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A Repurposing Approach to Target the PI3-Kinase Pathway of *Mycobacterium tuberculosis* $H_{37}R_v$ by Heavy Metal Ions

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Short Communication

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ABBREVIATIONS: TB: Tuberculosis; *EPTB: Extrapulmonary tuberculosis*; *HIV: Human Immunodeficiency Virus; MDR: Multi Drug Resistant;* PI3: Phosphoinositide; hVps34: Vacuolar Protein Sorting 34; LAM: Lipoarabinomannan; RTKs: Receptor Tyrosine Kinases; GPCRs: G-Protein Coupled Receptors; PDKs: Phosphoinositide Dependent Kinase1; Akt/PkB: Protein Kinase B; PH: Pleckstrin Homology; TGN: Trans Golgi Network; M. tuberculosis H37Rv: *Mycobacterium tuberculosis* H37Rv; PKC: Protein Kinase C

ABSTRACT

Tuberculosis (TB) is the leading cause of morbidity and mortality and is caused by *Mycobacterium tuberculosis* $H_{37}R_v$ (*M. tuberculosis* $H_{37}R_v$).TB is a disease comprising of intracellular trafficking pathways. Therefore, through understanding host signalling pathways and by targeting these pathways with heavy metals such as cadmium and cobalt might lead to the deterioration of bacterium and may help in the treatment of drug-resistant TB. As cobalt has been shown to induce the apoptosis in the cells, it may induce apoptosis in the infected macrophages by identifying *M. tuberculosis* $H_{37}R_v$ leading to the destruction of the bacterium. *M. tuberculosis* $H_{37}R_v$ survives by suppressing the activity of second messenger like calcium through inhibition of Protein kinase C (PKC), since calcium helps in gene expression therefore by replacing calcium by cadmium may subvert the pathogenicity of *M. tuberculosis* $H_{37}R_v$. PI3-kinase pathway subverted by *M. tuberculosis* $H_{37}R_v$ is studied because of its importance in the process of apoptosis, cell growth and survival. The knowledge of this mechanism may provide an insight view of *M. tuberculosis* $H_{37}R_v$ survival inside the macrophages and may be this hypothesis provides an efficient mechanism for the clearance of *M. tuberculosis* $H_{37}R_v$ by utilizing these metals as nutritional supplements which may help in the inhibition of the efficacy of this potent infection.

INTRODUCTION

Tuberculosis (TB) is one of the most infectious air-borne diseases caused by *Mycobacterium tuberculosis* $H_{37}R_v$ (*M. tuberculosis* $H_{37}R_v$) [1]. Although this infection primarily initiates in the lungs but it can also infect other parts of the body such as bones, eyes, joints, skeletons etc. which is considered as extra pulmonary *tuberculosis* (EPTB) [2]. Previously reported data had shown that 1.3 million Individuals died because of TB in 2016 (1.7 million cases in 2001). TB mortality rate is decreasing by approximately 3% per year globally [3] but this rate has to be increase by taking the population into consideration. Co-infection of TB along with HIV increases the rate of active TB which is the major cause of demises among all infectious disease [2]. TB is still being a global pandemic due to its resistance towards first line and second line drugs which leads to multi drug resistance TB (MDR-TB) and extensively drug resistance TB (XDR-TB) [4]. Some prokaryotic serine/threonine kinases and phosphatases have reported to modulate the host signaling system [5]. Among 11 kinases which were observed in *M. tuberculosis* $H_{37}R_v$, some of them were

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involved in cell division and stress responses while others in modulating the signaling cascades of host. In this review there is an overview of trafficking pathways and how *M. tuberculosis* $H_{37}R_v$ violates host signaling pathway by utilizing macrophages for its replication, survival and pathogenesis. The intracellular decrease in calcium and calcium dependent calmodulin helps in the survival of *M. tuberculosis* $H_{37}R_v$ through interference in PI3-kinase pathway and protects it from the phagocytic activity of macrophages. Therefore, by increasing the intracellular concentration of calcium ions, *M. tuberculosis* $H_{37}R_v$ survival might be hindered [4]. Phosphoinositide 3-kinase is the plasma membrane bound enzyme that binds to the intracellular tail of RTKs molecules. This kinase principally phosphorylates inositol phospholipids rather than proteins and both RTKs and GPCRs can activate it. It plays a central part in promoting cell survival and growth [6]. PI is unique among membrane lipids because it can undergo reversible phosphorylation at multiple sites on its inositol head group and generate a variety of phosphorylated PI lipids called phosphoinositides. The phosphorylation of activated PI3-kinase catalyzes the production of PI (3, 4, 5) P3 which serves as docking site for various intracellular signalling proteins. PI3-kinases which are activated by RTKs are class I type, they are heterodimers composed of a common catalytic subunit and different regulatory subunits. RTKs activate class Ia PI3-kinases in which the regulatory subunit is an adaptor protein that binds to two phosphotyrosines on activated RTKs through its two SH2 domain. Intracellular signaling proteins bind to PI (3, 4, 5) P3 produced by activated PI3-kinase via specific interaction domain such as pleckstrin homology (PH) domain which mainly functions as protein-protein interaction domain. Signal proteins bind to specific RTKs which activates PI3-kinase to produce PI (3, 4, 5) P3. The PIP3 recruits two protein kinases to the plasma membrane via their PH domains-Akt (also called protein kinase B or PKB) and phosphoinositide-dependent protein kinase 1 (PDK1) and this leads to the activation of Akt. Once activated, Akt phosphorylates various target proteins at the plasma membrane as well as in the cytosol and nucleus which leads to enhancement of cell survival and growth. The subversion of negative regulation of PI3-kinase signaling pathway may help in removal of *M. tuberculosis* $H_{37}R_v$ through enhancement of autophagy inside phagocytic cells and may provide an efficient platform for the treatment of drug resistant TB.

THE HYPOTHESIS

In this review it is hypothesized that by targeting the signalling pathways by using heavy metals as co-factors in the enzymatic pathways, we can subvert the effect of *M. tuberculosis* $H_{37}R_v$ and also hinders its survival. This will be done either by induction of apoptosis in the phagocytic cell through phosphorylation of the signalling pathway or by alteration of gene expression by using heavy metal that might interfere in the *M. tuberculosis* $H_{37}R_v$ survival inside the host cells and reduce its virulence. In various organisms, manifestation of heavy metals is a key regulatory molecule for their health. The survival of *M. tuberculosis* $H_{37}R_v$ may be interrupted by induction of autophagy so that *M. tuberculosis* $H_{37}R_v$ which resides in the phagosomes may get arrest in the autophagosomes by apoptosis through inhibition of Ca^{2+} dependent trafficking and also through generation of reactive oxygen species by using Cobalt. The modulation of host signal transduction cascades and gene expression is carried out by Cadmium, *M. tuberculosis* $H_{37}R_v$ residence in the phagosomes may be inhibited by activation of Ca^{2+} dependent trafficking and its survival could also be hindered [7]. Cobalt is a trace element which is present mainly in the form of cobalamin in host cells. It also acts as a cofactor in various metabolic pathways. Targeting Pi3-kinase pathway through inhibition of the serine /threonine kinase by Cobalt leads to phosphorylation of apoptotic protein Bad. Cobalt which acts as activator for Akt might be proved as inhibitor for Bad. Enhancement of apoptosis of the macrophages so that these phagocytic cells undergo programmed cell death and also *M. tuberculosis* $H_{37}Rv$ which resides inside the macrophage dies. This could be possible through selective targeting of the phagocytes, by recognizing only those macrophages in which *M. tuberculosis* $H_{37}R_v$ resides by using Cobalt.

DISCUSSION

PI3-kinase pathway of M. tuberculosis H37Rv

M. tuberculosis $H_{37}R_v$ has evolved numerous strategies to survive under phagocytic cells. Although these phagocytic cells are made for internalizing the bacteria, *M. tuberculosis* $H_{37}R_v$ utilizes these macrophages for its survival through various ways. *M. tuberculosis* $H_{37}R_v$ attributes to its survival through exploitation of host signaling pathways. PI3P which is formed by PI3-kinase on the membrane of early endosomes and phagosomes is also involved in the biosynthesis of phagolysosomes [7]. PIP3 may represent a docking site for several proteins which involved in the phagosome-lysosome maturation such as the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and the early endosomal autoantigen 1 (EEA1) [8]. In normal cells, PI3P generation regulates the delivery of phagocytosed antigens to lysosome. In case of *M. tuberculosis* H37Rv, it interferes with the generation of PI3P on the membranes of phagosome and hence inhibits further signaling event of the host that direct towards bacterial apoptosis [9]. This occurs in two steps; First, *M. tuberculosis* $H_{37}R_v$ inhibits the activity of the PI3 kinase hVPs34, and hence interferes in the production of PI3P on the membrane of phagosome and inhibits phagosome-lysosome fusion. This inhibition was accomplished by the major glycolipid cell-wall component lipoarabinomannan (LAM) [10]. However, since killed mycobacteria failed to inhibit phagolysosomal maturation but possess LAM, it still remains a question that how LAM-mediated inhibition of trafficking pathways would be regulated. LAM from *M. tuberculosis* $H_{37}R_v$, blocked increment in cytosolic Ca^{2+} which is fundamental

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requirement for generation of PI 3 phosphate (PI3P) on phagosomes in vivo. The second way through which *M. tuberculosis* inhibit PI3P accumulation on phagosomal membrane is by the means of a phosphatase termed SapM, a eukaryotic-like acid phosphatase secreted by *M. tuberculosis* H37Rv [11]. Along with lipid phosphatases, *M. tuberculosis* $H_{37}R_v$ also includes protein phosphatases PtpA and PtpB [12], which interferes in the host trafficking pathways, by modulation of vacuolar sorting proteins [13]. Lipoarabinomannan (LAM), the major cell wall glycolipid of *M. tuberculosis* $H_{37}R_v$ helps in the survival of *M. tuberculosis* $H_{37}R_v$ through inhibition of protein kinase C, a key signaling molecule inside the mononuclear cells that help in scavenging of cytotoxic oxygen free radicals [14]. LAM has been shown to interfere with phagosomal acquisition of late endosomal constituents. LAM and a number of other mycobacterial lipids have been reported to traffic within the infected macrophages and intercalate into the host cell endomembranes [15]. One of the *M. tuberculosis* $H_{37}R_v$ glycolipids is phosphatidylinositol mannoside (PIM), a biosynthetic precursor of LAM. PIM is abundantly produced by mycobacteria and represents 56% of all phospholipids in the mycobacterial cell wall [16]. In *M. tuberculosis* $H_{37}R_v$, LAM is present in the cell wall which leads to modulation of signaling pathways by dephosphorylation of apoptopic protein Bad through activation of serine /threonine kinase Akt and helps in survival *M. tuberculosis* $H_{37}R_v$ by escaping the apoptosis [17,18]. The inhibition of Ca^{2+}/calmodulin dependent PI3- kinase vacuolar protein sorting 34 (hvps34) pathway occurs by Lipoarabinomannan (LAM) through inhibition of phagosomes maturation and this is another prominent way of residing of *M. tuberculosis* $H_{37}R_v$ inside host cells through inhibition of phagolysosomal fusion [19,20]. There is a high need of identification and development of active compounds and drugs because of the *M. tuberculosis* H37Rv resistance to each line drugs [21-26]. *M. tuberculosis* $H_{37}R_v$ survives in the host macrophages through decrease acidification of phagosomal compartments, due to inhibition of VoH+ATPase and the absence of Cathepsins D like hydrolases [27]. To inhibit its survival in macrophages there is a need of generation of autophagic processes within the phagocytic cells. The induction of autophagy is either by starvation or by induction of oxidative stress inside the cells [28]. Autophagy is induced by activation of the PKC so that scavenging of free radical is not inhibited which may be the reason behind the induction of oxidative stress and the clearance of *M. tuberculosis* $H_{37}R_v$ from the macrophages.

Disadvantage of activated Protein kinase (Akt)

Bad, a proapoptotic protein which in its non-phosphorylated state promotes cell death by apoptosis but activated Akt phosphorylates Bad which creates phosphoserine-binding sites for a scaffold protein called 14-3-3, which sequesters phosphorylated Bad and hence promoting cell survival. *M. tuberculosis* $H_{37}R_v$ cell wall component mannosylated Lipoarabinomannan (Man-LAM) interacts with Akt and leads to phosphorylation of Bad and inhibition of apoptosis [29]. Man-LAM is a potent inhibitor of certain chemokines, cytokine as TNF-α and interleukins (IL-1α, IL-1β, IL-6 and IL-10) which induces inflammation and this inhibition leads to the inactivation of macrophages [30-33]. Macrophages avoid apoptosis due to inhibition of IFN-γ. The inhibition of cytokine, TNF-α helps in the survival of *M. tuberculosis* H37Rv by inhibition of apoptosis of infected macrophages [34-36]. It is hypothesised that an elevation in Ca^{2+} influx can induce apoptosis in the cells and in the case of *M. tuberculosis* $H_{37}R_v$, Ca^{2+} concentration gets reduced and this helps in pathogenesis by avoiding apoptosis (**Figure 1**) [37].

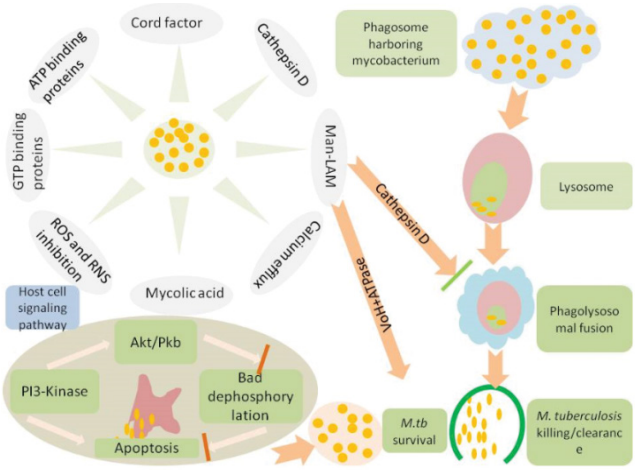

Figure 1: The Inhibitory Mechanism of PI3-Kinase Pathway By *M. Tuberculosis* H37Rv: There are Many Factors Which are Responsible for Bacterial Survival and Pathogenesis i.e., Cord Factor Which Inhibits PMN Migration, Mycolic Acid Which Prevents it from Lysozymes And Cytotoxic Oxygen Radicals and GTP-Binding Genes Which May Help in Virulence. Man-LAM, a Cell Wall Glycolipid Inhibits the Voh+Atpase and Cathepsins D Like Hydrolases and Helps in the Survival Through Reduced Acidification of Phagosomal Compartment. *M. Tuberculosis* $H_{37}R_v$ Violates the PI3-Kinase Pathway by Phosphorylating the Apoptotic Protein Bad through Activation of Akt/Pkb, a Serine /Threonine Kinase.

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Targeting the enzyme by Cobalt

Cobalt is an intrinsic factor of vitamin B12 and this vitamin is only synthesized by microorganisms. It is a water-soluble vitamin since its level may be maintained in the normal human serum and urine because of detoxification mechanism of living organism. The part of vitamin or we can say the amount of vitamin that would remained after its utilization by host could be excreted through urine for maintaining a balanced intracellular environment [38]. It may be predicted from the above conclusion that if any person is exposed to *M. tuberculosis* $H_{37}R_v$ infection the concentration of cobalt varies inside the host cells and it would be a parameter for the diagnosis of *M. tuberculosis* $H_{37}R_v$ infection and may be proved as revolutionised step in the field of medico logy. This may also be possible that increased dosage of cobalt as a dietary supplement to a *tuberculosis* patient raised some antibodies against it or *M. tuberculosis* $H_{37}R_v$ subvert its mechanism for survival inside the macrophages. Since Cobalt helps in pathogenesis, it is hypothesised that if any element is helpful in the pathogenesis of bacteria then its concentration becomes low because of its utilisation in the survival of bacteria [23]. Therefore, by measurement of levels of cobalt in normal person sera or urine and its elevated or suppressed level if there is an *M. tuberculosis* $H_{37}R_v$ infection in the diseased person may also help in the diagnosis of the disease at early stage. There are various genes present in *M. tuberculosis* $H_{37}R_v$ which are participating in the regulation of these heavy metals either by binding or by transportation (**Table 1**).

Table 1: List of metal ion transporter genes in *Mycobacterium tuberculosis* H37Rv.

				Orthologues			
S.No.	**Locus**	**Name**	**Substrate**	***M. bovis***	***M. leprae***	***M. smegmatis***	**UniProt (Function)**
1	Rv0092	ctpA	Heavy metal Cu^{2+}	Mb0095	ML1987	-	P9WPU1 (Involved in copper export)
2	Rv0103c	ctpB	Heavy metal Cu^{2+}	Mb0106c	ML2000c	-	P9WNP3 (involved in the hydration of fatty acids for production of polyhydroxylalkanoates)
3	Rv0265	fecB	Fe^{3+}	Mb0271c	ML2548	MSMEG_0438	L7N6B2 (ABC transporter substrate-binding protein)
4	Rv0362	mgtE	Mg^{2+}, Co^{2+} uptake	Mb0369	-	MSMEG_6269	O06312 (acts as a magnesium transporter)
5	Rv0908	ctpE	-	Mb0932	ML2115	MSMEG_5636	P9WPT1 (P-type ATPase involved in specific uptake of calcium)
6	Rv0924c	mntH	Mn^{2+}, Fe^{2+}, Zn^{2+}, H+ symporter	Mb0948c	ML2098	MSMEG_5589	P9WIZ5 (H+-stimulated, divalent metal cation uptake system. Transports zinc and iron. Can also interact with manganese and copper)
7	Rv0969	ctpV	Cu2+	Mb0994	-	MSMEG_5014	P9WPS3 (Necessary for copper homeostasis and likely functions as a copper exporter)
8	Rv1030	kdpB	K+ uptake	Mb1059	-	MSMEG_5393	P9WPU3 (Part of the high-affinity ATP-driven potassium transport or Kdp system)
9	Rv1239c	corA	Mg^{2+}/ Fe^{2+}, Co^{2+} uptake	Mb1271c	ML1090c	MSMEG_5056	O50455 (Mediates influx of magnesium ions, cobalt ion binding)
10	Rv1469	ctpD	Heavy metal (Cd^{2+})	Mb1504	ML1819	MSMEG_5403	P9WPT3 (Involved in heavy metal homeostasis. Probably exports nickel and cobalt ions out of the cell)
11	Rv1607	chaA	Ca^{2+}	Mb1633	ML1267	-	O53910 (Sodium and potassium: proton antiporter activity)
12	Rv1992c	ctpG	Heavy metal (Cd^{2+})	Mb2015c	-	-	P9WPS7 (cadmium ion transmembrane transporter activity)
13	Rv1997	ctpF	Ca^{2+}, Mg^{2+}	Mb2020	-	MSMEG_3926	P9WPS9 (calcium-transporting ATPase activity, metal ion binding)
14	Rv2025c	-	Cd^{2+}, Zn^{2+}, Co^{2+}	Mb2050c	-	-	P9WGF5 (cadmium ion, Zinc efflux transmembrane transporter activity)
15	Rv2084	arsA	AsO3- efflux	Mb2110	-	-	P9WLK1
16	Rv2287	yjcE	Na+, H+ antiporter	Mb2309	ML1792	-	P9WJI3 (Sodium and potassium: proton antiporter activity)
17	Rv2643	arsC	AsO3-	Mb2676	-	MSMEG_1172	I6X4W4 (arsinite and antimonite transmembrane transporter activity)
18	Rv2685	arsB	AsO3- efflux	Mb2704	-	MSMEG_0851	P9WPD7 (arsenite transmembrane transporter activity)

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19	Rv2856	nicT	Ni2+, Co2+	Mb2881	ML1571	-	I6YEJ7 (nickel cation transmembrane transporter activity)
20	Rv2877c	merT	Hg2+ uptake	Mb2902c	ML1585c	MSMEG_3541	I6YEL8 (Putative mercury resistance transport protein)
21	Rv3041c	-	Fe3+	Mb3067c	ML1726c	MSMEG_2326	I6YF11 (ATPase activity, coupled to transmembrane movement of substances)
22	Rv3044	fecB2	Fe3+	Mb3070	ML1729	MSMEG_2319	O53291 (Iron-siderophore ABC transporter substrate-binding protein)
23	Rv3236c	kefB	K+ or Na+, H+ antiporter	Mb3264c	ML0782	MSMEG_3664	O53291 (solute: proton antiporter activity)
24	Rv3270	ctpC	Heavy metal Cd2+, Fe2+	Mb3298	ML0747	MSMEG_6058	P9WPT5 (High affinity, slow turnover Mn2+ transporting ATPase, which is required for virulence)
25	Rv3578	arsB2	AsO3- efflux	Mb3609	ML0331	MSMEG_6072	I6YCG9 (arsenite transmembrane transporter activity)
26	Rv3743c	ctpJ	Heavy metal (Cd2+)	Mb3769c	-	-	P9WPT7 (metal ion binding, Probable cation-transporting P-type ATPase J)

Overall 24% of the total genes of *M. tuberculosis* $H_{37}R_v$ have code for metal ion transporter. In this way by measuring the level of cobalt in the blood or urine there is a chance of pre medicating the person who exposed to *M. tuberculosis* $H_{37}R_v$ infection or those in which *M. tuberculosis* $H_{37}R_v$ is in the latent form i.e., incapable of infecting the host but may be active at certain optimum conditions (**Figure 2**).

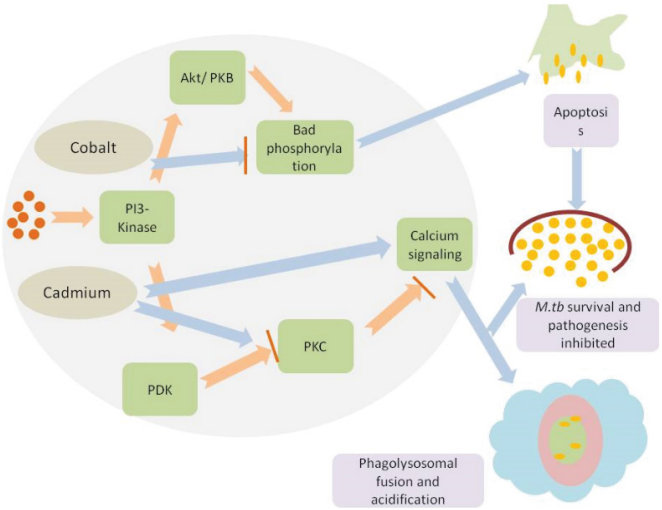

Figure 2: Regulation of PI3-kinase pathway by heavy metals: The phosphorylation of apoptotic protein Bad is inhibited by Cobalt because it induces apoptosis inside the macrophages in which *M. tuberculosis* $H_{37}R_v$ resides and eliminates it from the host macrophages. *M. tuberculosis* H37Rv also survives through inhibition of Calcium signalling through inhibition of PKC, therefore, by replacing the Calcium through Cadmium gene expression altered and *M. tuberculosis* $H_{37}R_v$ survival hindered.

Target LAM of *M. tuberculosis* H37Rv

In the virulent species of *M. tuberculosis* as *M. tuberculosis* $H_{37}R_v$, cell wall lipid LAM binds to the mannose ligands and exhibits its virulence. So by using the substrate analogue of Man-LAM which is also a glycolipid as lipoarabinofuranose or by using glucose as ligand for LAM its virulence may be reduced. Glucose and mannose are epimers and their structures are same, therefore they mimic the same conformation for Man-LAM but differ in functions. They may interfere with the cell wall structure, leads to its distortion and also reduced the virulence. This may cause the ultimate killing of *M. tuberculosis* $H_{37}R_v$. This may also induces autophagy inside the phagosomes which leads to the ultimate killing of *M. tuberculosis* $H_{37}R_v$ by increasing acidification of phagosomes by activation of Vo H+ATPase or by hydrolases as Cathepsins D and this may lead to phagosome-lysosome fusion and *M. tuberculosis* $H_{37}R_v$ survival also gets hindered. *M. tuberculosis* $H_{37}R_v$ survives and avoids phagocytic activity of macrophages due to the presence of lipids molecules in the cell wall and if this cell wall gets distorted its survival may be hindered and infection gets inhibited at the primary level.

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Interruption of phagolysosomal fusion

The inhibition of maturation of phagosomal compartment in *M. tuberculosis* $H_{37}R_v$ is due to a block in the trafficking pathway of PI3-kinase from the TGN to the phagosome which is also a reason for inability of effector molecules such as VoH+ATPases and Cathepsins D. This may be due to anomalous distribution of Rab molecules, a small GTPases involved in the intracellular trafficking from the endosomes to the TGN [15]. This is also through inhibition of calcium-dependent trafficking cascades. Ca^{2+} is a ubiquitous second messenger that could control multiple processes and evidenced to be involved in cellular activities like division, motility, stress response, signaling, etc. Calcium is a second messenger in the signalling pathway and its low level in the host cell helps in the pathogenesis of *M. tuberculosis* $H_{37}R_v$ [39]. Calcium also helps in the gene expression. If Calcium is replaced by Cadmium in the signalling pathway which has also the ability of alteration of gene expression, this may modulates the host signal-ling pathways and may also inhibit the infection of *M. tuberculosis* $H_{37}R_v$ (**Figure 2**). Alteration of gene expression may cause the formation of dysfunctional proteins which may be interfering with the *M. tuberculosis* $H_{37}R_v$ pathogenesis and infection capabil-ity. This may provide a future perspective for mutating the *M. tuberculosis* $H_{37}R_v$ protein through alteration of gene expression so that efficacy of infection is reduced.

CONCLUSION

As *M. tuberculosis* $H_{37}R_v$ modifies its pathogenic machinery day to day it leads to the worsen state of most of the tuberculosis infected persons through enhancement of infection. One of the effective mechanism through which *M. tuberculosis* $H_{37}R_v$ survives is by modulation of host signalling pathways. Therefore, to eradicate the morbid infection caused by this revolutionised bacterium it is necessary to target the signalling cascade of the host cell. We have focused our understanding towards subver-sion of the signaling pathways that are meant as a base for any living organism metabolism. Using cobalt in place of calcium may lead in the inhibition of several signaling pathways in which calcium is the key regulator and thus also hindered its survival and pathogenesis. On the other hand, significant intracellular calcium levels can induce apoptosis of the pathogen. The regulation of pathway may hinder *M. tuberculosis* $H_{37}R_v$ survival and also decreases its pathogenicity. In this perspective, the modulation of PI3-kinase pathway by heavy metals may provide a significant platform for elimination of this bacterium.

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