ROLE OF CALCIMYCIN IN REGULATION OF AUTOPHAGY IN THP-1 CELLS

THESIS SUBMITTED TO

NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA

FOR THE PARTIAL FULFILMENT

OF THE MASTER OF SCIENCE DEGREE IN LIFE SCIENCE

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CERTIFICATE

This is to certify that the thesis entitled "**ROLE OF CALCIMYCIN IN REGULATION OF AUTOPHAGY IN THP-1 CELLS**" which is being submitted by **Mr Uttam Chetan Muni**, Roll No. **413LS2040**, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by him under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DEDICATED TO

MY

BELOVED PARENTS

DECLARATION

I do hereby declare that the Project report entitled **"ROLE OF CALCIMYCIN IN AUTOPHAGY REGULATION IN THP-1 CELLS"** submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bona fide and original research work carried out by me under the guidance and supervision of **Dr. Rohan Dhiman**, Assistant Professor, Department of life Science, NIT, Rourkela.

Date: **UTTAM CHETAN MUNI**

Place:

ACKNOWLEDGEMENT

I express my deep sense of gratitude and reverence to my major advisor, **Dr. Rohan Dhiman**, Assistant Professor, Department of Life Science, NIT-Rourkela, for his excellent guidance, constant and untiring supervision, help and encouragement throughout the investigation and preparation of this manuscript.

I am extremely grateful and indebted to **Dr. Sujit Kumar Bhutia,** HOD, Department of Life Science, NIT-Rourkela, and **Dr. Surajit. Das**, **Dr. Samir Kumar Patra, Dr. Rasu Jaybalan**, **Dr. Bismita Nayak**, **Dr. Bibekananda Mallick**, **Dr. Suman Jha, Dr. Binod Bihari Sahu, Dr. Monalisa Mishra, Dr. Mohammed Saleem and Dr. Vidya Devi Negi** for their inspiring suggestions and valuable advice not only for this investigation but also in many other fronts without whom it would have been difficult to carry out this work.

I am highly obliged **Shradha Mawatwal** Ph.D. Scholar, Department of Life Science, NIT-Rourkela, for her constant help and encouragement during the period of my project. I am solely impressed by her great personalities.

My heartfelt thanks to my friend **Assirbad Behura, Pratibha Kumari** and all other classmates for their moral support, help and encouragement throughout the course of this work. I take the pleasure to acknowledge the constant help and support of my friends has always been cherished.

My sincere obligations are to the Staffs of Department of Life Science, NIT-Rourkela for their help during this period.

Lastly, I acknowledge with highest sense of regards to my parents, my brother, and other members of my family for their supreme sacrifice, blessings,

Unwavering support, love and affection without which the parent investigation would not have been successful in any sphere of my life.

At the end, I bow down my head to the almighty whose omnipresence has always guided me and made me energized to carry out such a project.

Date: **UTTAM CHETAN MUNI**

Place:

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ABBREVIATIONS

Adenosine triphosphate- ATP

Autophagy related genes-Atg

Chaperones mediated autophagy-CMA

Dimethyl sulfoxide-DMSO

Endoplasmic reticulum-ER

Ethylene diamine tetra acetic acid-EDTA

Fetal bovine serum-FBS

Interferons-IFNs

Inositol 1, 4, 5-trisphosphate -InsP3/IP3

Mycobacterium tuberculosis- M. tb

Pathogen-associated-molecular patterns-PAMP

Phorbol myristate acetate-PMA

Potassium chloride-KCl

Potassium Di hydrogen Phosphate- KH_2PO_4

Pre-autophagosomal structure-PAS

Sarcoplasmic/Endoplasmic Reticulum-SERCAs

Sodium chloride-NaCl

Sodium dodecyl sulfate-SDS

Sodium hydroxide-NaOH

Toll-like receptors-TLRs

Tumor necrosis factor-TNF

ABSTRACT

Mycobacterium tuberculosis (*M. tb*), is the known pathogen for causing tuberculosis. It infects approximately one-third of the world's population. According to the global tuberculosis report 2013 by WHO, Tuberculosis remains a major global health problem. In 2012, nearly about 8.6 million people developed TB & 1.3 million people died from the disease. This bug enters our body through the respiratory tract and macrophages are the first phagocytes to engulf it through phagocytosis leading to the formation of phagosome. Despite various efficient host immune barriers, the bug has a tremendous ability to circumvent these barriers like inhibition of phagosome-lysosome fusion and autophagy for its intracellular multiplication and survival. Autophagy is a host defense mechanism and various published reports have shown that calcium signaling in autophagy play an important role in curtailing the disease load. So the present study is to see the regulation of autophagy in human monocytic cell line, THP-1 by Calcimycin, an important calcium ionophore that helps to increases the intracellular calcium level inside the cell.

1. INTRODUCTION

The word autophagy is derived from two Greek word *auto* and *phagy*, where *auto* means self and *phagy* means eating. The term autophagy was first given by *Christian de Duve*. Autophagy is a highly regulated mode of cell suicide (Konstantinidis, 2012). Autophagy is a major metabolic process in which cell degradation occurs. Autophagy is induced under nutrient starvation conditions but in mammalian cells it is also invoked in response to specific hormones. Due to degradation of cellular components, it helps the cell to survive during starvation. Autophagy mainly occurs in the eukaryotic cells. It maintains a relatively controlled balance between anabolism and catabolism to get normal cell growth and development (Reggiori, 2002). The degradation of cellular components helps the cell to remove unnecessary organelles and to recycle the components for reuse (Kim, 2000; Klionsky, 2000). Autophagy plays a role as a specialized inflammatory response in immunologic effector function and It tregulates innate immunity to act as antimicrobial defense mechanisms in the cell. (Jae-Min et al., 2012). Autophagy has a great role to protect organisms against different diseases including infections like tuberculosis, follicular lymphoma, salmonellosis cancer, neurodegeneration and aging. Autophagic responses also affect bacterial invasion during infection in the host. Autophagy can target bacteria within the vacuole. Intracellular pathogens, such as *Mycobacterium tuberculosis* (*M. tb*), can actively live within host cells and exploit host defense by stopping of phagosomal maturation (Gutierrez et al., 2004; Yuk et al., 2009b). So, microorganisms inside the phagosome experience increased level of reactive oxygen and nitrogen species that leads to increase in the acidity of the phagosome. Then it activates the hydrolases enzymes that degrade bacterial lipids and proteins (Huynh & Grinstein, 2007) which lowers the metabolic rate of the bacteria. For bacterial destruction, it requires removal and clearance of phagolysosome fusion. So the process where cell directs autophagy against pathogens is called xenophagy.

The primary link between autophagy and calcium was shown by various groups, where they showed that autophagy can be increased by endoplasmic reticulum (ER) stress and ER calcium mobilization through agents like Vitamin D, ionomycin and thapsigargin up regulate autophagy (Berridge, 2000; Harr and Distelhorst, 2010). ER stress increases autophagy by negatively regulating the AKT/TSC/mTOR pathway leading to autophagosome formation (Jung et al, 2010). Study by Biswas et al., has shown that ATP-mediated autophagy is calcium-dependent

and associated with a decrease in cell viability of *M. bovis* BCG intracellular level. (Biswas et al, 2008)

So, the long term goal of one of the project in our lab is to see the effect of novel calcium-inducing compounds like Calcimycin on autophagy of mycobacteria infected macrophages and its effect on intracellular viability of mycobacteria. To accomplish this project, preliminary study was undertaken in the present thesis to see the cell viability and autophagy of THP-1 (Human monocytic cell line) cells in the presence of Calcimycin.

2. REVIEW OF LITRETURE

Autophagy is an evolutionary mechanism that begins by formation of an autphagosomes, enclosed within a double membrane that isolates the part of the cytoplasm. In mammalian cells, autphagosomes undergo a maturation process by merging with endocytic compartments and lysosomes. Autophagy helps in the degradation and recycling of cellular component (Eskelinen, 2005). Autophagy is a process that maintains cellular homeostasis. The degradation pathway mainly helps the cell to remove unnecessary organelles, misfolded protein, cellular remodeling, metamorphosis, aging and cell transformation. Autophagic feature is a sign of cell death. For autophagy it occurs in mainly occurs in four steps (1) Induction of autophagy (2) Autophagosome formation (3) Degradation (4) reuse. The autophagy induction occurs due to the nutrition starvation. Then it leads for the autophagosome formation. Autophagosome is an intracytoplasmic vacuole containing elements of a cell's own cytoplasm. Then in the degradation step autophagosome fuses with the lysosome. Lysosome is a major catabolic factory which contains different kind of hydrolases which are capable of degrading cellular constituent. These hydrolases can breakdown protein, carbohydrate and lipid. Lysosome has pH nearly of 4.8. These degrading structures are called "autolysosomes" or "autophagolysosomes". Once macromolecules are degraded in the vacuole. The Monomeric units (e.g., amino acids) are transported to the cytosol for reuse. For autophagic regulation it requires a specific group of molecules named Autophagy related genes (Atgs). Until now, more than 30 ATGs have been discovered in yeast (Nakatogawa et al., 2009). The proper collaboration of Atgs is essential for performing autophagy successfully. On the other hand, there are many signaling pathways involved in autophagic induction such as the mTOR pathway, endoplasmic reticulum (ER) stress signals, the insulin pathway, and calcium signaling (Yang and Carra et al., 2013).

Autophagy mainly occurred mainly by three main pathways. These are

1. Macroautophagy
2. Microautophagy
3. Chaperone-mediated autophagy

2.1.1. MACROAUTOPHAGY

Macroautophagy is a multistep process. In that vacuolar degradation pathway is terminated in the lysosomal and it is of fundamental role in maintaining homeostasis (Mehrpour, 2012). It mainly occurs to remove damaged organelles or misfolded proteins (Fig. 1). In the first step, there is initiation of autophagosome formation. It takes place by a pre-autophagosomal structure (PAS) (Noda et al., 2002). Autophagosome is a double membrane around the cytoplasmic components. PAS starts elongating for formation of autophagosome. Autophagosome initiation and formation is mainly controlled by the Atg1:Atg13:Atg17complex. When the autophagy is induced the autphagosomes are produced which fuse with lysosomes or late endosomes for formation of autophagolysosomes and provide constituents and energy for the cell. Then acidification and degradation occurs which breaks down the cellular components.

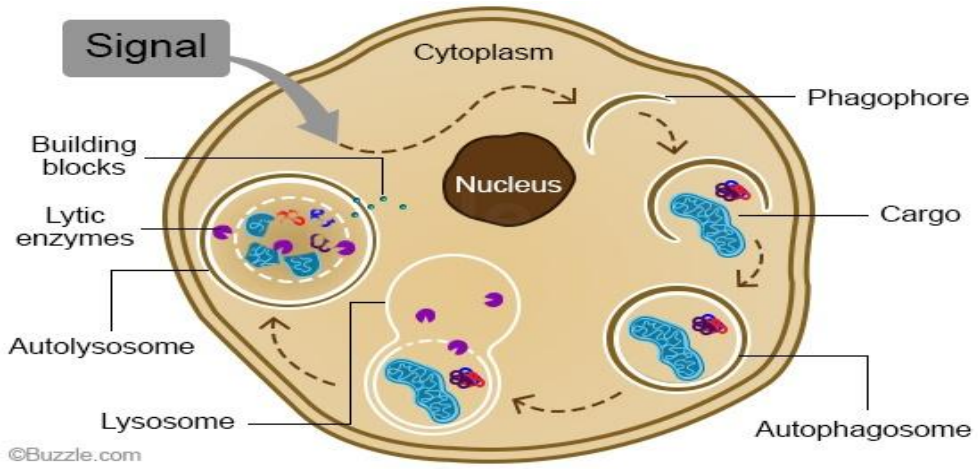


Fig. 1. Pathway of Macroautophagy (Adapted from- www.buzzle.com)

2.1.2. MICROAUTOPHAGY

It is a type of autophagic pathway where direct engulfment of cytoplasmic material into the lysosome takes place by direct invagination and folding of lysosomal membrane (Fig.2). Microautophagy is important for survival of cells under starvation. Both Macroautophagy and Microautophagy helps in recycling of nutrient in the cell. Microautophagy due to degradation of lipids incorporated into vesicles regulates the composition of lysosomal/vacuolar membrane (Wen-wen, 2012). Most of the autophagic bodies are produced by the help of Microautophagy (Muller et al., 2000). Microautophagy plays an important role as a route for delivery of glycogen into the lysosome (Takikita et al., 2009). Microautophagy helps in the size maintenance of organelle, composition of biological membranes and in restriction under cell survival (Wen-wen, 2012).

There are two types of Microautophagy. One is selective autophagy and another is nonselective autophagy. Nonselective autophagy can be seen in all kind of eukaryotic cells. But selective autophagy is only seen in yeast (Wen-wen, 2012). Nonselective microautophagy occurs mainly in four steps. In the first step, there is a formation of membrane invagination and autophagic tubes. Then it forms vesicles. After that, vesicle expansion occurs followed by vesicles degradation and recycling.

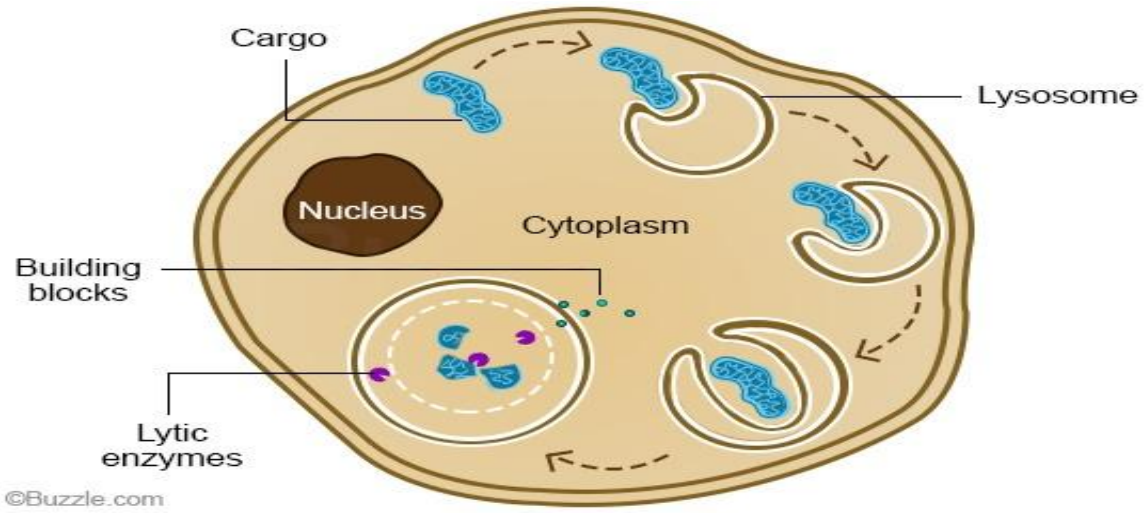


Fig. 2.Pathway of Microautophagy (Adapted from-www.buzzle.com)

2.1.3. CHAPERONES MEDIATED AUTOPHAGY (CMA)

Chaperones are proteins which help in the folding and unfolding of the proteins. The function of autophagy is mainly to degrade only specific kind of misfolded, or erroneously formed cytosolic proteins. When the protein is not correctly folded, it is recognized by a molecular chaperone called hsc70. It recognizes a penta peptide sequence motif KFERQ. Then, on the lysosome surface, it contains lamp-2A receptor that recognizes hsc70 (Fig.3). After recognition with the receptor, the misfolded protein is transported in to the lysosome. The misfolded protein is degraded by different kind of proteases present in the lumen, and the residual amino acids are released into the cytosol.

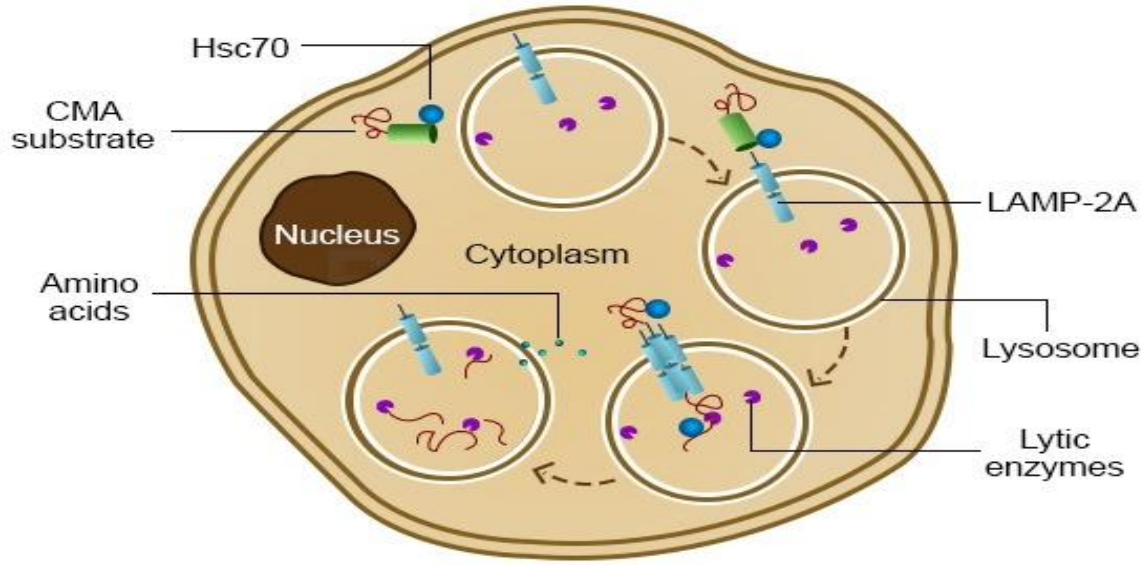


Fig. 3. Pathway of Chaperone-mediated Autophagy (Adapted from- www.buzzle.com)

2.2. ROLE OF AUTOPHAGY IN IMMUNE SYSTEM

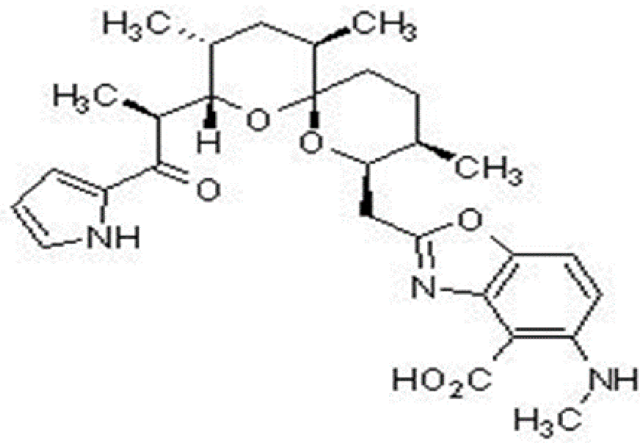
Autophagy during infections, acts as an innate immune defense mechanism and it has a complex link between the autophagy machinery and immuneresponses. On one hand the autophagy increases inflammatory responses and immune responses, while on the other hand immune and inflammatory signals increase or reduce autophagy (Songane and Kleinnijenhuis, 2012).

Different Published reports suggest that autophagy has a role in in the host defense against infectious agents including Group A *Streptococcus, Shigella flexneri, Listeria monocytogenes, Salmonella enteric, Francisella tularensis, Burkholderia pseudomallei* and *M. tb* (Songane and Kleinnijenhuis, 2012). It has been seen that knockdown of autophagy genes protects plants from viral, fungal and bacterial infection by preventing the uncommon increase in the programmed cell death during the plant innate immune system or hypersensitive response during infection (Liu et al., 2005). It induces autophagy with help of IFN-γ that leads to form phagosome-lysosome fusion, thus helps maturation of mycobacteria-containing phagosome to autophagolysosomes, which is mainly dependent on PI3K-dependent pathway, leading to killing and destruction of *M. tb* (Gutierrez et al., 2004; Kim et al., 2011).

M. tb reside in the macrophages of the host. It mainly acts as a habitat for the *M. tb* Upon *M. tb* infection multiple pathways are activated for regulate the cellular fates of host. After inhalation of *M. tb,* it attaches to phagocytic receptors and internalized into the alveolar macrophages (Bhatt and Salgame, 2007). Macrophages express Toll-like receptors (TLRs) that is mainly recognized by conserved pathogen-associated-molecular patterns (PAMPs) on *M. tb* for production of different kind cytokines such as tumor necrosis factor (TNF) and type I Interferons (IFNs) (Bhatt and Salgame, 2007; Hayashi et al., 2001 ; Killick et al., 2013). In addition, autophagy can be induced by different molecules called immune modulators such as vitamin D3, reactive oxygen species (ROS), including adenosine triphosphate (ATP). These have also shown their efficiency for elimination of *M. tb* in vitro condition (Songane and Kleinnijenhuis, 2012). Study by Biswas et al has shown that ATP-dependent autophagy is calcium-dependent pathway is related with the lowering of the intracellular viability of *M. bovis* BCG in the cell (Biswas, et al, 2008). Recent studies have shown that Vitamin D, IFN- gamma, ATP plays an important role in host defense mechanism against *M tb* by regulating autophagy innate immunity in the human macrophages, mainly through inducing autophagy (Liu et al., 2006; Shin et al., 2010; Xu and Wang et al., 2014). During the autophagy it is seen that it is activated by calcium signaling as shown in published reports. So to increase the calcium ion concentration we can use calcium ionophore like Calcimycin.

2.3. CALCIMYCIN AND AUTOPHAGY

Calcimycin is a calcium ionophore. Calcimycin acts as a mobile ion carrier and forms a stable complexes with divalent cations. It increases the intracellular calcium concentration and it also acts as an uncoupler of oxidative phosphorylation and inhibitor of mitochondrial ATPase activity. It is an antibiotic with weak antimicrobial activity against Gram-positive bacteria and fungi. The IUPAC name of the chemical compound is 5-(methylamino)-2-[[(2S,3R,5R,8S,9S)-3,5,9-trimethyl-2-[1-oxo-1-(1H-pyrrol-2-yl)propan-2-yl]-1,7-dioxaspiro[5.5]undecan-8-yl]methyl]-1,3-benzoxazole-4-carboxylic acid (Fig. 4).The molecular formula of Calcimycin is $C_{29}H_{37}N_3O_6$. The molecular weight of Calcimycin is 523.6 g/mole.



(Fig. 4. Structure of Calcimycin)

Calcium (Ca^{2+}) has a very diverse role in the cell. The organelles that regulate Ca^{2+} homeostasis are also the main place for apoptosis and autophagy regulation. Ca^{2+} is known as secondary intracellular messenger. The reservoir of the Ca^{2+} is endoplasmic reticulum (ER). The ER Ca^{2+} store is maintained by SERCAs (Sarcoplasmic/Endoplasmic Reticulum Ca^{2}+ ATPase). The concentration of Ca^{2+} is maintained mainly by two processes which are interlinked out of which one is slow sustained entry of extracellular calcium and other is rapid release of stored calcium (Berridge, 2000). So autophagy seems to be triggered by the ER stress and modulated by the ER Ca^{2+} content and release (Jaattela, 2007). Ca^{2+} release is only mediated by inositol 1, 4, 5-trisphosphate (InsP3/IP3) receptors (IP3Rs) in the ER that thus play a critical role in transient Ca^{2+} signaling (Berridge, 2000). The concentration of the calcium is directly related with the activity of calcium kinases and proteases (Hoyer-Hansen et al., 2007). So, it can also either

initiate or prevent autophagy in different mammalian cells (Kajitani and Kobuchi, 2007; Ding and Ni, 2007). As autophagy activation likely to be involved in controlling of *M. tb* growth, strategies targeted at stimulation of autophagy through different kind of therapeutic agents like Calcimycin, which induce calcium level may have pharma logical implications to treat TB.

3. OBJECTIVE

1. To study the effect of Calcimycin on cell viability of Phorbol myristate acetate (PMA) differentiated THP-1 cells.

2. To see the effect of non-toxic concentration of Calcimycin on autophagy of PMA differentiated THP-1 cells.

4. MATERIALS AND METHODS

4.1. CELL LINES

Cell line used in the study, THP-1 was a kind gift from Dr. Vivek Rai, Institute of Life Science (Bhubaneswar, India). Cells were maintained in RPMI-1640 growth medium, containing 10% heat inactivated FBS (Fetal bovine serum) and antibiotic cocktail (1X streptomycin and penicillin), at 37°Cin the presence of 5% CO_2. It is now well reported in the literature that THP-1 cells behave like human monocyte derived macrophages (MDM) in many ways (Stokes et al., 1999).

4.2. CHEMICALS

All the chemicals used in this study were of analytical grade and obtained from commercial sources. FBS and RPMI-1640 was purchased from GIBCO (Grand Island, NY). Antibiotic was purchased from GIBCO (Grand Island, NY). ECL western blotting detection reagents and analysis system was purchased from GE healthcare (UK). MTT cell assay kit was purchased from HiMedia Laboratory (Mumbai, India). BCA kit was purchased from Sigma Sigma-Aldrich. Following reagents were purchased from HiMedia Laboratory (Mumbai, India): Sodium chloride (NaCl), Sodium dodecyl sulfate(SDS), Acrylamide, Dimethyl sulfoxide (DMSO), hydrochloric acid (HCL), Tris base, Glycine, potassium chloride (KCl), Potassium Dihydrogen Phosphate (KH_2PO_4), Disodium phosphate (Na_2HPO_4), Ethylene diamine tetra acetic acid (EDTA), Triton X-100, glycerol, 2-mercaptoehtanol, Bromophenol blue, Sodium hydroxide (NaOH), Phorbol myristate acetate (PMA) and Bovine serum albumin (BSA). Following reagents were purchased from Sigma (St. Louis, Mo, USA): Bisacrylamide, Calcimycin and Sodium deoxycholate. All media and reagents used were endotoxin-free.

4.2.1. CELL CULUTURE MEDIA

Complete RPMI-1640 (with antibiotic)

RPMI media	500ml
FBS	50ml
Penicillin-Streptomycin (1X)	5.5ml

50 ml of heat inactivated FBS was added to RPMI medium. 5.5 ml of Penicillin-Streptomycin was added. Aliquots of 100 ml were prepared and stored at 4ºC.

4.2.2. BUFFER/ SOLUTIONS

10 X Phosphate Buffered Saline (pH 7.4)

NaCl	80.0 g
KCl	2.0 g
Na2HPO4	14.4 g
KH2PO4	2.4 g

The above components were dissolved in 800 ml of double-distilled water and pH was adjusted to 7.4 with HCl (0.1M). Final volume was made to 1 liter with double-distilled water.

1 M Tris (pH 6.8)

Tris base	121.1 g

Tris base was dissolved in 800 ml of double-distilled water and the pH was adjusted to the desired value by adding HCl (0.1 M). The volume of the solution is adjusted to 1 liter.

Reservoir Buffer 10 X (For SDS-PAGE electrophoresis)

Tris base	3.0 g
Glycine	14.4 g
10% SDS	10.0 ml

Above compounds were dissolved in 990 ml of double-distilled water and 10 ml of 10% SDS was added.

4 X Protein Sample buffer (For SDS-PAGE electrophoresis)

Upper Tris (1 M, pH – 6.8)	2.4 ml
SDS	0.8 g
Glycerol	4.0 ml

β-Mercaptoethanol	0.5 ml
Bromophenol blue	4 mg

Above components were dissolved in 3.1 ml of distilled water and final volume was made to 10 ml. The solution was dispensed into aliquots and stored at –20°C.

Transfer Buffer 10X (For Western blotting)

Tris base	10.8 g
Glycine	50.7 g

Above compounds were dissolved in 250 ml of distilled water and the final volume was made 360 ml by adding distilled water. The solution is sterilized by autoclaving. 300 ml of methanol was added to 1200 ml of 1X transfer buffer.

Acrylamide - bis-Acrylamide mixture

Acrylamide	30.0 g
Bis-Acrylamide	0.8 g

Acrylamide and bis-Acrylamide were dissolved in 70 ml double-distilled water final volume was adjusted to 100 ml. Then the solution is filtered and stored in -4°C.

10% Sodium dodecyl sulfate (SDS)

SDS	5 g

SDS was dissolved in 40 ml of double distilled water. The volume was adjusted to 50 ml with double distilled water.

10% APS

APS	0.1 g

0.1g APS was dissolved in 1ml distilled water.

PBS-T (For Western blotting)

10 X PBS	50.0 ml
Tween-20	2.5 ml

Distilled water	450.0 ml

These were mixed with distilled water.

Lower Tris (4 X) pH 8.8 (For SDS-PAGE electrophoresis)

Tris base	18.17 g
10% SDS	4.0 ml

Tris base was dissolved in 80 ml of double-distilled water and pH was adjusted to 8.8 using 0.1N HCl. Then 4 ml of 10% SDS was added and the final volume was made to 100 ml with double-distilled water.

Upper Tris (4 X) pH 6.8 (For SDS-PAGE electrophoresis)

Tris base	6.06 g
10% SDS	4.0 ml

Tris base was dissolved in 25 ml of double distilled water and pH was adjusted to 6.8 using 0.1N HCl. Then 4 ml of 10% SDS was added and the final volume was made to 100 ml with double-distilled water.

RIPA buffer

1% Triton X – 100	1 ml
150 mM NaCl	1.5 ml
0.5% Sodium deoxycholate	0.5 ml
50 mM Tris, pH – 8.0	0.5 ml
0.1 % SDS	0.1 ml
Protease inhibitor cocktail (1X)	0.1 ml
Distilled water	6.3 ml

All the components were added to distilled water except protease inhibitor cocktail (PIC) and the final volume of the solution was made to 9.9 ml by adding distilled water. PIC was added fresh to the solution.

5% SKIM MILK (For Western blotting)

Skim milk	5 g
PBS-T	100 ml

5g skim milk was dissolved in 100ml PBS-T.

4.3. METHODS

4.3.1. Cell culture

The human monocytic cell line, THP-1 was obtained from ILS, Bhubaneswar. It was cultured in RPMI-1640 (Gibco Laboratories, USA) containing 10% heat inactivated FBS at 37°C in the presence of 5% CO_2. Culture medium was changed when cell density was increased and pH became acidic. For changing the media, cells were centrifuged at 1000 rpm for 5 min and supernatant was discarded. Pellet was resuspended in 5 ml of fresh medium and transferred to the flask.

4.3.2. Cell viability

Cell viability assay was done using MTT cell viability kit according to the protocol supplied by the manufacturer. Briefly, 2.5×10^4 cells were seeded in triplicate overnight in the presence of PMA in each well of in 96-well plate. Next day, cells were washed and treated with varying concentration of Calcimycin for 24, 48 and 72 h. At appropriate time point, 10 µl of MTT was added in each well. Plate was gently swirled to mix the contents and incubated for 2-4 h at 37°C. Formazan crystals were formed and dissolved in 100 µl of lysis solution. For blank, cell lysis solution was added before adding MTT. The absorbance was read at 562 nm in plate reader.

The cell viability was calculated according to following formula:

% Cell viability = (OD of Test sample/OD of Control) x 100.

4.3.3. Western blotting

50 µg of protein from cell lysates after protein estimation by BCA reagent was electrophoresed on 15% SDS-PAGE. Protein bands were transferred to nitrocellulose membrane, blocked with skimmed milk followed by probing with anti-LC3 (Primary antibody) antibody that recognizes LC3-I and LC3-II bands for overnight at 4°C and later with secondary antibody for 45 min. at room temperature. Protein bands were visualized using enhanced chemiluminescence kit (Amersham, USA) as per manufacturer's instructions. Between each step membrane was washed three times with PBS-T. Blots were stripped and reprobed with β-actin antibody following same protocol as above for using as a loading control.

Cell lysis

10 µl of 100X PIC (protease inhibitor cocktail) is added in 1 ml of RIPA buffer for preparing cell lysis buffer. At appropriate time point, the supernatant of the cultured cells was removed and cells were washed twice with 1X PBS. Then cells were lysed in the wells with 0.1 ml of cell lysis buffer. Cell lysates were then transferred to ice-chilled micro centrifuge tubes. Tubes were then kept on ice for 30 minutes with intermittent vortexing after every 10 minutes. Tubes were then centrifuged at 15000 rpm for 15 min at 4°C and supernatants were dispensed into aliquots and stored at -20°C till protein estimation and SDS-PAGE electrophoresis.

Protein estimation by BSA reagent

Protein concentration of the sample was determined using BCA kit according to the protocol supplied by the manufacturer. Briefly, according to the number of samples 1:50 BCA working solution was prepared and mixed with 5 µl of standards, blank and test cell lysate samples in 96-well plate. Plate was incubated for 15-20 min at 37°C before taking absorbance at 562 nm. Readings were calculated and protein was estimated.

5. RESULT

5.1.1. Calcimycin kills THP-1 cells at concentration more than 0.8 µM at 24 h of treatment

PMA differentiated THP-1 cells were treated with different concentration of Calcimycin (0.001-1.0µM) for 24 h. Cell viability was decreased to 78±8.2% at 1µM Calcimycin as shown by MTT assay in Figs. 5 and 8.

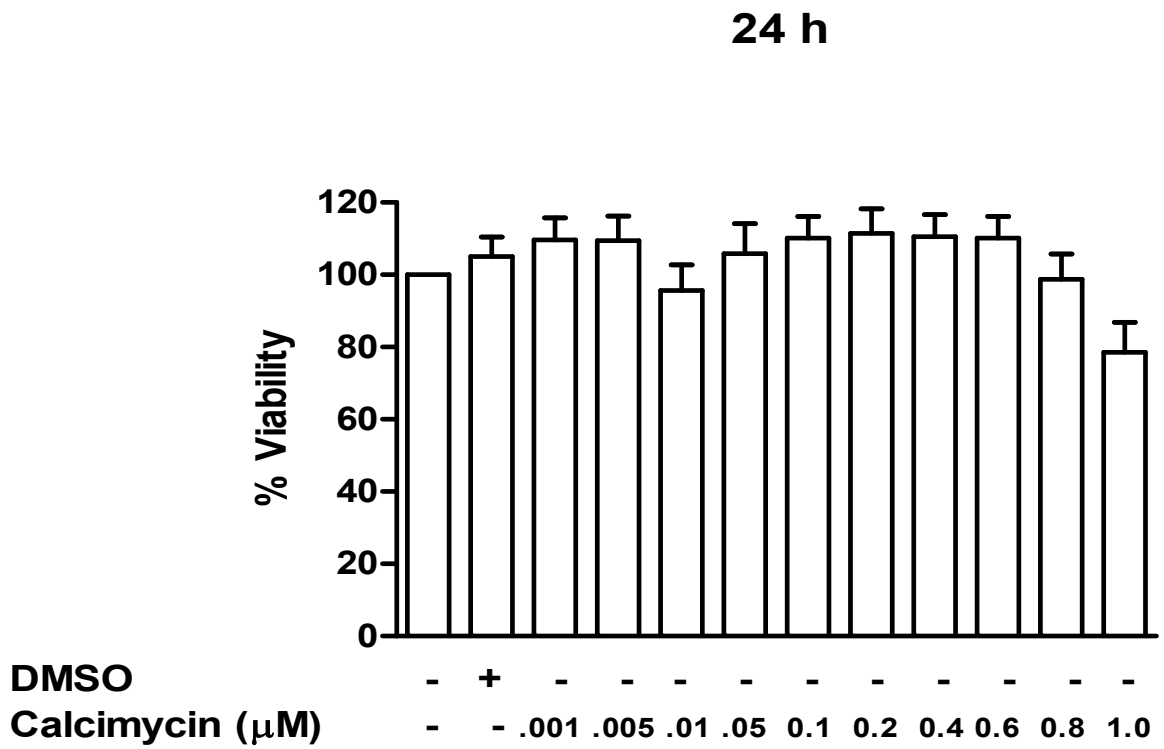


Fig.5. Effect of varying concentration of Calcimycin on cell viability of THP-1 cells after 24 h of treatment. The cytotoxicity was measured by MTT assay as described in materials and methods. Briefly, THP-1 cells were seeded in triplicate in 96 well plate and differentiated over night with 50ng/µl of PMA. Next day, cells were washed and subjected to different concentration of Calcimycin as shown in the figure after 24 h of treatment, MTT was added and cells were incubated for 3 h. After incubation, cell lysis buffer was added and OD was taken at 562 nm. Data are expressed as percentage cell viability over control cells.

5.1.2. Calcimycin kills THP-1 cells at concentration more than 0.6μM at 48 h of treatment

PMA differentiated THP-1 cells were treated with different concentration of Calcimycin (0.001-1.0μM) for 48 h. Cell viability was decreased to 85.4±2.1% and 69.7±4.2% at 0.8 μM and 1μM Calcimycin as shown by MTT assay in Figs. 6 and 8.

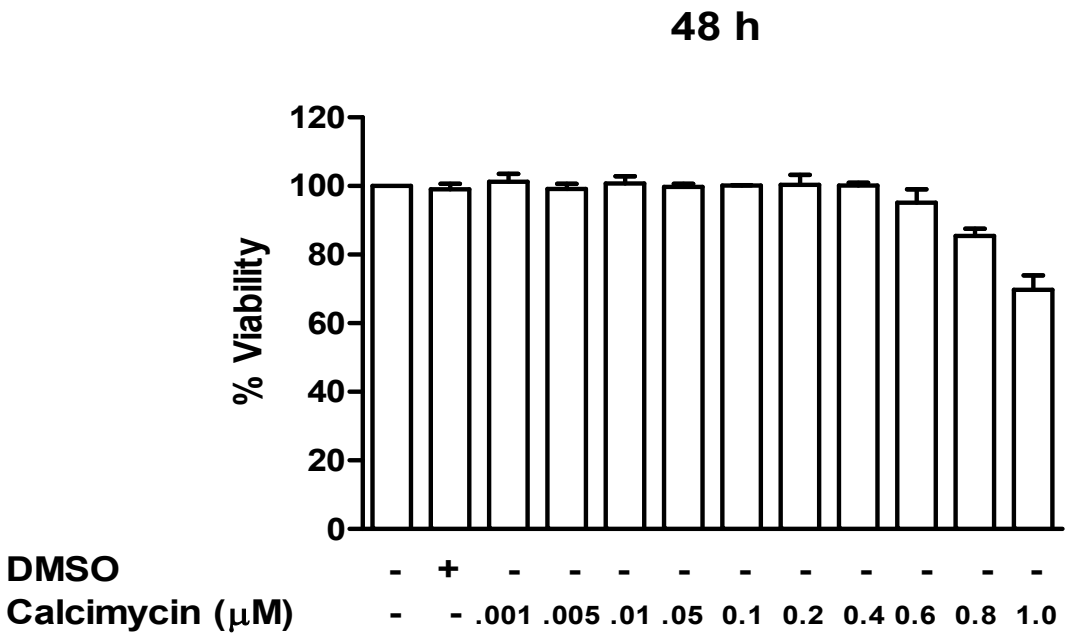


Fig.6. Effect of varying concentration of Calcimycin on cell viability of THP-1 cells after 48 h of treatment. MTT assay was employed to check the cell viability. Briefly, as written above, differentiated THP-1 cells were treated with Calcimycin for 48 h. After 48 h, MTT reagent was added and plate was incubated for 3 h and then lysis buffer was added. OD was taken at 562 nm. Data are expressed as percentage cell viability over control cells.

5.1.3. Calcimycin kills THP-1 cells at concentration more than 0.4μM at 72 h of treatment

PMA differentiated THP-1 cells were treated with different concentration of Calcimycin (0.001-1.0μM) for 72 h. Cell viability was decreased to 83.6±3.5%, 76.4±3.6% and 60±2.2% at 0.6 μM, 0.8 μM and 1μM Calcimycin as shown by MTT assay in Figs. 7 and 8.

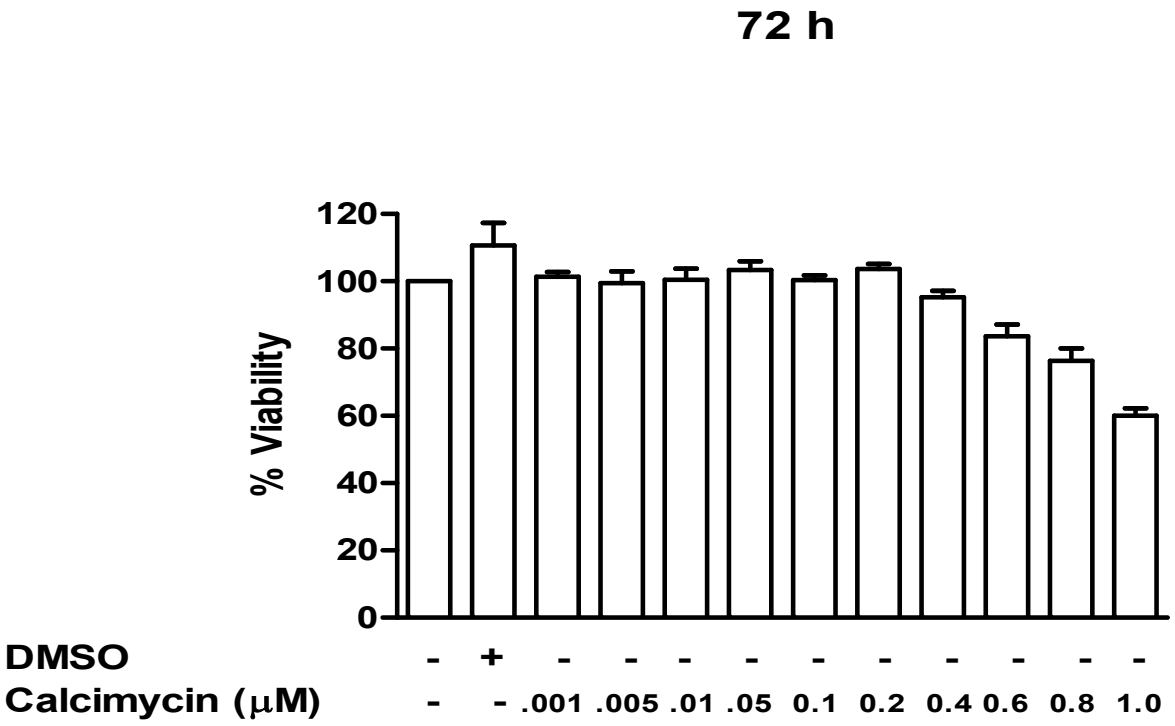


Fig.7. Effect of varying concentration of Calcimycin on cell viability of THP-1 cells after 72 h of treatment. MTT assay was employed to check the all viability. Briefly as written in pervious legend differentiated THP-1 cells were treated with Calcimycin for 72 h. After 72 h, MTT reagent was added and plate was incubated for 3 h and then lysis buffer was added. OD was taken at 562 nm. Data are expressed as percentage cell viability over control cells.

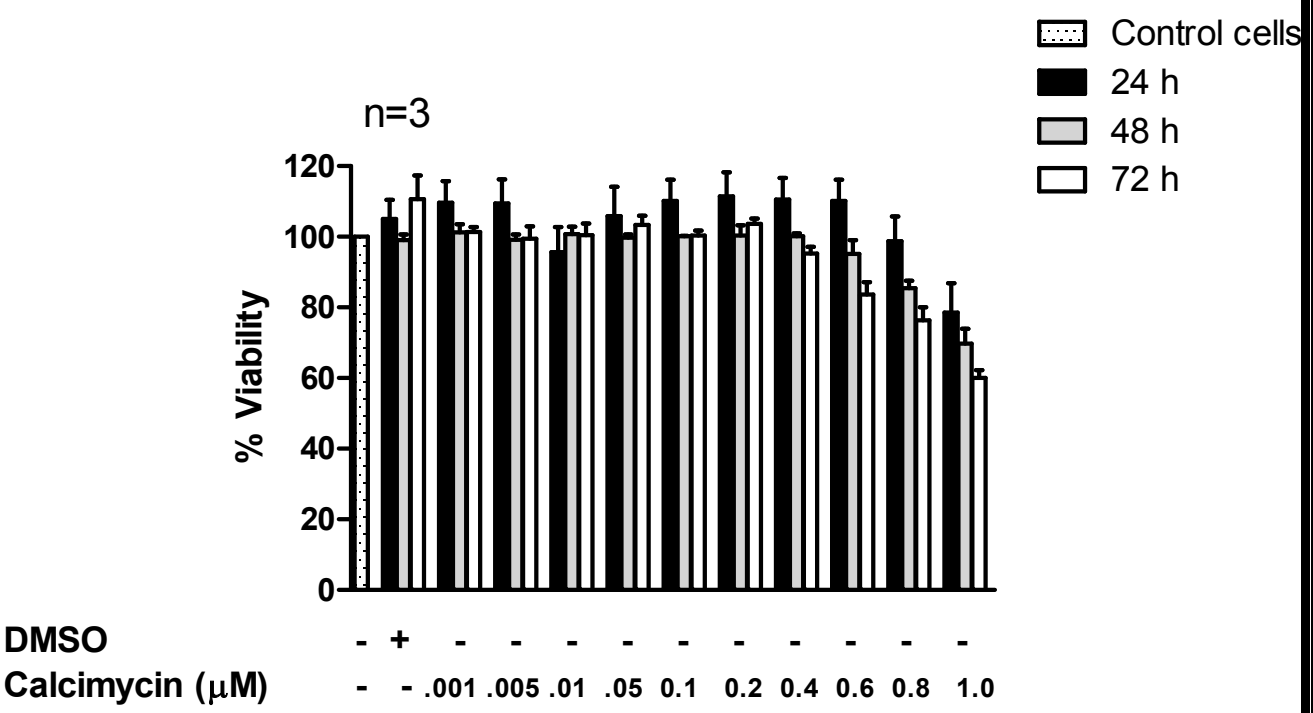


Fig.8. Effect of cumulative frequency varying concentration of Calcimycin on cell viability of THP-1 cells after 24, 48 and 72 h of treatment MTT assay was done to check the cell viability. Briefly, as written above, differentiated THP-1 cells were treated with Calcimycin for 24, 48 and 72 h. The cumulative % viability at different concentration of Calcimycin is shown here. OD was taken at 562 nm. Data are expressed as percentage cell viability over control cells.

5.2. The non-toxic concentration of Calcimycin (0.4μM) has an effect on autophagy of PMA differentiated THP-1 cells.

The effect of non-cytotoxic concentration of Calcimycin (0.4μM) on autophagy in differentiated THP-1 cells was studied by western blotting. We found that Calcimycin treatment led to accumulation of LC-3-II and in THP-1 cells compared untreated cells. This result suggests that Calcimycin is a potent inducer in THP-1 cells.

The expression of Atg7 and beclin1 expression shows that autophagy is occurring in THP-1 the cell at 0.4 μM

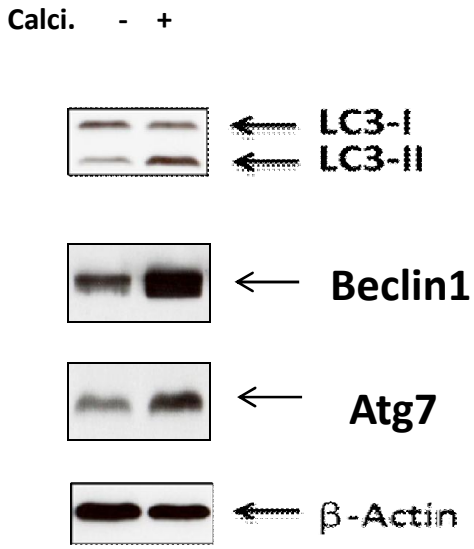


Fig.9. Effect of Calcimycin (0.4μM) on LC-3, Atg7 and Beclin1 degradation in differentiated THP-1 cells at 48 h of treatment. Differentiated THP-1 cells were treated with 0.4 μM of Calcimycin. After 48 h, whole cell lysates were prepared as described in materials and methods. Lysates were subjected to western blotting with an antibody against LC-3, Atg7 and Beclin1. The blot was stripped and reprobed with an antibody against β- actin (lower panel).

6. DISCUSSION

Better treatments are urgently needed for elimination and for cure of *M. tb.* Several studies shows that in TB patients that autophagy plays an important role in host defense against *M. tb*, which is the most detailed in vivo studied gene in autophagy have been shown to be associated with susceptibility to TB in different geographic regions such as China, West-Africa, and United States (Intemann et al., 2009; King et al., 2011; Che et al., 2010). Over the last few years, a number of genes like *P2X7*, *VDR*, *NOD2*, *TLR8*, which are associated with autophagy have been tested as candidate genes in tuberculosis patients. Recently, therapeutic modulation of autophagy by antiprotozoal drug nitazoxanide and its metabolite tizoxanide has been shown to lower intracellular *M. tb* proliferation in infected macrophages by inhibiting a major negative regulator of autophagy (Lam et al., 2012). So, by inducing autophagy through pharmacological drugs may help in TB therapy that involves molecules of the host responses in order to affect the host-pathogen interaction to get a new outcome that is helpful to the host. Since pharmacological agents are only inducing a host response and not directly affecting the bug so chances of acquired resistance in the bug would be minimal which a major bottleneck in the drug discovery area is. Our results show that Calcimycin is a potent autophagy inducer in THP-1 cells at concentration of 0.4 μM. Future studies will see the autophagic potential of this compound against mycobacteria infected macrophages and its effect on the mycobacterial viability. Understanding the mechanism of autophagy by calcium ionophores may provide an attractive target for the control of mycobacterial infection, which will help in developing novel vaccines and therapeutics against TB.

7. CONCLUSION

From different concentration of Calcimycin out of that 0.4 μM concentration or above that is inducing autophagy in the cell. But 0.4 μM is non cytotoxic to the cells. By the expression of LC-3- II, Atg 7 and Beclin1 show that autophagy is occurring at 0.4 μM concentration.

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