Polyphenolic acetates: A newer anti-Mycobacterial therapeutic option

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ABSTRACT: The objective of our research project was screening of various highly specific substrates of Acetoxy Drug: Protein Transacylase (M.TAase) for ant mycobacterial activity. Mycobacterial culture was done in Middlebrook’s 7H9 media. Protein purification (Mycobacterial Tranacetylase, M.TAase) was done by ion exchange chromatography and its demonstration was done on SDS- polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. Middlebrook’s 7H9 broth was procured from Becton Dickinson. CM-Sepharose, DEAE-Sepharose and Q-Sepharose were purchased from Amersham Pharmacia. Anti acetyl lysine polyclonal antibody was purchased from Cell Signaling. The Middlebrook 7H9 medium was used for M. smegmatis culture. The media was prepared according to the manufacturer’s instructions. The various Polyphenol acetate compounds were tested for their ant mycobacterial activities. Minimal inhibitory concentrations (MIC) were calculated by Alamar blue dye assay method. The GST protein was used as a receptor protein and purified Mycobacterial Glutamine Synthetase (GS) as TAase for acetylation by DAMC. To demonstrate the TAase catalyzed acetylation of GST by DAMC, purified M.TAase (GS) was preincubated with GST and DAMC followed by western blot using anti acetyl lysine antibody, which avidly react with the acetylated proteins. The growth pattern of M. smegmatis was diminished under the influence of various polyphenolic acetates (PA) tested for their anti mycobacterial activity. DAMC and DAMC-5-carboxylic acid was found to have MIC of 40µg/ml whereas DAMC-6-carboxylic acid was reported to have MIC value of 35µg/ml and for ellagic acid tetra acetate (EATA) it was 60µg/ml. Previous work in our lab has led to discovery of a novel enzyme acetoxy drug: protein transacetylase (TAase), catalyzing transfer of acetyl group from various polyphenolic peracetate (PA) to certain receptor proteins such as cytochromes P-450, NADPH cytochrome reductase, nitric oxide synthase (NOS) has been established in various eukaryotic as well as prokaryotic sources. PA(s) irreversible inhibitors of mammalian CYP linked MFO, possibly due to modification of cytochrome p-450 by acetylation in a reaction catalysed by M.TAase (GS) utilizing PA(s) as a donor of acetyl groups. Accordingly, it was hypothesized that the CYP51 of mycobacteria involved in the cell wall sterol synthesis could possibly be modified by our PA(s) through the novel unknown action of GS as transacetylase leading to the death of mycobacterial cell by way of acetylation catalyzed by acetoxy drug: protein transacetylase (M.TAase or GS).

KEY WORDS: Transacetylase; Glutamine synthetase; Mycobacterium smegmatis; Polyphenolic acetates; Acetoxy drug: protein transacetylase
INTRODUCTION

Mycobacterium tuberculosis causes more human death than any other single infectious organism. According to the WHO global Tuberculosis report 2011, there were 8.8 million (range, 8.5–9.2 million) incident cases of TB, 1.1 million (range, 0.9–1.2 million) deaths from TB among HIV-negative people and an additional 0.35 million (range, 0.32–0.39 million) deaths from HIV-associated TB. The prevalence of tuberculosis in India is 3100,000 and the mortality due to this deadly disease is 320,000.

An apparent synergy between the epidemiology of AIDS and tuberculosis, with the HIV pandemic has intensified the growing problem in TB control. Current anti-tubercular drug regimens are lengthy, usually lasting up to 6 months and includes 3-4 components resulting in patient’s poor compliance is one of the major factor causing multi-drug resistance TB. Due to multi-drug resistance, treatment of TB is entering a new challenging era where effective control requires the identification of new drugs and novel therapeutic targets.

Recently polyphenol acetate compounds have been a growing area of research to develop novel therapeutic agents to combat the emergence of drug resistance tuberculosis.

In our lab, a novel enzyme Acetoxyc Drug: Protein Transacetylase (TAase), catalyzing transfer of acetyl group from various polyphenolic peracetate (PA) to certain receptor proteins such as cytochromes P-450, NADPH cytochrome reductase, nitric oxide synthase (NOS) has been established. Recently, TAase has been purified to homogeneity from rat liver and human placenta.

The N-terminal amino acid sequence analysis of TAase when aligned with nonredundant Swiss -Prot Database, sequence revealed a perfect match with N-terminal sequence of mature Calreticulin, a prominent Ca2+ binding protein of endoplasmic reticulum. Further studies confirmed the identity of TAase as Calreticulin Transacetylase (CRTAase).

Again, studies were undertaken in our lab to examine the existence of TAase in prokaryotic organism i.e. M. smegmatis. The N-terminal sequence of purified TAase from M. smegmatis was found identical with Glutamine Synthetase (GS). Since nothing was known whether GS of M. smegmatis has TAase activity.

The objective of our research project was screening of various highly specific substrates of TAase for antimycobacterial Acitivity.

METHODOLOGY

The study was conducted jointly in Department of Biochemistry, VP Chest Institute and Department of Chemistry, University of Delhi, India.
(Sorvall), for obtaining the cell free extracts. The supernatant was taken and the pellet containing the cell debris was discarded. The supernatant thus obtained was centrifuged at 42,000 rpm for 2.30 hr. in ultracentrifuge (Beckman). The pellet was kept for further use. The pellet was dissolved in minimum amount of 0.1 M NaH$_2$PO$_4$, according to the pellet obtained. The sample was stored at 20°C.

**Demonstration of Mycobacterial GS catalyzed protein acetylation by DAMC using anti acetyl lysine antibodies**

The acetylation or acetylated lysine residues were detected by using Anti acetyl lysine Polyclonal antibody. Purified GS (50 µg) was incubated with GST as a receptor protein, DAMC (100 µM) and 0.25 potassium phosphate buffer (pH 6.5) and incubated for 30 minutes at 37°C in water bath. After the completion of reaction, sample buffer (loading dye) was added to the reaction mixture to stop the reaction. This reaction mixture was used to detect the acetylated protein using western blot. For western blot, electrophoretically separated proteins were transferred onto nitrocellulose sheets at 300mA for 3 hrs at 4°C. Non-specific sites on the nitrocellulose sheet were blocked with blocking reagent. Primary antibody dilution was prepared in TBST containing 1% BSA and incubation was carried out at 4°C for 12 hrs with shaking at intervals. The nitrocellulose sheets were extensively washed with TBST (TBS with 0.05% Tween). Goat anti-rabbit-HRP (horseradish peroxidase) conjugated secondary antibody (Bangalore Genie), appropriately diluted in TBST (TBS with 0.05% Tween).  After 5 minutes incubation reagents were drained, and sheets were kept in water.

**Screening of various polyphenolic acetates against Mycobacterium smegmatis**

Several compounds synthesized in our laboratory were tested for their antimycobacterial activity. The compounds were tested against *Mycobacterium smegmatis*. The mycobacterium was grown in Middlebrook's 7H9 media, depending on the growth conditions of the bacteria, which gave an optimum growth of the test bacteria. Cell suspensions were prepared and diluted in 7H9 Middlebrook's liquid broth so that their turbidities matched that of McFarland no.1 turbidity standard. Each compound was dissolved in Dimethyl sulfoxide (DMSO 10mg/ml) sterilized by filtering through membrane filters (0.25 µm). Stock solutions were diluted in 7H9 broth to two times the maximum desired final testing concentrations prior to their addition to micro plates. 200µl of sterile deionized water was added to all outer perimeter wells of sterile 96-well (round bottom) plates (Tarsons) to minimize evaporation of the medium in the test wells during incubation. The wells in rows B to G in columns 3 to 11 received 100µl of 7H9 Middlebrook's broth. 100µl of 2X drug solutions were added to the wells in rows B to G columns 2 and 3. By using an autopipette, 100µl was transferred from column 3 to 4 and the contents of the wells were mixed well. Identical serial 1:2 dilutions were continued through column 10, and 100µl of excess medium was discarded from the wells in column 10. Final drug concentration ranges were 10 to 100µg/ml. 100µl of *M. smegmatis* inoculum was added to the wells in rows B to G in columns 2 to 11. Thus the wells in column 11 served as drug free (inoculum only) controls. The plates were sealed with Parafilm and were incubated at 37°C for 72 hrs. Fifty microliters of a freshly prepared 1:1 mixture of 10X Alamar Blue reagent and 10% Tween 80 was added to all the wells. Color changes were recorded visually after 24 hrs. Blue color denotes no growth whereas pink color shows growth.

**RESULT**

**Demonstration of Taase catalyzed acetylation of proteins by DAMC**

The GST protein was used as a receptor protein and Mycobacterial GS as Taase for acetylation by DAMC. To demonstrate the Taase catalyzed acetylation of GST by DAMC, purified Taase was preincubated with GST and DAMC followed by western blot using anti acetyl lysine antibody, which avidly react with the acetylated proteins. **Figure 1** shows Taase catalyzed acetylation of Glutathione-S-transferase by DAMC.

![Acetylated GST](image)

**Figure 1**: GS (MTase) catalysed acetylation of GST by PA (DAMC)

*Western blot analysis using anti-acetyl lysine polyclonal antibodies*

**Lane 1** Protein markers; **Lane 2** GST+DMSO; **Lane 3** GST + GS + DAMC; **Lane 4** GST +DAMC
Growth inhibition of *Mycobacterium smegmatis* under the influence of various PA

The growth pattern of *M. smegmatis* was diminished under the influence of various PA tested for their anti-mycobacterial activity. DAMC and DAMC-5-carboxylic acid was found to have MIC of 40µg/ml whereas DAMC-6-carboxylic acid was reported to have MIC value of 35µg/ml and for ellagic acid tetra acetate (EATA) it was 60µg/ml (Figure 2).

**DISCUSSION**

The role of protein modification in control of cellular processes is well known. Among protein modification, the acetylation of protein assumes the crucial role in cellular metabolism. The acetylation of protein is catalyzed by Acetyl CoA-specific acetyl transferases. The importance of histone acetyl transferases, p53 specific acetyl transferase etc. are some of the well known examples of enzymatic protein acetylation involving Acetyl CoA as the acetyl group donor.

The earlier work carried out in our laboratory on the biochemical action of polyphenolic acetates, PA(s) focused our attention on the role of enzymatic acetylation of protein independent of Acetyl CoA. PA(s) were found to acetylate certain proteins mediated by an enzyme localized in rat liver microsomes. Since, a large number of polyphenolic acetates were found to be substrates, the aforementioned enzyme was named Acetoxy drug: protein Transacetylase (TAase). An assay procedure for TAase was developed based on the irreversible inhibition of Glutathione-S-transferase (GST) by PA, the extent of inhibition of GST was considered proportional to the activity of TAase.

Using this assay procedure, TAase was purified to homogeneity from buffalo liver, rat liver, human placenta and buffalo lung. Then the efforts were made to examine the possible existence of TAase in prokaryotic organisms and *Mycobacterium smegmatis* was the chosen organism. Considerable TAase activity was found in the cell free extract of *M. smegmatis*.

In-depth studies were conducted to decipher the protein identity of M. TAase. For this purpose, the N-terminal sequencing of purified M. TAase was carried out. Further, the N-terminal sequence of M. TAase when aligned with non-redundant Swiss Prot database revealed 100% identity with N-terminal sequence of Glutamine Synthetase (GS) of *M. smegmatis*.

The present study was carried out to screen various polyphenol acetates (PA) for their antimycobacterial activity. GS catalyzed protein acetylation was further confirmed by demonstration of acetylation of a Receptor proteins such as GST when incubated with DAMC at 37°C followed by Western blot using anti-acetyl-lysine antibody which avidly react with acetylated receptor GST protein (Figure 1). Thus, we concluded that TAase activity is a novel, unknown function of GS.

The observation of various investigations highlighted CYP51 linked MFO as the crucial enzyme involved in sterol biosynthesis and could serve as a suitable target for the development of drug candidates. Sterol 14α-demethylase of *M. tuberculosis* was found to be CYP51 linked MFO. Several well-known azoles, which were known to be the inhibitors of fungal CYP linked MFO, were considered as suitable drugs to target sterol 14α-demethylase of *M. tuberculosis*. The earlier work carried out in our laboratory projected PA(s) as irreversible inhibitors of mammalian CYP linked MFO, possibly due to modification of p-450 by way of acetylation catalyzed by acetoxy drug: protein Transacetylase.

Accordingly, it was hypothesized that the CYP51 of mycobacteria involved in the cell wall sterol synthesis could possibly be modified by our PA(s) through the novel unknown action of GS as transacetylase leading to the death of mycobacterial cells. The result of the various investigations conducted in our lab substantiated the TAase activity of the purified GS of *M. smegmatis*. Hence, it was thought pertinent to examine whether TAase of mycobacteria utilizing PA(s) as a donor of acetyl group could be culminating in the acetylation of mycobacterial CYPs.

Such an approach was thought novel and innovative to examine the anti-mycobacterial effects of PA. We then set forth to screen, in the first instance, to examine several acetoxy coumarins for their ability to inhibit the growth of *M. smegmatis*. The DAMC-6-carboxylic acid gave a surprisingly low MIC value of 35µg/ml in comparison to the 40µg/ml given by DAMC. The order in which the PA were effective against mycobacterial growth can be evaluated as 7,8-DAMC-6-carboxylic acid >EATA (Figure 2).
CONCLUSION

To conclude from our research project, purified mycobacterial GS exhibited Protein: acetyltransferase (TAase) activity. The TAase activity of Mycobacterial GS was found useful to target certain receptor protein by PA in Mycobacteria. PA(s) were effective against mycobacterial growth in the following order: 7,8-DAMC-6-carboxylic acid > 7,8-DAMC > 7,8-DAMC-5-carboxylic acid > EATA. The action of PA(s) could possibly be utilized in the development of effective anti-TB drugs in future.

Further study with more number of acetoxy drugs and their antimycobacterial effect on human tuberculosis (M. tuberculosis, M. bovis) is needed.

REFERENCES