LUCIFERASE REPORTER ASSAY

Introduction

Firefly beetle [Photinus pyralis] luciferase is one of the popular reporter molecules used in molecular biology and biochemistry. Luciferase can be used to monitor promoter response activity in bacteria, cultured cells and transgenic plants or animals. The luciferase assay replaces the ¹⁴C chloramphenicol acetyl transferase assay because it possess lower cost, faster result and over a 1000 fold increase in sensitivity. Peak height and integrated total light output from a reaction is proportional to the amount of functional luciferase enzyme. If it is performed under optimal conditions, could result in a direct relationship between the amount of light emitted from the sample and the transcriptional activity of the regulatory elements (3).

The oxidative carboxylation of luciferin a known bioluminescence reaction is catalysed by firefly luciferase. At the optimal reaction pH of 7.8 light emission peaks at 562 nm. This form of light emission yields a very sensitive non-radioactive assay. Firefly luciferase is a 62,000 dalton protein which is active as a monomer and does not require subsequent process for its activity. Several factors may affect the sensitivity and success of the assay including pH, temperature, and substrate concentration. To ensure maximum sensitivity, the assay is performed in the presence of excess ATP, luciferin and Mg²⁺ in a buffer that will maintain a pH of 7.8.

Luciferase is extracted from transflect cells through cell lysis. A typical firefly luciferase assay is then carried out in an assay cuvette. ATP, Mg²⁺ and buffer are added to the lysate either separately or as a preformulated solution. The luminescent reaction is then
triggered by an injection of luciferin and the emitted light is recorded. When luciferin is added to sample containing luciferase, there is an immediate light flash that reaches peak intensity at 0.3-0.5 seconds that decays rapidly. This rapid exponential decay is caused by the reaction product, oxyluciferin which inhibits luciferase activity. Coenzyme A is added to overcome this rapid extinction. It displaces the inhibiting oxyluciferin product substrate from the enzyme.

**Caution**

Prior to reconstitution, all the reagent were stored at -20°C. It can be stored at -20°C for six months or at 4°C for 5 days. All the reagents were brought to room temperature before performing assay.

Infection with *Mycobacterium tuberculosis* affects much of the world's population, despite the fact that drugs for treating tuberculosis are available for over half a century. Each year, it is estimated that 9.2 million new cases appear, of which many leads to death. The World Health Organization has estimated that approximately 2 billion people worldwide are latently infected with *M. tuberculosis* and approximately 10% of them develop the active disease during their lifetime. In addition, tuberculosis is a frequent HIV co-infection and a major cause of death among people living with HIV-AIDS. In recent years, multidrug-resistant and extensively drug-resistant tuberculosis strains have emerged and so tuberculosis is considered as one of the most significant threats to global health.

Enoyl-acyl carrier protein reductase (ENR) is a key enzyme of the type II fatty acid synthesis (FAS) system. ENR is an attractive target for narrow spectrum antibacterial drug discovery because of its essential role in metabolism and its sequence conservation across many bacterial species. In addition, the bacterial ENR sequence and structural organization
are distinctly different from those of mammalian fatty acid biosynthesis enzymes [VIII]. So ENR inhibitors can be designed for the development of new and potent antitubercular drugs. Isonicotinic acid hydrazide (isoniazid, INH) belongs to the group of the first line antitubercular drugs in clinical practice over 50 years. INH is believed to kill mycobacteria by inhibiting the biosynthesis of mycolic acids, critical component of the cell wall. Thus, it was thought of interest to replace the pyridine ring of INH with its pyrimidine bioisoster.

The luciferase reporter mycobacterio phage technique has been described as an efficient system to decrease the time required for diagnosis and drug susceptibility testing of M. tuberculosis and other mycobacteria. The first luciferase reporter phage (LRP), phAE40 (10), was constructed from the mycobacteriophage TM4, a lytic phage able to infect mycobacteria of clinical importance including M. tuberculosis. However, the lytic nature of the phage produces a rapid mycobacterial cell lysis resulting in a loss of detectable light output and limiting the sensitivity of detection to 10^4 mycobacterial cells. In contrast, a second reporter phage, phGS18, made from the temperate phage L5 forms lysogens after infection of M. smegmatis, resulting in accumulation of the luciferase protein, sustained increases in light output and up to a 1,000-fold-improved limit of detection. However, L5 fails to efficiently infect and lysogenize M. tuberculosis limiting its clinical utility. D29, a relative of L5 does infect M. tuberculosis but like TM4 it is lytic, and the LRPs derived from D29 have similar sensitivity characteristics that the position of the luciferase gene within the phage genome affects the luciferase activity, that host-range mutants of TM4 that plaque more efficiently reduce luciferase activity, and that conditionally replicating mutants which plaque less efficiently produce more luciferase signal.

These constructs showed about a log increase in RLU output but still fell short in their sensitivity to be used for diagnosis. The poor sensitivity may be attributed to the lytic nature
of the existing phage constructs, which lyse the bacteria leading to rapid disintegration of ATP.

Furthermore, solubility plays an important role for the development of drug in tuberculosis. The docking study was performed to rationalize the possible interactions between the synthesized compounds and the active site. Compounds which were found promising in the docking study were evaluated for their antimycobacterial activity by luciferase reporter phage (LRP) assay method against *M. tuberculosis* H37Rv strain.

**MATERIALS AND METHODS**

Luciferase reporter phage (LRP) assay Standard strain H37RV, a clinical sensitive strain was grown in Middlebrook 7H9 complete medium 12 with and without test compounds for 3 days at 37°C. Luciferase Reporter Phage Assay was done using different concentration of test compounds. DMSO was used as the solvent control. LRP phage AETRC21 was added and the samples were incubated for four hours. Equal volume of the cell phage mixture was mixed with 0.3 Mm D-Luciferin in 0.05 M sodium citrate buffer of pH 4.5 and light output was immediately measured as RLU (Relative light units) in the luminometer at 10 seconds integration. Compounds exhibiting a reduction of 50% or more in RLU in the test vials compared to that of the control were considered to have anti mycobacterial activity. These LRP assays offer an elegant means of detecting viable mycobacteria and provide a rapid tool for drug susceptibility screening (2).

**RESULTS**

**Table 16. Luciferase reporter phage assay report**
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DISCUSSION

Compounds containing pyrimidine moiety have been reported as antiviral, antihypertensive, anticancer and antimicrobial etc. It has bioisosteric similarities with isoniazid. These observations led me to an attempt to choose pyrimidine derivative against the antitubercular activity. It was thought of interest to replace the pyridine ring of isoniazid with its pyrimidine bioisoster. This replacement followed the molecular docking of the designed compound into crystal structure of *Mycobacterium tuberculosis* enolyl reductase INHA [PDB ID: 2H7M] because it has better resolution over many other target. Compounds which were found promising in the docking studies were evaluated for their antimycobacterial activity by luciferase reporter phage assay method. Glide and molegro are the software chosen for docking. Since the entire target compounds contained some core moiety with little modification on the phenyl ring. The binding energy was found to be similar. A compound which is considered to be an active agent if 50 % reduction in the relative light units is observed when compared to control using a luminometer. Results of anti mycobacterial activity are summarised in the Table No. 16.
REFERENCES


6.  ICMR bulletin september 2002, vol 32, No 9 ISSN 0377-4910

