (TB) disease and 1.5 million TB-related deaths worldwide. Recent increasing incidence of multidrug-resistant (MDR; resistance to at least rifampicin (RIF) and isoniazid [INH]) and extensively drug-resistant (MDR resistance plus resistance to a fluoroquinolone and an aminoglycoside) makes TB a serious concern. Lot of research is needed to deal with this infectious disease for a better alternative in treatment or modification of these older TB drugs. The present study aimed at evaluating antimycobacterial activity of norbornene (NOR) derived INH copolymer with poly ethylene glycol (NOR- polyethylene glycol [PEG]-INH) a novel nanocarrier along with the anti-TB drug using luciferase reporter phages (LRPs).

**Methods:** NOR derived INH accounts for 74% of INH, 24% of NOR, and 2% of PEG. H37Rv control strain, a sensitive, and a resistant strain of *Mycobacterium TB* (MTB) used in this study. The *in vitro* activity of the drug was evaluated using absolute concentration method. The resistant strain was evaluated using LRP assay to observe the minimum inhibitory concentration of the drug.

**Results:** Reduction in light units was observed for the resistant strain exposed to plain INH and NOR-PEG-INH separately. 24% of reduction was observed in strains exposed to plain INH whereas 37% of reduction was observed in strains exposed to NOR-PEG-INH.

**Conclusion:** NOR-based INH had better antimycobacterial activity compared to plain INH and RIF. Antimycobacterial activity of INH and RIF increases even with very low dosage with NOR conjugate.

**Keywords:** Norbornene, Antimycobacterial activity, Minimum inhibitory concentration, *Mycobacterium tuberculosis*.

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**INTRODUCTION**

Tuberculosis (TB) is considered as the deadly disease of the past and present in the earth [1]. The current TB chemotherapy under directly observed therapy short course even though highly efficient, control of TB is highly challenging [2]. Long duration and complexity of the chemotherapy results in non-adherence to the treatment leading to crucial response such as failure or relapse or multidrug-resistance TB and extensively drug-resistant TB in patients [3]. Newer anti-TB drugs, namely, delamanid, bedaquiline, and pretomanid has a good synergistic effect but attempts to shorten the course of treatment with these alternative drugs resulted in severe adverse effects [4,5].

At present, there is a pressing need to improve treatment by either enhancing the application of existing agents with nanocarriers or introduction of the new drugs which shorten the treatment. The drug delivery systems have produced novel solutions to reduce the side effects and duration of treatment using these four drugs through nanotechnology [6].

Nanotechnology is a technology which uses materials of nanometer scale length (1-100 nm) can be used for a broad range of applications and the creation of various types of nanomaterials and nanodevices [7]. Nanotechnology has provided a huge development to pharmacology through the designing of drug delivery systems. Polymers act as a nanocarriers for drug delivery [8,9]. Norbornene (NOR) derived doxorubicin polymeric micelle with hydrazone

linker shows excellent performance inside the cell as an efficient drug delivery system and longer bioavailability. NOR derived thiobarbiturate homopolymeric symbolize themselves as promising carriers for the stimuli triggered intracellular delivery of hydrophobic drugs [10-12].

NOR derived anti-TB drugs can be a multi frontline TB drugs for potential therapeutic treatment. It has stability under physiological condition and depending on acidic pH condition the drug releases and has the feasibility of potential drug delivery in macrophage compartments [13]. The interaction of NOR along with isoniazid (INH) toward *Mycobacterium TB* (MTB) has to be studied detailed with drug susceptibility testing (DST). Various phenotypic methods are utilized for DST of MTB including absolute concentration method (ACM), proportion method, resistance ratio method, alamar blue assay, and luciferase reporter mycobacteriophage (MP) assay [14].

Luciferase reporter phage (LRP) assay involves viable mycobacteria infected with reporter phages (phAETRC21) expressing firefly luciferase gene. Light production requires metabolically active MTB cells, in which reporter phages replicate and luciferase gene will be expressed. Drug-susceptible MTB strains with specific anti-TB drugs, fail to produce light after infection with LRPs but drug-resistant strains are unaffected by the drugs and produce light similar to the strains which are not exposed to drug (control) [15]. The aim of the study was to evaluate the efficacy of NOR anti-TB drugs susceptibility testing on MTB using Luciferase reporter MP.

## METHODS

The plain nanocarrier with INH was procured from Department of Polymer Chemistry, IISER, Kolkata, India. INH attached to the NOR monomers through a stimuli responsive linker by covalent binding method. NOR covalent binding with INH is shown in Fig. 1.

H37Rv strain, of MTB received from National institute for Research in TB (NIRT) was used as the standard strain. The Institute Ethical Committee approval was obtained and informed consent were received from the TB patients. A sensitive strain and resistant strain were identified from the sputum received from the patients. H37Rv, sensitive strain and resistant strain were grown in Lowenstein Jensen (LJ) medium.

### Drug preparation

As per the ICMR MANUAL 20 mg of NOR-polyethylene glycol [PEG]-INH was dissolved in 20 ml of sterile distilled water to make up 10,000 µg/ml which serves as a stock solution, from the stock solution working solution was prepared by further dilution. The stock solution was maintained at -20°C, working solution was used for the incorporation of the drug into LJ's medium.

### ACM

To calculate efficacy of the drugs, DST was performed using ACM. The following concentration of anti-TB nanodrugs was used in this study; 0.025, 0.05, 0.1, 0.2, 0.5, 1, 2.5, and 5 µg/ml for INH. Drug-free media and media containing graded concentration of the drugs were inoculated with the standard strain H37Rv of MTB, sensitive strain and a resistant strain and readings were taken on 28<sup>th</sup> day [14].

### Phage propagation (MP)

MP buffer was prepared. 500 µl of phage buffer was added in cryovial. 50 µl of the phage was added in the first vial and 50 µl to the next. The phages were diluted up to the desired dilution in the same buffer. 500 µl of bacterial suspension (*Mycobacterium smegmatis*) equivalent to 2-3 McFarlands standard to the last three dilutions were added and incubated at 37°C for 30 minutes. After incubation, 200 µl from each dilution was taken and mixed with 5 ml of soft agar separately and poured on to the 7H9 base medium, the plates were incubated at 37°C for 18-24 hrs. The plates showing lacy pattern of plaque formation was chosen to harvest the phages. 5 ml of MP buffer was added to the plates, shaken for 2 hrs at room temperature in rotatory shaker (~ 80RPM). The buffer was aspirated and filtered using 0.45 µm membrane filter. The dilution was marked in which phages harvested with date and stored at 4°C [15].

### LRP DST

From the culture suspension prepared for DST LRP (DLRP), 100 µl was transferred to 4 sterile cryovials. 400 µl of 7H9 medium was added to all the four vials (Two vials serves as blank and third vial for INH and fourth vial for NOR+PEG+INH). Mix and incubate at 37°C for 72 hrs. 50 µl of phAETRC21 and 20 µl of 0.1 M CaCl<sub>2</sub> were added to all vials. Incubated at 37°C for 4 hrs. 100 µl of the cell-phage mixture was transferred to a luminometer cuvette. 100 µl of D-Luciferin was added and reduction in light units (RLU) was immediately measured at 10 seconds integration. Luminometer cuvette was discarded in to the lysol bath [16].

The percentage reduction in RLU was calculated using:

$$\% \text{ Reduction in RLU} = \frac{\text{Control RLU} - \text{Test RLU}}{\text{Control RLU}} \times 100$$

## RESULTS AND DISCUSSION

The nanocarriers with anti-TB drugs were validated using scanning electron microscope and atomic force microscopy for their size in IISER Kolkata [13]. INH was dissolved in distilled water.

Minimum inhibitory concentration (MIC) of three strains with plain TB drugs and NOR based TB drugs showed that NOR+PE+INH showed anti-TB activity with lower concentration. The LJ medium bottles

used for ACM were shown in Fig. 2. The significant antimycobacterial activity of NOR+PEG+INH of the sensitive strains and resistant strains in comparison with INH were explained in Table 1. NOR based nanocarriers have a capacity to interact with antituberculous drugs and form a copolymer. The presence of antimycobacterial activity of NOR+PEG+INH against MTB indicates that the formed copolymer does not affect the original mechanism of anti-TB activity of the drug INH. The copolymer and the anti-TB drugs directly interact with MTB the results were similar to that of another study done using analogue of INH [16].

In ACM NOR+PEG+INH on resistant strain, no inhibitory action was observed. INH loses their activity while binding to egg proteins, which are the ingredients of LJ medium [17]. Alternative DST method of MP with luciferin was used in this study. The plate which is used for harvesting phages was shown in Fig. 3. LRP has various advantages such as less time-consuming and do not involve any tedious

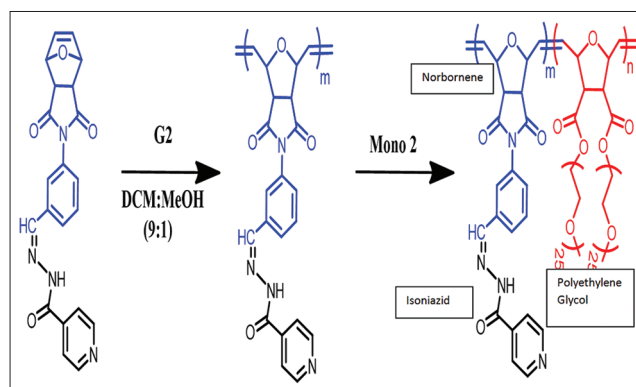


Fig. 1: Chemical structure of norbornene linked with isoniazid



Fig. 2: Drug susceptibility testing performed in Lowenstein Jensen medium using absolute concentration method

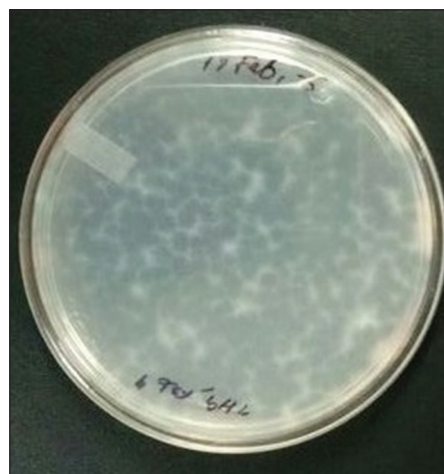


Fig. 3: Plate showing lacy pattern

**Table 1: MIC of plain and NOR conjugate with INH by ACM**

Control and clinical strains	MIC of Anti-TB drugs	
	Plain INH	NOR+PEG+INH
H37Rv	0.025 µg/ml	1 µg/ml
Sensitive strain	0.5 µg/ml	1 µg/ml
Resistant strain	2.5 µg/ml	5 µg/ml

TB: Tuberculosis, MIC: Minimum inhibitory concentration, NOR: Norbornene, PEG: Polyethylene glycol, INH: Isoniazid, ACM: Absolute concentration method

**Table 2: DST for the resistant strain using luciferase reporter MPs**

Strains	Solvent control		INH (0.2 µg/ml)	
	Control 1	Control 2	Plain INH	NOR+PEG+INH
Resistant strain	1269 RLU	750 RLU	760 RLU (24%)	625 RLU (37%)

DST: Drug susceptibility testing, INH: Isoniazid, NOR: Norbornene, PEG: Polyethylene glycol, RLU: Reduction in light units, MPs: Mycobacteriophages

work [18-20]. Emission of light in a sample infected with phages and supplemented with luciferin indicates the presence of viable bacterial cells [21]. In our study, viable cells were less compared to drug-free control; this proves that the compound is active.

LRP assay results shows that INH along with NOR has a better antimycobacterial activity compared to plain INH which is shown in Table 2 and proved that anti-TB drugs along with nanocarrier are stable under extracellular conditions. Strains exhibiting a reduction of less than 50% relative light units in the drug containing vial compared to control were classified as resistant. The resistant strain was inhibited by NOR+PEG+INH at a rate of 37% compared to plain INH which had an inhibition rate of 24% thus by further standardizing the inhibitory activity of NOR+PEG+INH can be increased at a higher rate.

Cytotoxicity assay of anti-TB drugs done in IISER proved that at lower concentrations of the drug, the cytotoxicity could not attribute to damage to cytoplasmic, endosomal and lysosomal membranes, and normal human cell death can be prevented when exposed to NOR [11]. The nanodrug conjugates have high drug loading efficiency up to 58-95%, so that more amounts of drugs which is proposed to act on target site can be loaded [22]. *In vivo* activity using Thp1 cell line for the promising drug delivery is in process.

## CONCLUSION

The antimycobacterial susceptibility testing of nanocarrier with anti-TB drugs revealed that NOR-based INH with 74% of INH has lower MIC value. The predominant anti-TB drug INH plays a major role in treatment of TB infection both in intensive and continuous phase. Thus, enhancing activity of INH with NOR conjugates appreciated as a welcoming step. The nanosized polymers with anti-TB drugs are expected on future treatment for TB infection.

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