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Antimycobacterial Activity of Norbornene-Polyethylene Glycol, Isoniazid and Rifampicin Nanocarrier towards *Mycobacterium tuberculosis*

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A B S T R A C T

The digital world that we live today is filled with innovations and advanced technologies, but still the role of medical sectors in preventing infectious diseases like Tuberculosis is feeble- minded. One of the advanced technologies so called Nanotechnology plays a vital role in research for the diagnosis and treatment of Tuberculosis, thus preventing side effects and drug resistance. The present study aimed at evaluating, Norbornene derived Isoniazid Co polymer (NOR-PEG-INH) and Norbornene derived Rifampicin Co polymer (NOR-PEG-RIF), a novel nanocarrier along with the drug for Tuberculosis with targeted drug delivery and longer bioavailability. The nanocarrier along with the drug preparation and their property were studied by the collaborated institute, Indian Institute of Science Education and Research (IISER) by covalent binding method. The invitro activity of the drug was evaluated using absolute concentration method to observe the Mininium Inhibitory Concentration (MIC) of the drug against H37Rv control strain of Mycobacterium tuberculosis (MTB) and clinical isolates of Mycobacterium tuberculosis from patient samples. The results showed that NOR-PEG-INH and NOR-PEG-RIF, the nanocarriers of Isoniazid and Rifampicin were able to show the lower MIC to inhibit Mycobacterium tuberculosis.

Introduction

India is the country with highest burden of Tuberculosis (TB) patients with 2. 2 million cases annually (WHO, 2015). The current tuberculosis treatment regimens under Directly observed therapy short course (DOTS) although highly effective, are far from ideal (Alliance, 2015).

Using the optimal combination of available four drugs (Isoniazid, Rifampicin, Pyrazinamide and Ethambutol) the duration of treatment required for curing patients cannot be reduced below 6 months (Richard *et al*., 2005). Long duration and complexity of the treatment results in non-adherence to

the treatment which leads to critical response like failure or relapse or Multidrug resistance Tuberculosis (MDR-TB) in patients (Jing *et al*., 2008).

Since 1960 new drugs for Tuberculosis are being developed but still we could not find a better alternative for the effective four drugs (Isoniazid, Rifampicin, Pyrazinamide and Etambutol). Isoniazid and Rifampicin plays an important role in inhibition and killing of Mycobacterium tuberculosis this indicates that lot of research is needed in this arena for a better alternative or modification of these two drugs. The development of newer TB treatment regimens have several limitations like the new compounds when they get to the animal testing stage the number of laboratories capable of in vivo testing is sadly minimal (Ormer, 2011). The new compound should show activity against drug sensitive and drug resistant mycobacterium, active against non replicating mycobacterium (Latent Tuberculosis) and absence of cross resistance with existing therapeutic agents for TB (Ming *et al.,* 2014).

Food and drug administration(FDA) and European Medicine Agency(EMA) has recently approved two anti- TB drugs namely Bedaquiline and Delamanid but the duration of treatment and side effects of drugs has to be studied further (Skripconda *et al*., 2013; Diacon *et al*., 2014). Currently there is an urgent need to improve treatment by either enhancing the application of existing agents with nanocarriers or introducing new drugs. The evolution of drug delivery systems has produced novel solutions to reduce the side effects and duration of treatment using these four drugs through nanotechnology (Svetlana, 2005).

Nanotechnology works on matter at dimensions in the nanometer scale length (1-100 nm) and thus can be used for a broad range of applications and the creation of various types of nano materials and nano devices (Pratima, 2015). Nanotechnology has provided a huge improvement to pharmacology through the designing of drug delivery systems able to target phagocyte cells infected by intracellular pathogens, such as Mycobacteria, for an example invitro study was done on Tyloxapol niosomes for encapsulation, stabilization and dissolution of anti-tubercular drugs formulation and the study showed that reduce dose-related drug toxicity but bioavaibility of the drugs was shorter (Mehta and Neha, 2013).

Norbornene derived doxorubicin polymeric micelle with hydrazone linker shows excellent performance inside the cell as an efficient drug delivery system and longer bioavailability (Shivashankar *et al*., 2014a). Amphilic Norbornene derived thiobarbiturate homopolymeric vesicles have great scope in the field of medicine as they symbolize themselves as promising carriers for the stimuli triggered intracellular delivery of hydrophobic drugs (Shivasankar *et al*., 2014a; Shivasankar *et al*., 2014b).

Narborornene derived antituberculosis drugs demonstrated that this can be a multi frontline TB drugs for potential therapeutic treatment (Shivasankar *et al*., 2011). 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 but the interaction of Norbornene along with isoniazid and rifampicin towards Mycobacterium tuberculosis has to be studied detailed (Vijayakameswara, *et al*., 2012).

Thus the aim of the study was to demonstrate Invitro antimycobacterial activity of Norbornene-Polyethylene glycol - Isoniazid and Rifampicin nanoparticles towards *Mycobacterium tuberculosis*.

Materials and Methods

The plain nanocarrires and the Isoniazid and Rifampicin drugs with nanocarriers were received from Department of polymer chemistry, IISER, Kolkata, India. Isoniazid and Rifampicin separately were attached to the Norbornene monomers through a stimuli responsive linker by Covalent binding method. Figure 1 and 2 shows the binding of anti-TB drugs with Norbornene.

Antimycobacterial susceptibility testing-Inoculum preparation

H37Rv strain of *M. tuberculosis* received from NIRT (National institute for Research in Tuberculosis) was used as the standard strain and 60 isolates from the patients who were clinically diagnosed with pulmonary tuberculosis were included in this study. The Institute ethical committee approval and consent from the pulmonary tuberculosis patients were obtained. Two sputum samples, spot sample and early morning samples were collected in a universal container to identify and isolate the organism.

The tubercle bacilli were identified in sputum samples by Ziehl–Neelsen staining and cultured on Lowenstein Jensens Medium (LJ) after purification with Modified Petroffs Method using 4 % NaoH. H37Rv, the control strain was grown within 3 weeks in LJ; other clinical isolates were grown in different period from 2 weeks to 8 weeks. All the Mycobacterium tuberculosis isolates were confirmed by Niacin, Catalase and Nitrate test with control medium of Para nitro benzoic acid (PNB) Medium

Drug preparation

As per the ICMR Manual 20 mg of NOR-PEG-INH and NOR-PEG-RIF was dissolved in 20 ml of sterile distilled water and 20 mg of Dimethyl formamide respectively to make up 10,000μg/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 Lowenstein Jensen's Medium.

Absolute concentration method

To calculate efficacy of the drugs, drug susceptibility test was performed using absolute concentration method. The following concentration of anti-TB nano drugs was used in this study; 0.025, 0.05, 0.1, 0.2,0.5,1,2.5, 5 μg/ml for INH 1,2, 4, 8, 16, 32, 64, 128μg/ml for RIF. Drug free media and media containing graded concentration of the drugs were inoculated with the standard strain H37Rv of MTB and clinical isolates of Mycobacterium tuberculosis. Readings were taken on 28th day (NIRT manual, 2010).

Control study

Antimycobacterial activity of Plain Norbornene, dimethyl formamide, polyethylene glycol were separately tested as a control with different concentration from 0.01 to 100 μg/ml using H37Rv strain of MTB to show the nanocarrier has any antimycobacterial activity.

Statistical analysis

The MIC results were presented as mean value. Parametric t test was used to employed to calculate the significance of difference between Plain Isoniazid: NOR-PEG-INH and Plain Rifampicin: NOR-PEG-RIF for clinical isolates of MTB and H37Rv strain of MTB. Data were analyzed at 95% confidence interval.

Results and Discussion

Minimum Inhibitory concentration tested from 0.01- 100 µg/ml. The nanocarrier showed different MIC on the control H327Rv strain explianed in Table 1.

Table 2 explains about the drug susceptibility testing of newer drug and plain drugs towards H37Rv strain of MTB Test were done repeatdly for two times to get better results. the MIC of Norbornene based INH was around 1 µg/ml and for plain INH was around 0.025 µg/ml. the MIC of Norbornene based RIF was around 4 µg/ml and for plain drug RIF was around 32 µg/ml.

Table 3 shows the Minimum inhibitory concentration (MIC) of clinical isolates with plain TB drugs and Norbornene based TB drugs. It was observed that NOR-PEG-RIF showed anti-TB activity with lower concentration . By using correleration study we found that there is no significant antimycobacterial activity of NOR-PEG-INH (P=.14) but there is a significant antimycobacterial activity of NOR-PEG-RIF (P= .00) in comprison with Rifampicin.

The result contains the Minimum Inhibitory Concentration obtained by absolute concentration method of antimycobacterial susceptibility testing of control strain of MTB, H37Rv and 60 clinical isolates of MTB with plain nanocarriers, plain INH and RIF and co polymer of nanocarrier with Isoniazid and Rifampicin.

Norbornene based nanaocarriers have a capacity to incorporate with antituberculous drugs and form a copolymer. So it is possible to demonstrate the antimycobacterial susceptibility activity invitro. The presence of antimycobacterial activity of INH-NPS against Mycobacterium tuberculosis indicates that the formed co-polymer does not affect the original mechanism of anti -TB activity of the drugs Isoniazid and Rifampicin activity. The copolymer and the anti TB directly interacts with Mycobacterium tuberculosis the results were similar to that of another study done using analogue of Isoniazid (Svetlana, 2005).

Isoniazid and rifampicin are the main drugs involved in MDR-TB, the nanocarrier of these drugs will inhibit the growth of the organism and thereby lower the MIC to use effectively as Anti-TB drugs. Also Norbornene nanocarrier with Rifampicin was found to be 3 times more active than plain Rifampicin.

If the minimum inhibitory concentration of the nanocarrier based drug is lesser than the cut off value of 64 µg/ml for Rifampicin, 0.2 µg/ml for isoniazid, then we can interpret that the organism is susceptible to the drug (NIRT Manual, 2010).

Table.1 Antimycobacterial testing of nanocarriers with H37Rv control strain of MTB

Componenents	Strain	MIC in (µg/ml)
Norbornene	H37Rv	25
Polyethylene glycol	H37Rv	100
Dimethyl formamaide	H37Rv	50

Table.2 Drug susceptibility testing of nanocarrier with drug and plain drugs towards H37Rv strain of MTB

Antimycobacterial drugs	Isoniazid Plain drug I	NOR-PEG-INH	Rifampicin Plain drug II	NOR-PEG-RIF
Concentration of drugs used in µg/ml	0. 025, 0. 05, 0. 1, 0. 2,0.5,1,2.5, 5	0. 025, 0. 05, 0. 1, 0. 2,0.5,1, 2.5,5	1,2,4,8,16,32,64,128	1,2,4,8,16,32,64,128
MIC of H37Rv strain (µg/ml)	0.025	1	32	4

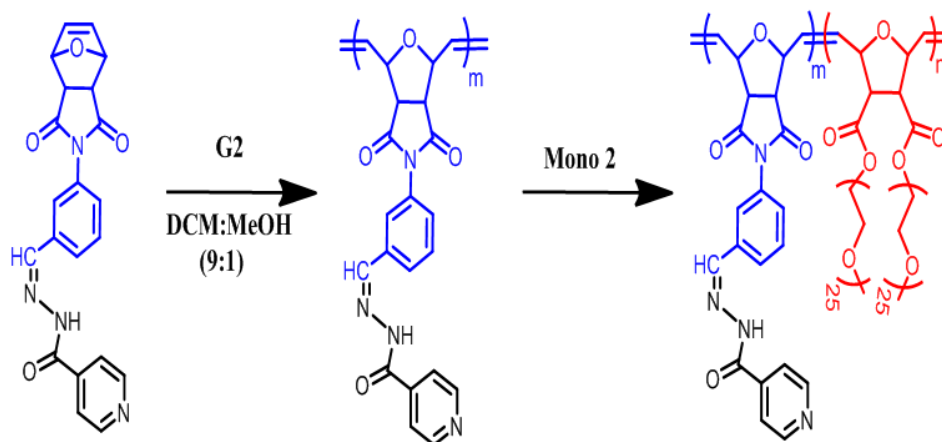NOR- Norbornene, PEG- Polyethylene glycol, INH- Isoniazid, RIF- Rifampicin

Test were done repeatdly for two times to get better results. the MIC of Norbornene based INH was around 1 µg/ml and for plain INH was around 0.025 µg/ml. the MIC of Norbornene based RIF was around 4 µg/ml and for plain drug RIF was around 32 µg/ml.

Table.3 Standardisation / Minimum inhibitory concentration (MIC) of clinical isolates with plain TB drugs and Norbornene based TB drugs (n= 60)

Drugs	Isoniazid Concentration in µg/ml								Rifampicin concentration in µg/ml							
	0.025	0.05	0.1	0.20	0.5	1	2.5	5	1	2	4	8	16	32	64	128
MIC of Plain TB Drugs for 60 clinical sample	2	18	23	3	7	2	3	2	0	0	32	0	19	4	2	3
MIC of Norbornene based TB drug for 60 clinical samples	4	3	4	1	12	2	21	13	10	16	15	8	5	4	1	1

It was observed that NOR-PEG-RIF showed anti-TB activity with lower concentration . We found that there is no significant antimycobacterial activity of NOR-PEG-INH ($P=.14$), but there is a significant antimycobacterial activity of NOR-PEG-RIF ($P= .00$) in comprison with plain Rifampicin.

Fig.1 Norbornene derived Isoniazid Copolymer
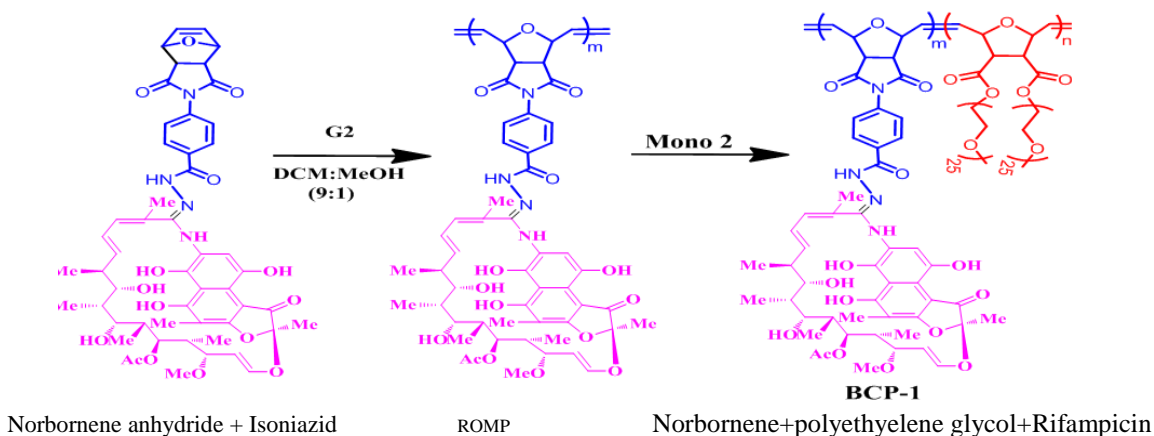

Norbornene anhydride + Isoniazid   ROMP   Norbornene+polyethyelene glycol+ Isoniazid

G2- Genetration 2, DCM- Dichloromethane, MeoH- Methanol, Mono2- Monomer 2, ROMP-Ring opening metatheses polymerization.

Fig.2 Norbornene derived Rifampicin Copolymer

Norbornene anhydride + Isoniazid   ROMP   Norbornene+polyethyelene glycol+Rifampicin

G2- Genetration 2, DCM- Dichloromethane, MeoH- Methanol, Mono2- Monomer 2, BCP-1-Block copolymer 1.

The MIC of Norbornene derived INH was around 1 µg/ml and for plain drug INH was around 0.025 µg/ml, the MIC of Norbornene derived RIF was around 4 µg/ml and for plain RIF was around 16 µg/ml. 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 (Tatiany, 2012).

The invitro study it is proved that anti-TB drugs along with nanocarrier are stable under extracellular conditions (Vijayakameshwari, 2012). Norbornene derived isoniazid has showed lower MIC compared to plain drug Isoniazed, Some of the drugs losses their activity while binding to egg proteins, the ingredients of LJ medium (Yu, 2011). Since Rifampicin shows lower MIC, Rifampicin resistance also called Multidrug resistance can be prevented by use of this nanocarrier which will additionally reduces the duration of treatment. Further studies are necessary to know the activity of Norbornene derived isoniazid and Rifampicin on lung tissue and cell line.

Cytotoxicity assay was done already (Shivasankar *et al*., 2014) and proved that at a lower concentrations of the drug, the cytotoxicity could not be attributed to damage to cytoplasmic, endosomal and lysosomal membranes, thus preventing normal human cell death.

In conclusion, the standardization of antimycobacterial susceptibility of nanocarrier with antituberculosis drugs revealed that Norbornene based isoniazid has lower MIC value thus it will gives better penetration into cell for inhibition. The MIC value was lower for Rifampicin with nanocarrier also for the inhibition. As the isoniazid and the Rifampicin are the main drugs utilized for the treatment of Tuberculosis infection and more concern on Multi drug Resistance Tuberculosis, if nanosized polymer with anti-tuberculosis drugs has an effective anti-mycobacterial activity then these drugs are of focus on future treatment protocol for Tuberculosis infection.

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