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Mycobacterial Excretory Secretory-31 (ES-31) Protein with Serine Protease and Lipase Activities- A Potential Drug Target against TB Infection

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Abstract

Tuberculosis is the second leading cause of death worldwide. The researchers still finding way to eradicate TB. There is an urgent need to discover new drug which is more effective and less toxic to combat drug resistance. This article reports potential of ES-31 antigen as a new drug target. The characterization of ES-31 antigen showed that ES-31 is a 31 kDa protein antigen and has serine protease as well as lipase activities and shown to be a chymotrypsin-like protein which is having catalytic triad responsible for both activities. Addition of serine protease inhibitors (53-76%), metallo protease inhibitor (46-61%), lipase inhibitor (61%) or anti-ES-31 serine protease antibody (89%) strongly inhibited the MTB H37Ra growth in axenic culture. The importance of excretory secretory ES-31 antigen for the survival of MTB H37Ra and H37Rv bacilli has been shown by 77% and 78% growth inhibition in macrophage culture by protease inhibitor pefabloc. Inhibition of ES-31 leads to growth inhibition of MTB bacilli, suggests that it may be an important drug target for exploring new drugs for tuberculosis.

Keywords: Excretory secretory protein antigens; ES-31; Drug target; Serine protease; Lipase

Introduction

Tuberculosis (TB) has been the major cause of morbidity and mortality throughout the world. Even after declaration of TB as a global emergency by World Health Organization in 1993, it continues to be serious health problem with 9.6 million new patients and 1.5 million deaths reported in the year 2014 including 0.4 million deaths among human immunodeficiency virus (HIV) positive individuals [1]. The major problems responsible for the dreadful situation are lack of precise diagnosis and failure in control program for various reasons.

Although the existing drug treatment has immense value in controlling the disease. It also has some pitfalls, major being "drug resistance in tuberculosis". Increasing resistance to anti-tuberculosis drugs has driven the urge for developing new drugs. Prophylactic BCG vaccine failed to reduce the prevalence of infection in adults in the countries where the disease is endemic [2]. Most available ant mycobacterial drugs do not act upon latent forms of *Bacillus*. HIV-TB co-infection is again the complication which adds a new challenge in TB control and hence there is need for more effective and less toxic drugs. Unexpectedly development of new anti-TB drugs with good therapeutic or prophylactic potential has not been progressed, though there is urgent need to prevent mortality and morbidity related to tuberculosis.

Mycobacterium tuberculosis bacilli are known to secrete number of proteins which play important role in pathogenicity and also have been explored for prophylactic and diagnostic usefulness. In *Mycobacterium tuberculosis*, conserved general secretion (Sec) and twin-arginine translocation (Tat) the two pathways perform the major protein export, it also possess specific pathway for specific subset of proteins. The export of protein is essential for MTB growth and survival. Targeting SecA2, ESX and ESX3 pathways will lead to development of new drug against TB [3]. The most popular group of secreted proteins of *Mycobacterium tuberculosis* is Ag 85 complex. The role of these proteins is being illustrated as essential aspect of immune response after infection, development of protective immunity and in complications of the disease [4]. For more than two decades, our laboratory has been actively involved in immune screeing of both pulomonary and extra pulmonary tuberculosis using various ES proteins. In this context many excretory secretory proteins were isolated, characterized and were being evaluated for serological diagnosis routinely in 1000 bedded Kasturba Hospital attached to Mahatma Gandhi Institute of Medical Science. ES-31, ES-41, ES-43, ES-6, ES-20, ES-100 and EST-6 [5-7] are extensively being studied extensively and found to be essential in screening suspected cases of tuberculosis infection. Among all these proteins, ES-31 protein was observed to be a good immunogenic and diagnostic marker in pulmonary and extra pulmonary tuberculosis [8,9]. Further, ES-31 protein showed serine protease and lipase activities with good drug target potential [10,11].

Isolation and Characterization of ES-31 Antigen

M. tuberculosis H37Ra bacilli were grown in thyroxine supplemented Sauton medium for 10 days to obtain culture filtrate proteins [12]. ES-31 was isolated by ammonium sulphate precipitation followed by SDS-PAGE and FPLC cation exchange [13]. ES-31 can also be isolated by affinity chromatography using anti ES-31 antibody (raised in goat) coupled to the Sepharose 4B column (1 cm) [13]. In short, culture filtrate protein (1 mg) was applied to the column and washed with 0.01 M phosphate buffer saline (PBS pH 7.2). Bound

ES-31 serine protease was eluted with Glycine-HCL buffer (0.01 M, pH 2.5), neutralized with Tris-HCL buffer (0.01 M, pH 8.6), concentrated and stored at -20˚C [13]. ES-31 showed protease activity under non reducing conditions using 10% SDS-PAGE gels copolymerized with 0.1% bacteriological gelatin [13]. ES-31 has also shown to have zinc containing serine protease activity using azocasein as a substrate at pH 7.5 [13,14]

Drug Target Potential of ES-31

In-vitro studies

Confirmation of metallo serine protease activity was demonstrated by enzyme inhibition studies. In azocasein assay as shown in Table 1, serine protease inhibitor inhibited the serine protease activity i.e., 92% by 0.5 mM Pefabloc, 90% by 0.1 mM 3,4 dichloroisocoumarin and 90% by 1 mM PMSF and metallo protease inhibitors i.e., 65% inhibition by 1 mM EDTA and 78% by 0.1 mM 1,10 phenanthroline [14].

Inhibitor	Optimum concentration	ES-31 serine protease activity (units/mg protein)a (% inhibition)
Control	-	17.5
Pefabloc[b]	0.5 mM	1.4 (92)
3,4 dichloroisocoumarin[b]	0.1 mM	1.75 (90)
Phenyl methyl sulfonyl fluoride (PMSF) [b]	1 mM	3.50 (80)
Ethylene diamine tetracetic acid (EDTA) [c]	1 mM	6.125 (65)
1,10 phenanthroline [c]	0.5 mM	3.85 (78)
Isoniazid	0.2 mg/ml	0.88 (95)
Anti-ES-31 serine protease antibody	100 mg	0.70 (96)

Table 1: [a] Units of activity represent A440_1000/mg protein/min, [b] Serine protease inhibitors, [c] Metalloprotease inhibitors.

As it was hypothesized that chymotrypsin-like proteases possess both serine protease as well as lipase activity with the specific catalytic triad present in the active site of the enzyme [15-17]. We observed that ES-31, a serine protease also has lipase activity shown by titrimetric assay [10]. ES-31 showed presence of 44.5 U/mg pr of lipase activity. The lipase activity of ES-31 was 100% inhibited by serine protease and metallo protease inhibitors (PMSF (1 mM) and EDTA (1 mM)) as well as lipase inhibitor (Orlistat). The inhibition of serine protease activity by lipase inhibitor i.e., 1 mM Orlistat was found to be 89.4%, suggesting common active site for serine protease as well as lipase activity of ES-31 thus with drug target potential. Thus ES-31 antigen is having a catalytic triad which is responsible for its serine protease as well as lipase activity and ES-31 can be categorized as a chymotrypsin-like enzyme [10].

We tried to evaluate the effect of isoniazid and orlistat in different combination of concentrations in order to check the synergistic effect. Orlistat (250 ng/ml) and isoniazid (200 ng/ml) combination showed maximum inhibition of 86% *in vitro* while 73% inhibition was observed by combination of orlistat (250 ng/ml) and isoniazid (200 ng/ml) on bacterial growth in axenic culture [18,19]. Orlistat can be served as an alternative to isoniazid in isoniazid resistance or intolerance cases due to its significant inhibitory action on MTB *Bacilli*. Whereas, inhibition MTB H37 Ra and MTB H37 Rv growth by 0.01 mM Pefabloc by 77% and 78% respectively in macrophage culture [14].

In-vivo studies

Addition of serine protease inhibitors (53%-76%) or metallo protease inhibitor (46%-61%) or anti-ES-31 serine protease antibody (89%) strongly inhibited the mycobacterial growth in MTB cell culture (Table 2). 0.01 mM Pefabloc (76%) showed maximum inhibition of MTB H37 Ra growth was in axenic culture (Table 2). Lipase inhibitor orlistat showed maximum 61% inhibition of MTB H37Ra bacilli in axenic culture [18,19]. Whereas, inhibition of MTB H37 Ra growth by 0.01 mM Pefabloc by 77% in macrophage culture. Further extended study of MTB H37Rv *Bacilli* culture showed maximum inhibition by 0.01 mM Pefabloc by 78% in macrophage culture. This study shows the importance of secretion of ES-31 antigen in the survival of the bacilli and growth [14]. Figure 1 illustrates how addition of ES-31 to macrophage culture had enhanced the entry of *Bacilli* and their multiplication in human macrophages [14].

Inhibitor	Optimum Concentration	Concentration of ES-31 serine proteasec/ml of culture filtrate (% inhibition of secretion)	*M. tuberculosis* H37Ra CFUd Count (% growth inhibition)
Control		75 ng	$16X10^5$
Pefabloc[a]	0.1 mM	15 ng (80)	$3.8 X 10^5$ (76)
3,4 dichloroisocoumarin[a]	0.02 mM	25 ng (67)	$7.6 X 10^5$ (53)
Phenyl methyl sulfonyl fluoride (PMSF)[a]	0.2 mM	30 ng (60)	$7.2 X 10^5$ (55)
Ethylene diamine tetracetic acid (EDTA)[b]	0.1 mM	40 ng (47)	$8.6 X 10^5$ (46)

1,10 phenanthroline[b]	0.1 mM	20 ng (73)	6.2 X 10^5 (61)
Isoniazid[c]	0.2 mg/ml	10 ng (87)	2.2 X 10^5 (86)
Anti-ES-31 serine protease antibody[d]	100 mg	_	1.8 X 10^5 (89)

Table 2: [a]Serine protease inhibitors, [b]Metalloprotease inhibitors, [c]Quantitation of mycobacterial ES-31 serine protease was carried out by peroxidase sandwich ELISA. Amount of anti-ES-31 serine protease antibody coated_50 mg/well. Goat anti-ES-31 serine protease antibody IgG peroxidase conjugate dilution = 1:1000, [d]CFU: colony forming units per ml of culture medium.

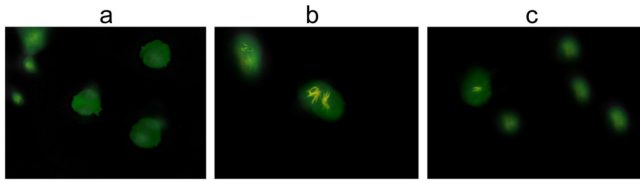

Figure 1: (a) Macrophage culture without *Bacilli* (control). (b) Macrophage culture infected with virulent *M. tuberculosis* H37Rv *Bacilli*. (c) Macrophage culture infected with virulent *M. tuberculosis* H37Rv bacilli in the presence of Anti-ES-31 serine protease antibody [14].
•Slide shows a higher magnification views (X1000) of *M. tuberculosis* H37Rv bacilli in macrophages after staining with aruamine. A 517 nm filter was used to view the slide.
•The bacilli are bright yellow and the macrophages are green.

Conclusion

The data suggests that ES-31 antigenic protein with protease and lipase activities can be targeted by many drugs as *in-vitro* and *in-vivo* studies so that it can have good drug target potential and also suggest that it may help in the replacement of routinely used anti-TB drugs in resistant cases in particular.

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