EFFECT OF CALCIMYCIN REGULATED AUTOPHAGY ON NUCLEAR RECEPTOR HNF-4α EXPRESSION IN THP-1 CELLS

THESIS SUBMITTED TO:

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CERTIFICATE

This is to certify that the thesis entitled "***Effect of Calcimycin regulated autophagy on Nuclear Receptor HNF -4α Expression in THP – 1 Cells***" which is being submitted by **Miss Pratibha Kumari**, Roll No. **413LS2033**, 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 her 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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Last, but not the least, I would thank the Almighty and my parents, whose dedicated and untiring efforts towards me has brought me at this stage of my life.

Pratibha Kumari
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DECLARATION

I do hereby declare that the Project Work entitled "**Effect of Calcimycin regulated autophagy on Nuclear Receptor HNF - 4α Expression in THP – 1 Cells"**, submitted to the Department of Life Science, National Institute of Technology, Rourkela is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Rohan Dhiman, Asst. Professor, Department of Life Science, National Institute of Technology, Rourkela, Odisha.

Date:

Place:

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ABBREVIATIONS

1) HNF-4α - Hepatocyte nuclear factor 4 alpha
2) ER - Endoplasmic reticulum
3) T.B. - Tuberculosis
4) *M. tb* - *Mycobacterium tuberculosis*
5) LBD - Ligand Binding Domain
6) DBD - DNA Binding Domain
7) RAR - retinoic acid receptor
8) RXR - retinoid X receptor
9) NR2A - nuclear receptor subfamily 2, group A
10) INF-γ - Interferon gamma
11) TNF-α - Tumor necrosis factor alpha
12) TLR - Toll like receptor
13) NOD 2 - Nucleotide-binding oligomerization domain-containing protein 2
14) IL-1β - Interleukin 1 beta
15) FBS - Fetal Bovine Serum
16) DMSO -Dimethyl sulfoxide
17) PMA - Phorbol myristate acetate
18) PIC - Protease Inhibitor Cocktail
19) EDTA - Ethylene diamine tetra acetic acid
20) NaOH - Sodium Hydroxide
21) KCl - Potassium Chloride
22) KH_2PO_4 - Potassium Dihydrogen Phosphate
23) HCl - Hydrochloric acid
24) Na_2HPO_4 - Disodium phosphate
25) SDS - Sodium dodecyl sulphate

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ABSTRACT

Nuclear receptors (NRs) include 49 members and belong to family that function as ligand-inducible transcription factors. The basic structure of nuclear receptors involves the n-terminal transactivation domain and c-terminal ligand binding domain. Transactivation domain that is DNA binding domain contains highly conserved zinc finger region whereas ligand binding domain is responsible for the transactivation within the targeted tissue. It has been reported that PPARγ, one of the nuclear receptor play an important role in mycobacterial infection. *Mycobacterium tuberculosis* is the bug that causes tuberculosis which is one of the deadly diseases of humankind. This bacterium gains entry through air passage and internalized by alveolar macrophages for efficient clearance. In spite of these barriers, bug has the tremendous ability to abrogate these responses for its own multiplication like inhibition of autophagy. Autophagy is one of the host defence mechanisms that have gained popularity in last 4-5 years for their anti-microbial responses. Various factors like ATP, Vitamin D and cytokines have been shown to induce autophagy. ATP is reported to induce autophagy by increasing intracellular calcium level. In our lab we are trying to study the response of one of the calcium ionophore that increases intracellular calcium level, Calcimycin in inducing autophagy in THP-1 cells. So, in this study we tried to link the role of calcimycin induced autophagy with the expression of another nuclear factor, HNF-4α.

INTRODUCTION

Nuclear receptor binds to non - polar regulatory molecules that are activated by ligands. These are the proteins and they belong to a family which contains transcription factors which are highly conserved. Transcription is regulated by the nuclear receptor in response to small lipophilic compounds. There are three domains in case of nuclear receptors:

- Ligand binding domain (LBD)
- Transcriptional regulation domain
- DNA binding domain (DBD)

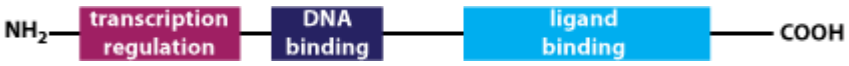

Figure 1: Structure of nuclear receptor (Adapted from: 1)

The DNA binding domain (DBD) binds to specific DNA sequences whereas LBD targets small lipophilic ligands. These two domains of the nuclear receptors are the highly conserved domains. Zinc motifs are present in the N - terminal of the DBD (1). There are 48 nuclear receptors known in human genome. Out of which ligand dependent transcription factors are 24. Since, it has an important role in the discovery of various drugs and controls the functions related to various diseases such as diabetes, cancer, infertility and obesity etc. hence, we can say that nuclear receptors helps to reach the pharmacological targets. The metabolic and reproductive diseases are the result of dysfunctioning of the nuclear receptors (2). There are many organisms that live in our bodies which are harmless or even helpful but sometimes under certain conditions they show they harmful effect and some organisms may cause some diseases. The disorders caused by the organisms like viruses, parasites, bacteria and fungi are known as infectious diseases. These infectious diseases can be transmitted from one person to another person. Fever and fatigue are the common symptoms of the infectious diseases. Other symptoms vary depending on the organism causing the infection. There are various infectious diseases like influenza, smallpox, chickenpox, measles, avian influenza and tuberculosis (T.B.) etc. and these diseases can be prevented through vaccines.

T.B. is most common infectious and dangerous disease and *Mycobacterium tuberculosis* (*M. tb*) is it's the causative agent. T.B. was one of the most devastating diseases and it was first explained in 1882 by Robert Koch. Recently it has been found that the expression of PPARγ increases due to Mycobacterial infection. PPARγ is one of the types of nuclear receptor. Due to increase in expression of PPARγ, the formation of lipid droplet also increases and the response shown by the macrophage decreases, this might helps the *Mycobacteria* in escaping from the host immune mechanism. Whereas, when the expression of the PPARγ is inhibited the capacity to eliminate the *Mycobacteria* through macrophages increases. This suggests that PPARγ plays an important role in the chronic infection (3, 4).

Phagosome maturation pathway is interfered through *M. tb* in host phagocytic cell, since it is an intracellular pathogen which persist within the phagosomes because of which the phagosome - lysosome fusion is inhibited. Hence, *M. tb* prevents itself from the antigen-processing and bactericidal environment of phagolysosome (5). Autophagy is involved in nutrient regeneration, protein and organelle degeneration but also in clearance of intracellular pathogens such as *M. tb.* There are mainly three forms of autophagy such as macroautophagy, microautophagy and chaperone mediated autophagy. In case of macroautophagy mainly damaged cell organelles or unused proteins are removed. There is a formation of double membrane around cytoplasmic substrates. In case of macroautophagy which results in the formation of Autophagosome. Autophagosome fuse with the lysosome and the contents are degraded via acidic lysosomal hydrolases. In case of microautophagy the cytoplasmic materials are directly engulfed into the lysosome through inward folding of the lysosomal membrane. Chaperone-mediated autophagy involves the hsc-70 containing complex recognition. Because of this substrate/chaperone complex is formed due to binding of protein with that of the chaperone. After entering into the cell the substrate protein gets unfolded and it is translocated across the lysosomal membrane in the presence of lysosomal hsc-70 chaperone. This process of autophagy is extremely selective. Calcium signaling regulates the autophagy. ER stress induction also initiates the autophagy. Negative regulation of AKT/TSC/mTOR pathway leads to formation of Autophagosome which in turn increases the autophagy through ER stress. Calcimycin is a calcium ionophore which increases the calcium ion concentration. Therefore in this study we try to interlink the connection between calcimycin mediated autophagy and nuclear receptor mainly HNF-4α.

REVIEW OF LITERATURE

NUCLEAR RECEPTORS

Nuclear receptors are the intracellular transcriptional protein which is an important factor for the expression of gene in response to lipophilic molecules. Key role is played by the nuclear receptors in the study of many diseases like cancer, diabetes and obesity etc. they are also important for metabolism and cell death, for maintaining the cellular phenotypes and also essential for the development of embryo. If the nuclear receptors will not function properly then this will leads to certain metabolic and reproductive diseases like diabetes, infertility, obesity and cancer etc. (3). Lipophilic hormones are suitable to serve as regulators for the control of gene expression because they are able to diffuse from a source and permeate to a target. There are several kinds of lipophilic hormones such as steroid, vitamin D3, thyroid hormones and retinoid etc. (6). By the middle of the century glucocorticoids became a popular therapeutic agent and also many of lipophilic hormones were related with various human diseases (6). According to the classical steroid hormone action model, some allosteric changes occurs due to binding of hormones to its receptor as a result of which hormone receptor complex binds to the high affinity sites in chromatin and which starts the process of transcription. We can broadly divide nuclear receptors into four classes:

- Class I receptor
    - Contains steroid hormone receptors and function as ligand induced homodimers.
    - Also includes androgen (AR), progestin (PR), estrogen (ER), glucocorticoids (GR) etc.
- Class II receptor
    - They contain all retinoic acid receptor, RAR, vitamin D3 and retinoid X receptor, RXR.
    - Also includes hetrodimerization with RXR when ligands are absent some of these receptors acts as repressors.

- Class III receptor
    - These bind to direct repeats as homodimers and also called as orphan receptors.
- Class IV receptor
    - Binds to extended core sites as monomers.

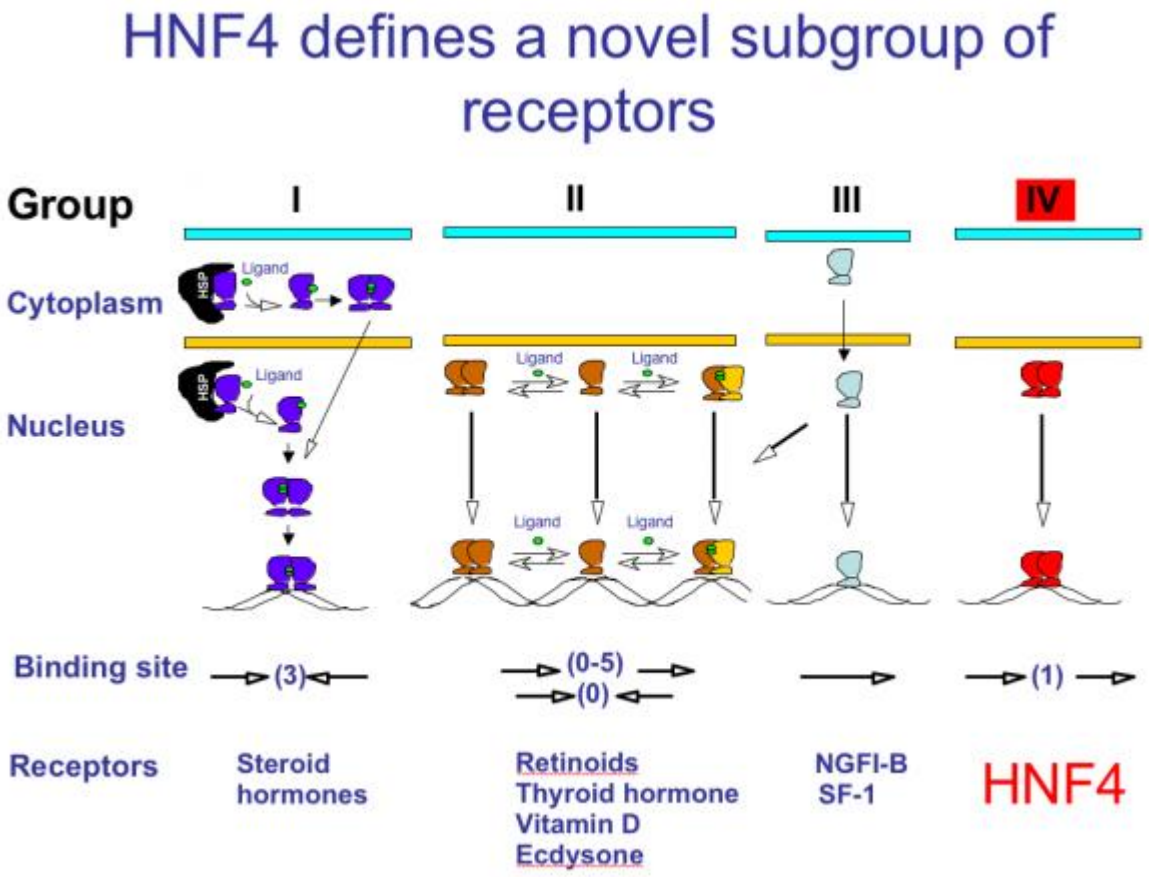

Figure 2: Nuclear receptor subfamily (Adapted From: 7).

HNF - 4A gene encodes HNF - 4α (Hepatocyte nuclear factor 4 alpha) nuclear receptor protein in human. It is also known as NR2A (nuclear receptor subfamily 2, group A, member 1). It binds to DNA as a homodimers. The major targets of this gene are intestines, liver and kidney (8, 9). Expression of HNF4-α can be up regulated through alkaloid berberine.

Any changes or mutation in HNF4-α gene decreases the amount of insulin produced which leads to diabetes.

MAIN ROLE OF NUCLEAR RECEPTORS:

- The regulatory signals are propagated within the effector cell from outside through nuclear receptor.
- Homeostasis is maintained and also helps to adapt to the changes.
- Function as a key factor in the development of drugs.
- Act as a transcription factors by controlling the cellular process by regulating the targeted gene expression. As a result synthesis of specific mRNA takes place due to which specific protein synthesis occurs, so that the targeted cells activity are regulated (10).
- Also plays a major role in the embryonic development.

INFECTIUOS DISEASES

There are several infectious agents that cause infectious diseases such as protozoan, fungus, bacteria, virus and helminths. These infectious agents cause disease like smallpox, influenza, bird flu, AIDS and T.B. etc. T.B. is the most common and dangerous infectious diseases. The causative agent of tuberculosis is *M. tb*. T.B. spreads by droplet transmission, via the air when a contaminated patient coughs or sneeze. Humans are the reservoirs for this bacterium. *M. tb* is a non-motile rod shaped and a intracellular parasite, usually of macrophages (11). The *bacterium* used to reside in our alveoli (in lungs) and remain dormant throughout a long time. A host can carry this particular bacterium throughout his life without any apparent symptoms but in certain individuals it may reactivate to cause the disease. Usually the bug resides inside the phagocytic cells or macrophages. Our immune system is well equipped to protect us against different bacterial cells or infection and important component of this system is macrophages. For the growth and survival of *M. tb*, macrophages act as a host cell niche. Macrophages phagocytose all those bacteria like *Mycobacterium* and have the capability to kill the same after internalization. But *M. tb* hijack these immune system cells for its own multiplication and growth (12). By this virtue, bug attains the power of staying inside the cells for long time without being degraded by the host cell machinery by suppressing various host defence mechanisms like inhibition of autophagy.

NUCLEAR RECEPTORS PLAYS AN IMPORTANT ROLE IN AUTOPHAGY

Autophagy is a mechanism which affects the immune responses. It is a cell autonomous defense mechanism which acts against *M. tb*. There are several factors such as "Atg proteins" like Atg5 and Beclin1 (Atg6) responsible for the execution of the process of autophagy. Autophagy is the process which kills the intracellular *M. tb*. One of the most important roles of autophagy is presentation and processing of antigen. These are modulated by cytokines and T helper type 1 cytokines like tumour necrosis factor (TNF)-α and interferon (INF)-γ that stimulates autophagy. Th2 cytokines interleukin (IL)-4, IL-13 and IL–10 inhibits the autophagy. Other factors like Vitamin D, NOD-like receptor 2 (NLR-2), Toll- like receptor (TLR) and ATP have been shown to induce autophagy. The expression of cathelicidin increases through binding of vitamin D to the vitamin D receptors (13). Cathelicidin are the antimicrobial peptide and have the ability to kill the *M. tb* (14). Autophagy can also be induced by vitamin D through cathelicidin in human monocytes and macrophages. Calcitriol (1, 25 vitamin D3) is produced in kidney and at a very low concentration it circulates in the serum. IFN-γ induced autophagy requires Calcitriol (15). Calcitriol can be produced through its precursor 2, 5–hydroxyl vitamin D.

Beclin 1 and Atg5 are the important factors for the IFN-γ and Calcitriol induced autophagy in human cells (15). The IFN-γ plus Calcitriol induce autophagy in human cells promotes maturation of phagosomes harbouring *M. tb* and facilitates their fusion with lysosomal compartments that contain antimicrobial peptide (16).

NLR-2 is also known for the induction of autophagy. Intracellular NLR-2 recognises *M. tb* and regulates secretion of IL-1β (17).

Engagement of NLR-2 by muramyl dipeptide activates autophagy, promotes bacterial trafficking to the autophagolysosome and enhances antigen presentation (18). On the entry of the bacteria the Atg16L1 recruitment to the plasma membrane is done through Nucleotide-binding oligomerization domain-containing protein 2 (NOD 2) (19). ATP has also been shown to induce autophagy by increasing intracellular calcium level.

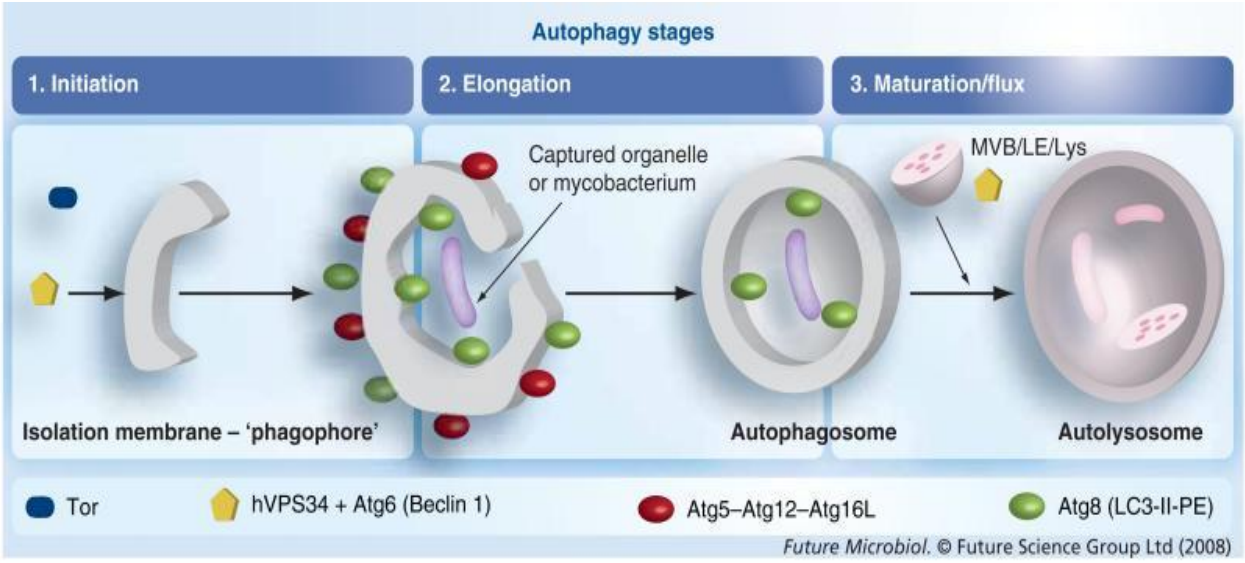

Figure 3: PI3 kinase hVPS34 executes the process of autophagy and at initiation P13-phosphate is formed. Autophagy also requires the Beclin (Atg6) which is the subunit of Hvps34 complex. Autophagosome wraps around organelles or a phagosome containing Mycobacteria. Due to Atg factors, the membrane of the Autophagosome elongates and bends to form two complexes. One is Atg5 which is conjugated to Atg12 and the other is Atg8 which conjugated to PE (phosphatidylethanolamine).

Adapted from: (20)

Calcimycin is a calcium ionophore that increases intracellular calcium level and it forms stable complexes with ions having charge of +2 and can be obtained by fermentation of *Streptomyces chartreusensis*. Its molecular formula is $C_{29}H_{37}N_3O_6$ and it is also having antibiotics properties against gram positive bacteria and fungi. Calcimycin inhibits mitochondrial ATPase activity and in some cells it induces apoptosis. Calcimycin is commercially available as free acid, Ca^{2+} salt. The IUPAC name of Calcimycin 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.

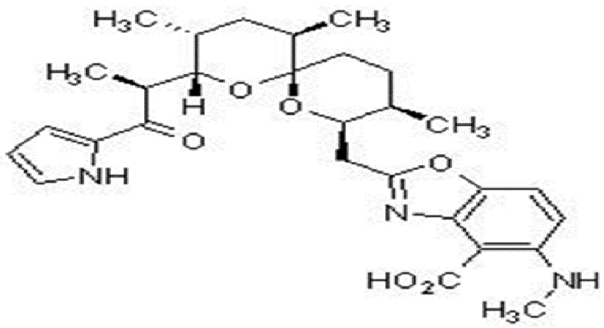

Figure 4: calcimycin structure

Adapted from: www.wikipedia.com

PPARγ expression has been reported to increase during mycobacterial infection. PPARγ is one of the types of nuclear receptor. Due to increase in expression of PPARγ, the formation of lipid droplet also increases and the response shown by the macrophage decreases might helps the mycobacteria in escaping from the host immune mechanism (21). Whereas when the expression of the PPARγ is inhibited the removal of the mycobacterium increases by macrophages. This suggests that PPARγ plays an important role in the chronic infection.

In our study, we tried to elucidate the role of calcimycin induced autophagy on HNF-4α expression in THP-1 cells.

OBJECTIVES OF MY WORK

1) To see Calcimycin effect on the viability of THP-1 cells.
2) To see non-cytotoxic effect of Calcimycin concentration on HNF-4α expression.
3) To see the interplay between calcimycin induced autophagy and HNF-4α expression.

MATERIALS AND METHODS

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).

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.

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.

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 litter with double-distilled water.

1 M Tris (pH 6.8 or 7.4 or 8.0 or 9.0)

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 concentrated HCl (0.1M). The volume of the solution is adjusted to 1 litter.

Reservoir Buffer 10X (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 Buffer10X (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.

METHODS

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.

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, 10µl 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.

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 BCA 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.

RESULT

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

PMA differentiated THP-1 cells were treated with different concentration of Calcimycin (0.001-1.0 μM) for 24 h. Cell viability was decreased to 74.14 ± 6.6% at 1 μM Calcimycin as shown by MTT assay in Figs. 5 and 8.

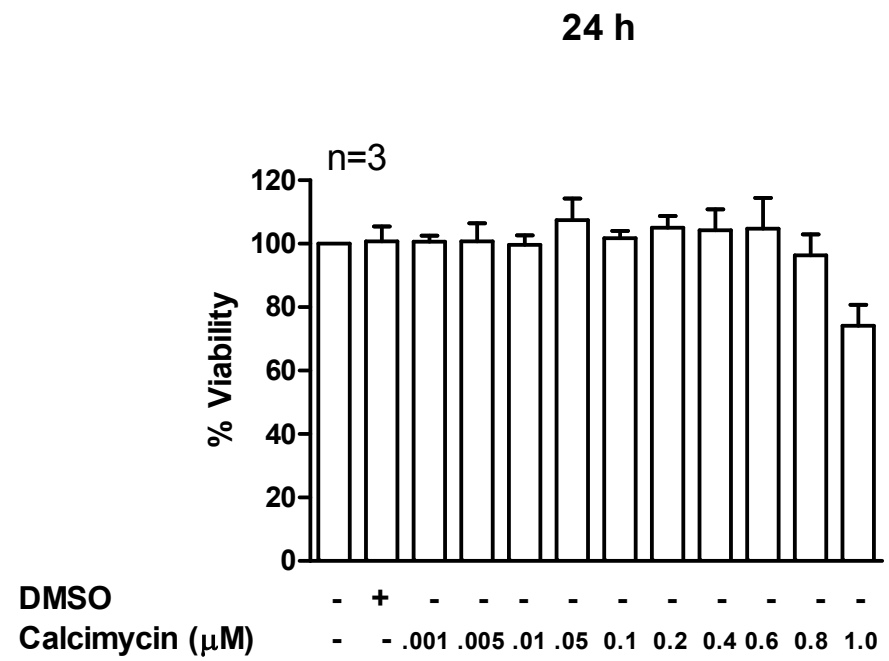

Figure 5: Effect of varying concentration of Calcimycin on cell viability of THP-1 cells after 24 h of treatment. The cytotoxicity was measured by MTT assay as described in materials and method. Briefly, THP-1 cells were seeded in triplicate in 96 well plates and differentiated over night with 50 ng/μl 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.

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

PMA differentiated THP-1 cells were treated with different concentration of Calcimycin (0.001-1.0 μM) for 48 h. Cell viability was decreased to 83.07 ± 4.8% and 59.7 ± 2.6% at 0.8 μM and 1μM Calcimycin respectively as shown by MTT assay in Figs. 6 and 8.

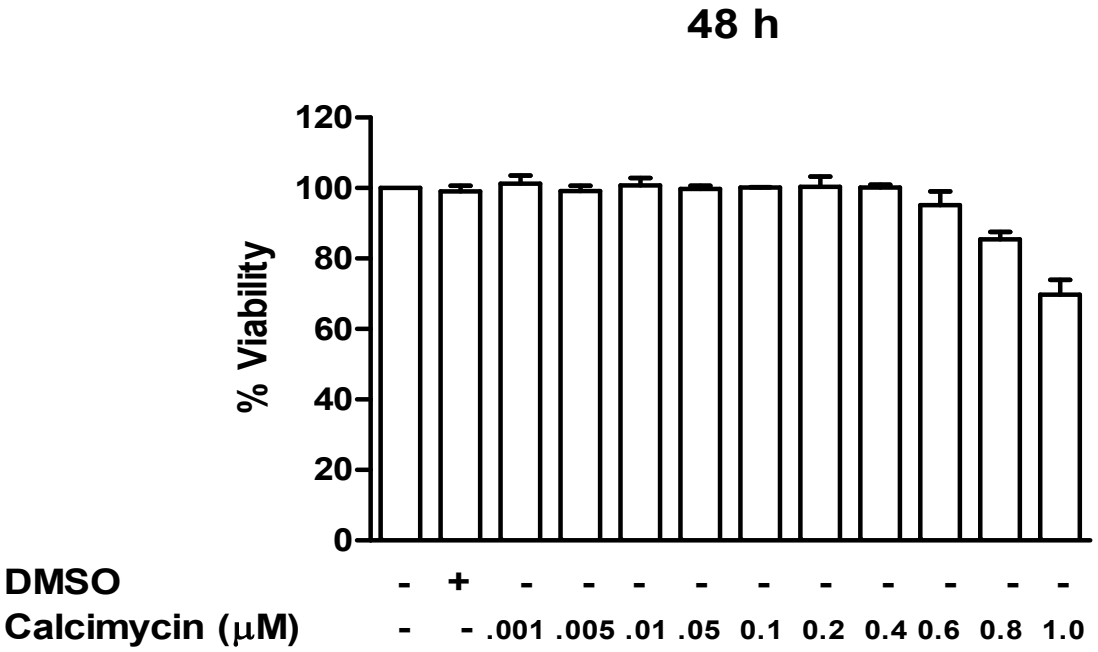

Figure 6: Effect of varying concentration of Calcimycin on cell viability of THP-1 cells after 48 h of treatment. MTT assay was employed to check the all viability. Briefly as written in pervious legend differentiated THP-1 cells were treated with Calcimycin for 48 h. After 48 h, MTT reagent was added, plates were 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.

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

PMA differentiated THP-1 cells were treated with different concentration of Calcimycin (0.001-1.0 μM) for 72 h. Cell viability was decreased to 80.32 ± 4.6%, 68.03 ± 3.7% and 50.01 ± 2.2% at 0.6 μM, 0.8 μM and 1 μM Calcimycin as shown by MTT assay in Figs. 7 and 8.

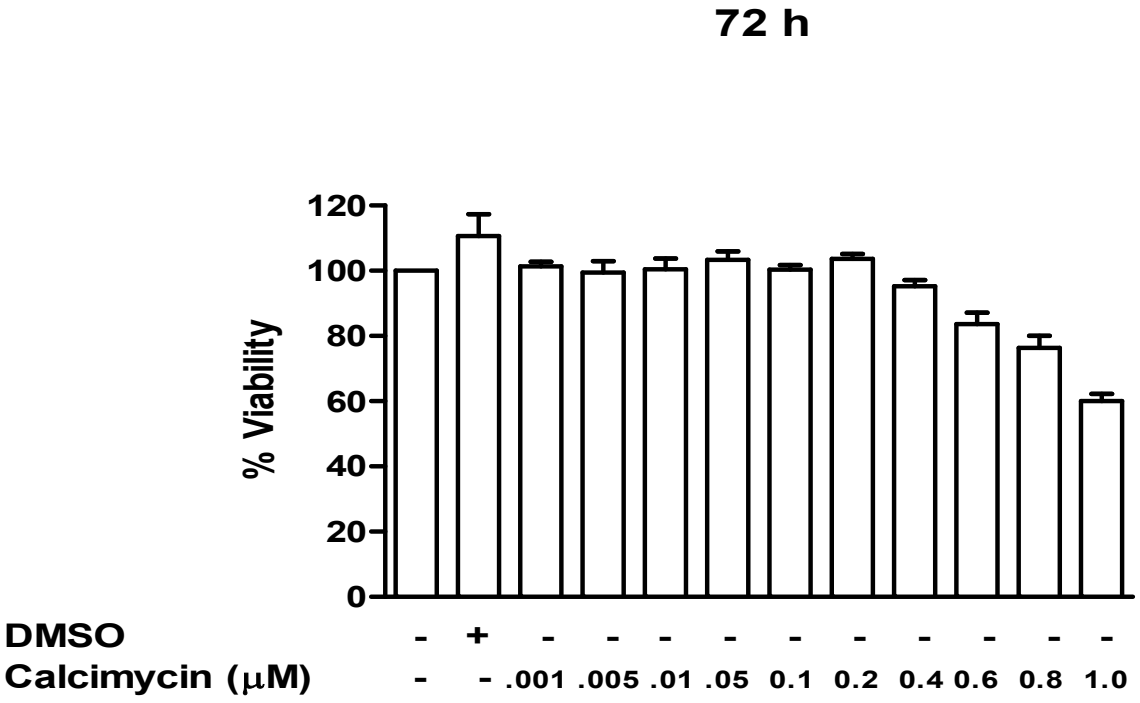

Figure 7: Effect of varying concentration of Calcimycin on cell viability of THP-1 cells after 72 h of treatment. MTT assay was employed to check the cell viability. Briefly, as stated above, differentiated THP-1 cells were treated with Calcimycin for 72 h. After 72 h, MTT reagent was added, plates were 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.

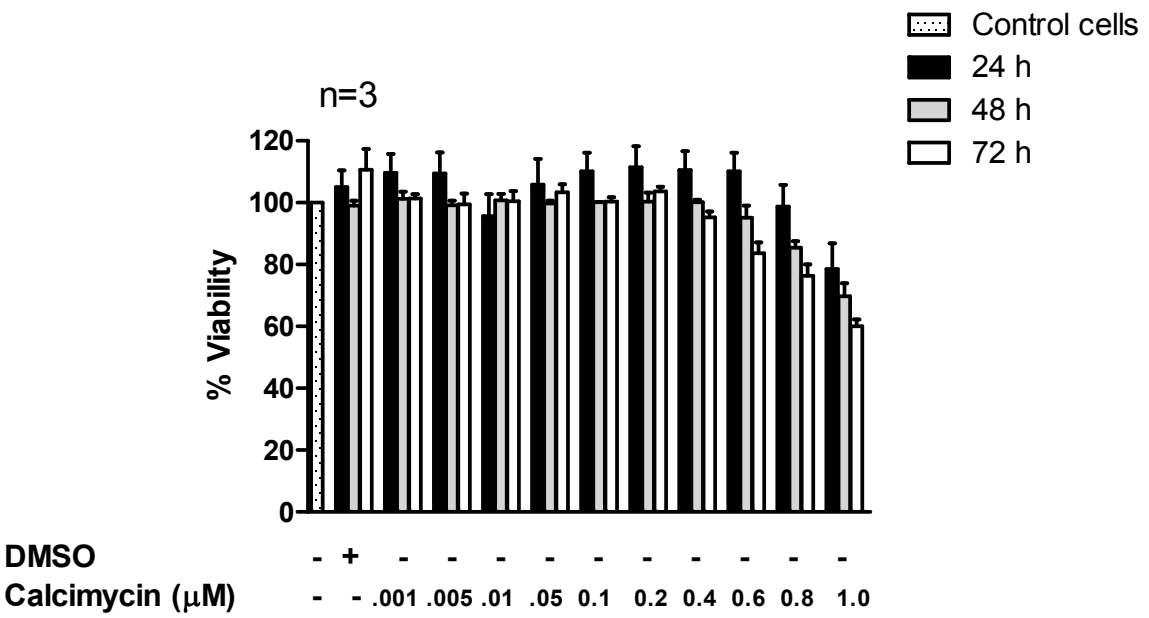

Figure 8: Effect of varying concentration of Calcimycin on cell viability of THP-1 cells after 24, 48 and 72 h of treatment. MTT assay was employed to check the cell viability. Briefly, as written above, differentiated THP-1 cells were treated with Calcimycin for 24, 48 and 72 h. At appropriate time points, cell viability was measured by taking OD at 562 nm. Data are expressed as percentage cell viability over control cells.

Calcimycin treatment increases HNF-4α expression in THP-1 cells.

PMA differentiated THP-1 cells were treated with 400 mM of Calcimycin. After 48 h cell lysates were prepared and western blotting was done to detect HNF-4α expression upon Calcimycin treatment.

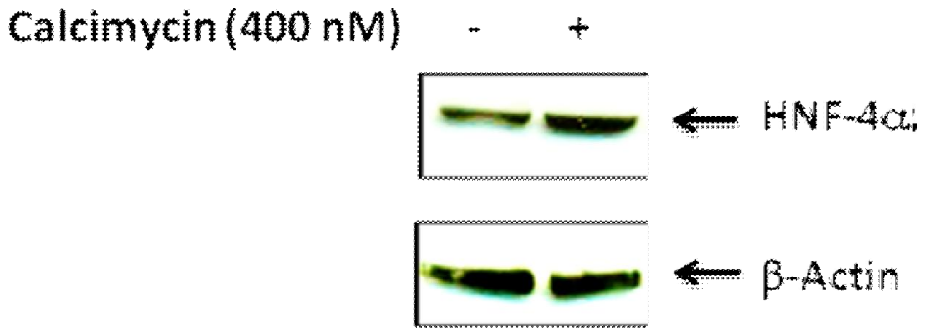

Figure 9: Effect of calcimycin (0.4 µM) on HNF-4α expression in differentiated THP-1 cells at 48 h of treatment. PMA differentiated THP-1 cells were treated with calcimycin (0.4 µM). After 48 h of treatment, whole cell lysates were electrophoresed and western blotting was done to detect the HNF-4α expression. Blot was stripped and for loading control, β-actin expression was detected using anti- β-actin antibody.

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

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

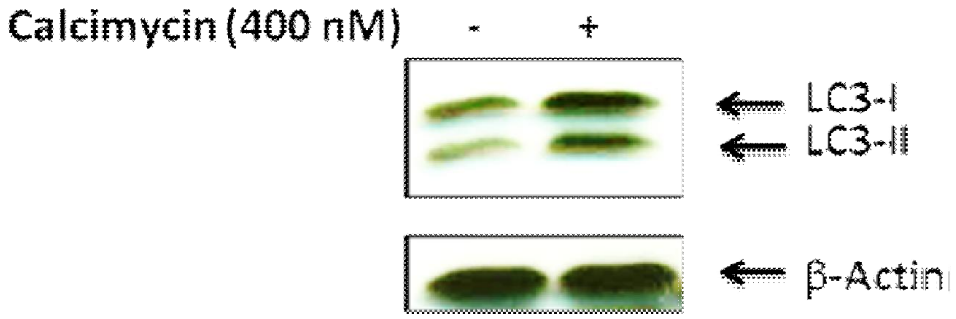

Figure 10: Effect of Calcimycin (0.4 µM) on LC-3 degradation in differentiated THP-1 cells at 48 h of treatment. Differentiated THP-1 cells were treated with 0.4 µM of Calcimycin. After 48 h, whole cell lysates were prepared as described in materials and methods. Lysates were subjected to western blotting with anti-LC3 antibody (upper panel). Blot was stripped and for loading control β-actin expression was detected using anti-β-actin antibody.

3-MA abrogates Calcimycin induced HNF-4α expression in THP-1 cells.

In differentiated THP-1 cells 3-MA, an autophagy inhibitor was used to see the role of autophagy in HNF-4α expression. We found that addition of 3-MA inhibits the Calcimycin induced HNF-4α expression in THP-1 cells.

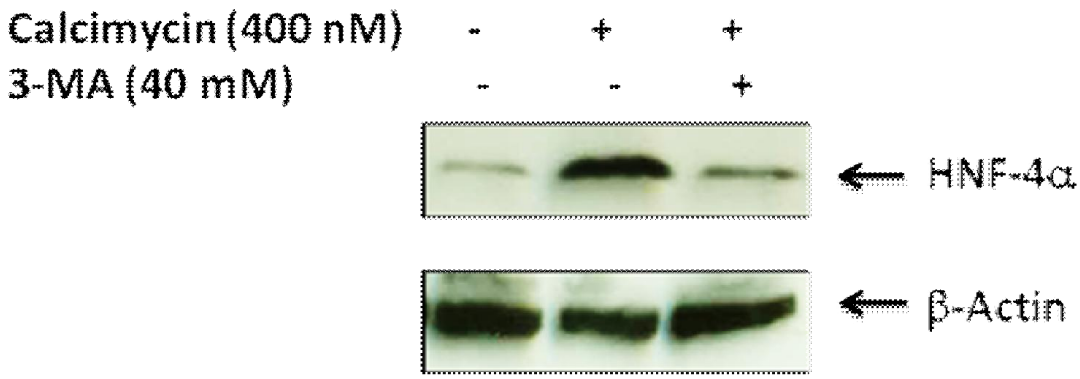

Figure 11: Effect of Calcimycin and 3-MA on HNF-4α expression in THP-1 cells. PMA differentiated THP-1 cells were pre-treated with 3-MA (Methyl Adenine) before treating with Calcimycin (400 nM). After 48 h of Calcimycin treatment, whole cell lysates were prepared. Cell lysates were then electrophoresed and western blotting was done to detect HNF-4α expression. Blot was stripped and for loading control β-actin expression was detected using anti-β-actin antibody.

DISCUSSION

Various inducers of autophagy are being discovered every day. Amongst important autophagy inducers, ATP has been shown to belong to these classes of compounds by increasing intracellular calcium level.

In our lab we are currently using Calcimycin, an calcium ionophore to study its antimycobacterial effect. Nuclear factor like PPARγ has been shown to play an important role in tuberculosis. In one of the experiment we found increased expression of HNF-4α in THP-1 cell line upon Calcimycin treatment. This result is surprising because THP-1 cells share close proximity to human alveolar macrophages and HNF-4α expression has been shown in literature to be limited in the organs like liver and kidney.

Since we found Calcimycin induces autophagy in THP-1 cells so to further see the interplay between Calcimycin induced autophagy and HNF-4α expression we used autophagy inhibitor (3-MA)in the presence and absence of Calcimycin. We found that 3-MA abolished the HNF-4α expression induced by Calcimycin. Clearly suggesting autophagy regulates HNF-4α expression in THP-1 cells. Further experiments are required to elucidate the mechanism of HNF-4α expression in the presence of Calcimycin and its role in tuberculosis.

CONCLUSION

1) Concentration 0.4 μM of Calcimycin didn't show any cytotoxicity in THP-1 cells after 24, 48 and 72 h of treatment.
2) 0.4 μM of Calcimycin induced HNF-4α expression in THP-1 cells.
3) Autophagy inhibitor (3-MA) abolished the increased HNF-4α expression by Calcimycin.

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