SAC ameliorates TNF-α induced inflammation by inhibiting p38 and JNK phosphorylation in HaCaT cells

M.Sc. SUMMER TRAINING PROJECT WORK

UNDER THE GUIDANCE OF

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(SUDESHNA MONDAL)

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1. Introduction:

Inflammation is a part of body's immune response (innate immunity) which protects our body from physical injury, pathogen's attack, chemical substances or radiation by the increased movement of plasma and blood leukocyte from blood into the site of injury (1). In the case of chronic inflammation, various disease is developed e.g. psoriasis, rheumatoid arthritis, cancer, ischemia, type III hypersensitivity etc. Various inflammatory mediators are considered to play key role in the development of an acute and chronic response with IL-1 and TNF-α being the primary cytokines propagating this process.

In some cases, e.g. autoimmune disease, the body's own protective immune system causes damage to its own tissues when there are no foreign invaders such as bacteria and virus.

Causes of inflammation:

1. Pathogens like bacteria, virus, fungi (2)
2. External injuries (3)
3. Chemical or radiation (2)

HaCaT, a model to study keratinocyte

HaCaT (Ha- human, Ca- calcium, and T- temperature) is an immortalized non tumorigenic cell line used to study the keratinocyte differentiation and skin inflammation disease such as psoriasis, atopic dermatitis because its phenotype resembles of normal human keratinocyte. HaCaT maintain their epidermal differentiation capacity and reform a structured and differentiated epidermis (4). Exogenous growth factor and GF depletion regulate the proliferation and differentiation of HaCaT inducing apoptosis.

TNF- α

TNF-α is a pro-inflammatory pleotropic cytokine which mediates inflammation in different inflammatory skin diseases (5). TNF-α is expressed by many immune cells such as macrophage, monocytes, T cells, NK cells, neutrophils and other immune cells. In 1975 Carwell et al first described this as a cytokine showing its cytotoxicity activity and tumor necrosis (6). It acts by binding to cell receptors with high affinity and high specificity and induce some proinflammatory cytokines e.g IL-1β, IL-6, IL-8. It has two transmembrane receptors TNFR1, also known as p^{55} or p^{60} and TNFR2, known as p^{75} or p^{80} which is a part of TNF receptor super family (TNFRSF) having an intracellular segment, a transmembrane domain and an extracellular ligand binding domain characterized with cytokine rich motif helps TNF-α to elicit the biological response. TNF binds with almost equal affinity to this receptor. Mainly TNFR2 activates 1. ERK (extracellular regulated kinase) and 2. C Jun N terminal kinase, two member of MAP kinase (6).

It was considered for a long time that bacterial lipopolysaccharide triggers the production of TNF-α. Beside this many other cytokine, enterotoxin, superantigen, interleukin and also TNF-α itself as an autocrine fashion stimulate TNF-α production (7).

Activation of MAP kinase pathway by TNF-α

Mitogen activated protein kinases (MAP kinase) are composed of a family of protein kinases which regulate several important cellular activities such as gene expression, mitosis, metabolism, programmed cell death by phosphorylating specific serines and threonines of target proteins. For this activity, MAP kinase have been extensively studied to detect their effect in many human disease.

TNF-α induces inflammation by activating MAP kinases.Three groups of MAP kinases yet have been proposed by previous studies: the extracellular signal-regulated kinases (ERK); the p38 MAP kinases and the c-Jun NH_2-terminal kinases (JNK). MAP kinase has an important role in production of pro inflammatory substances after transcription and translation.

Three protein kinases act sequentially in this pathway - a MAP kinase kinase (MAP2K or MKK) and a MKK kinase (MAP3K or MKKK) by phosphorylation of pThr-Xaa-pTyr motif. The sequence of this motif is Thr-Glu-Tyr (ERK); Thr-Gly-Tyr (p38); and Thr-Pro-Tyr (JNK) (8).

TNF-α induced JNK and p^{38} MAP kinase pathway

The JNKs are known as stress activated protein kinases and P^{38} kinases play a major role in activation of immune response. They are a key regulator of inflammatory cytokine expressions.

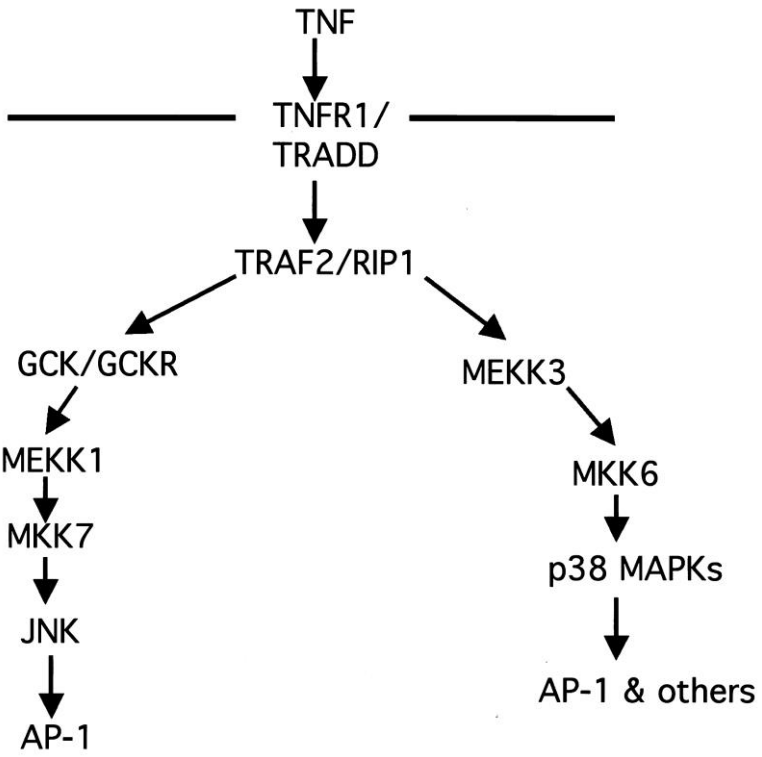
P^{38} MAP kinase is activated by MKK3 and MKK6 and c-jun N-terminal kinases (JNK) is activated by MKK4 and MKK7 which are induced by the members of MAP3K protein kinase family. TNF-α is an activator of this protein kinase family. The members of MAP3K protein kinase family are:

1. Apoptosis sensing kinase 1 (ASK1)
2. Map and ERK kinase kinase (MEKK)
3. Transforming growth factor β- activated protein kinase 1 (TAK-1)
4. Mixed- lineage protein kinase (MLK)
5. Tumor progression locus 2 (TPL 2)

ASK1 has an important role in activation of JNK and p^{38} caused by TNF-α. In ER stress condition reactive oxygen species (ROS) production is stimulated by TNF-α and ASK1 is activated by forming complex with TRAF2 in the TRADD-RIP-1-TRAF2 complex and activate MKK4, MKK7, MKK3, MKK6.

TAK1 has a significant role in ubiquitin mediated signal transduction by TNF receptor. It forms a complex with this receptor and mediates the activation of MKK4,MKK7(JNK) and MKK3, MKK6(p^{38}).

TNF-α stimulate ROS production which triggers RAC/Cdc42 activation (Rho family). Then RAC/Cdc42 activate MLK by binding CRIB motif. MLK stimulate the activation of MKK3, MKK6 (p^{38} kinase) and MKK4, MKK7 (JNK kinase) by phosphorylation and transmit the signal further level. (8)



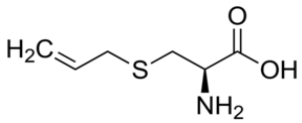
P^{38} and inflammation

From previous studies it was postulated that there was a strong link between p^{38} and inflammation. This pathway plays a major role in production of proinflammatory cytokines (IL-1β, IL-6) and induction of VCAM-1 and other inflammatory molecules. This MAP kinase pathway also induce expression of enzyme e.g iNOS which act in regulation of oxidation. P^{38} has a regulatory role in proliferation and differentiation of immune cells such as CD-40, CSF, EPO (9).

SAC

Garlic (*Allium sativam*), a member of family of Liliaceae contains many organo sulfer compounds, was used in medicinal purpose to protect against several diseases due to their anti-inflammatory, antioxidant activity which was previously reported (10). Aged garlic extract (AGE) in ethanol solution contains an anti-inflammatory compound S- Allyl- L-Cysteine. SAC

is produced from γ-Glutamyl-S-allylcysteine by γ-Glutamyltransferase enzyme. SAC can inhibit transcription of cytokine genes such as TNF-α, IL-1β by inhibiting transcription factor NF-κβ. From several studies in vitro, it was confirmed that SAC can scavenge the reactive oxygen species (ROS) and inhibit ROS dependent signal regulated kinase such as ERK, JNK, p^{38} MAP kinase, AKT(11). It was investigated that AGE decreases TNF-α level which induce inflammation and COX 2 (cyclo oxygenase) protein expression. So it is clear that SAC from AGE act as an anti-inflammatory compound.



S-allyl cysteine

Objectives

From previous studies in this lab revealed that SAC exerts anti-inflammatory effects in TNF-α induced HaCaT cells via down regulation of mRNA levels of TNF-α and IL-1β cytokines, that have been implicated in progression of inflammatory pathways. Now we will investigate in our present study whether SAC mediated anti-inflmmatory effects in TNF-α induced HaCaT cells is via modulation ERK, p^{38} and JNK MAP kinase pathway.

1. Investigation of the mRNA levels of TNF-α and IL-1β by real time PCR.
2. Investigation of the p38 and JNK phosphorylation by western blot.

2. Materials and methods:

Materials

All antibodies were purchased from Elabscience. Primers were purchased from Integrated DNA Technologies. DMEM, FBS, penicillin-streptomycin, amphotericin B and L-glutamine were purchased from HiMedia. Random hexamer primer, RiboLock RNase inhibitor, RevertAid reverse transcriptase and dNTPwere purchased from Thermo Fisher Scientific, USA. PowerUpTM SYBR Green Master Mix for real- time PCR was purchased from Applied Biosystems, Life Technologies, USA.

Cell Culture Techniques

An immortalized human keratinocyte cell line, HaCaT were grown in cell culture flask which was maintained in DMEM (Dulbecco's Modified Eagle Medium) growth medium supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin, 1% amphotericin B and 1% L-glutamine at 37°C under 5% CO_2 in incubator.

Cell thawing

This process was used for retrieving the previously stored cells. Cryovials containing HaCaT cells were taken and a quick thaw was performed. Next, the suspension containing cell and media was the centrifuged at 1000 rpm for 2 minutes. The supernatant was removed and the cell pellet was re-suspended in fresh medium. Cells were then transferred into a cell culture flask and an appropriate volume of growth medium was added to allow cell growth.

Cell Passage

Cell confluency was observed under light optical microscope. Cell passage was then performed by the following way: After removal of the media from the flask, trypsin-EDTA solution was added to the monolayer of the HaCaT cells and incubated in 37°C under 5% CO2 in the incubator for 1-2 minutes for the detachment of the monolayer from the matrix component of the cell culture flask. After that, complete media was added to the flask when trypsinization was complete. Next, cell suspension was centrifused to pellet down the cells. After that the pellet was collected and resuspended in fresh medium. Then cells were counted using hemocytometer and trypan blue viable count method. Finally, required amount of cells were seeded to the flask for further growth. This whole process was performed in laminar air flow under appropriate aseptic condition.

Cell Freezing

Cells were collected via trypsinization and 10% FBS and DMSO were added to it. Cells were immediately kept in cryovials and placed in -80ºC freezer followed by transfer to liquid nitrogen.

Cell seeding for experiment

HaCaT cells at the density of 1.6×10^6 were plated in 60 mm plates in presence of complete medium and incubated for 24 h. After overnight incubation in complete medium serum starvation was performed for 24 h followed by treatment with drugs and inducers.

Cell treatment

HaCaT cells were either left untreated or treated with SAC for 1 h followed by treatment with TNF-α (100 ng/ml) for indicated time period. For PCR analysis of the mRNA levels of the pro-inflammatory cytokines (TNF-α and IL-1β), SAC treatment was followed by 24 h TNF-α (100 ng/ml) induction. For western blot analysis, cells were treated with SAC (150 μM) for 1 h followed by treatment with TNF-α (100 ng/ml) for 1 h.

RNA extraction

Media was removed from cells followed by washing of the cells with PBS buffer. Next, 1 ml of trizol reagent was added in each plate to lyse the cells and then collected in different eppendorfs. The next steps were performed in a RNA hood after UV treatment. 200μl chloroform was added per 1 ml of trizol in each eppendorf. Then the eppendorfs was shaked 80 times and incubated at room temperature for 2-3 minutes followed by centrifugation at 11500g for 15 minutes at 4°C in a cold centrifuge for phase separation. Following centrifugation the mixture was separated into 3 phases – a lower pink phenol chloroform phase where proteins remain, an interphase where cell debris was present and a colourless aquous phase where DNA and RNA were present. So, the aquous phase was transferred carefully into fresh eppendorfs without disturbing the interphase. For precipitating the RNA from aquous phase, 500 μl isopropanol per was added to each eppendorf and incubated for 15 minutes followed by centrifugation at 11500g for 10 minutes at 4°C. The supernatant was removed completely and the pellet was washed with 80% ethanol. The samples were gently taped to loose the pellet and centrifuged at 8000 g for 10 minutes at 4°C. The supernatant was discarded and the RNA pellet was kept to air dry for 5-10 minutes. The pellet was the dissolved in nuclease free water.

RNA concentration determination

The concentration and purity was determined by spectroscopy at 260 and 280nm. A_{260}/A_{280} (A=Absorbance) ratios were calculated for each sample. The value nearly 2 was perfect for further experiment.

cDNA synthesis

cDNA was synthesized from each of the RNA samples using reverse transcription. Reverse transcriptases use an RNA template and random hexamer primer to direct the synthesis of the first strand cDNA which can be used as a template for the Polymerase Chain Reaction (PCR). The RNA sample was transferred in required amount in different PCR tubes and marked them. To each of the sample 0.5µl of random hexamer and nuclease free water were added. The mixture was placed in a thermal cycler for 1 cycle for 5 minutes at 65°C. Then 2µl of 5X cDNA reaction buffer, o.5µl of ribolock, 1µl dNTP and 0.5 µl reverse transcriptase (RTase) enzyme was added to each PCR tubes and placed to thermal cycler for 1 cycle of each at 25°C for 5 minutes, 42°C for 60 minutes and at 70°C for 5 minutes. The samples were then stored at 4°C until PCR was performed.

REAL TIME PCR

Polymerase chain reaction is a technique which is used to amplify DNA. In this process primers are used which are complimentary to the sequence of interest in a mixed DNA, extended by DNA polymerase.

In real time PCR the amount of PCR products is quantitatively measured at early states of the reaction process in a real time detection mode. The fluroprobes bind to a specific target regions of amplicons and produce fluorescence. When the target sequence has been amplified, the cleavage of probe was occurred ensuring polymerization. The process is performed in a thermal cycler. When the PCR cycles are initiated a change in flurosecene is arised which is called baseline. Thresold cycle (CT) is a parameter which is defined as a fractional PCR cycle number in which florescent signal that is detected above the threshold considering a real signal. In real time PCR in order to avoid error which was occurred due to differences in the starting amount of RNA, efficiency in CDNA synthesis and PCR amplification, housekeeping genes are required for normalization.

Real time PCR was performed using cDNA as template, primers for TNF-α, IL-1β and β-actin and SYBR Green Master Mix. The primer sequences for human TNF-α gene, human β actin gene and IL-1β are as follows :

TNF α forward primer	5'-CAT GTT GTA GCA AAC CCT CA-3'
TNF α reverse primer	5'-CTT GGT CTG GTA GGA GAC G-3'

IL-1β forward primer	5’-AAAAGCTTGGTGATGTCTGG-3’
IL-1β reverse primer	5’-TTTCAACACGCAGGACAGG-3’

β-Actin F	5-CTTCCTTCCTGGGCAT-3’
β-Actin R	5-CAGGGTACATGGTGGTG-3’

Real time PCR was performed using Applied Biosystems StepOne Plus Real-Time PCR System . The PCR conditions were as follows: 95° C for 10 min followed by 40 cycles of 95° C for 15 s, 55° C for 1 min and 60° C for 1 min. Expression of target gene was analyzed in triplicate samples and was normalized to β-actin expression levels using comparative ΔΔCt method.

Whole cell protein lysate preparation

After treatment wells were washed with PBS and RIPA lysis buffer (Tris-HCL, NaCl, EGTA, NP-40, sodium deoxycholate, β-glycerophosphate, leupeptin and Na_3VO_4) containing three ionic and non ionic detergents was added to the eppendorfs to separate the protein from cell mixture. Next the solution was centrifuged to pellet down the cells and extraction of protein. The supernatant were collected in eppendorfs which was containing protein.

Protein estimation: Bradford assay

Bradford protein assay is a spectroscopic analytical process for measuring the protein concentration in a solution. In this process a dye, coomassiae brilliant blue G-250 is used. The dye interacts with the carboxyl group of protein by vanderwaals force and amino group by electrostatic interaction. The measurement of protein concentration is based on the absorbance shift of the dye at 595nm.

A mixture was prepared by adding 190 microlit distilled water, 10 microlit GeNei Bradford reagent and 1 ml previously collected supernatant and incubated at room temperature in dark. Next OD values were measured in spectrophotometer at 595nm and from this protein concentration was determined.

Western Blot

Western blot (also called immunoblotting) is a technique for protein analysis which enables a target protein extracted from cell lysate to be separated and identified in a complex protein mixture based on molecular weight (through Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis) and specificity of antigen antibody interaction.

- **SDS PAGE**

1. Gel preparation:

Components	**10% resolving gel (15ml)**	**5% stacking gel (10ml)**
30% acrylamide	5ml	3.4ml
Doubledistilled water	5.9ml	830µl
1.5M Tris-Hcl ph 8.8	3.8ml	
1M Tris-Hcl ph 6.8		630µl
10% SDS	0.15ml	50µl
10% APS	0.15ml	50µl
TEMED	0.006ml	6µl

2. Gel casting

Gel plates were washed thoroughly and rinsed with double distilled water with ethanol and air dried. The plates were assembled by cello tape and tightened by clamps. Leakage was tested with water. Early prepared resolving gel solution were added with 1 ml pipette man.100%ethanol was added to the above layer for avoiding bubbles. It was kept for 15-30 mints to solidify. After solidified alcohol was removed and stacking gel was added in similar way. Instantly the comb was placed. After polymerization, the comb was removed and glass plates were placed in electrophoresis tank. 1X SDS PAGE running buffer was poured into the tank. 30µg of sample and 4µl of ladder was loaded into the wells. Gel was run first in 90 V up to stacking /resolving junction and in 100 V gel was run until the dye front reaches the bottom of the gel.

Composition of SDS-PAGE running buffer: 10X (1000 ml)

Tris	30 g
Glycine	144 g
SDS	10 g

- **Blotting**

When gel run is in progress 8 blotting papers were immersed in 1X transfer buffer. Wearing gloves and using forceps PVDF membrane was cut and was immeresed in NaOH for 5 to 10 secs. After washing with double distilled water it was immersed again in transfer buffer. The gel was cut appropriately discarding the stacking gel. In the semi dry transfer apparatus a transfer sandwich was created as follows:

Sponge
4 blotting paper (buffer was added)
PVDF membrane (avoiding bubble it was rolled with a glass tube)
Gel (roll again)
4 blotting paper (roll again)

Then the whole system was transferred in 8V/4° c for 1.5 hr(for 8%gel). When transferred was completed the membrane was cut according to specific molecular weight ladder and washed 3 times in 1X TBST buffer.

Composition of transfer buffer: (1X/500ml)

COMPONENT	AMOUNT	CONCENTRATION
Tris	1.5g	15Mm
Glycine	7.2g	192Mm
Methanol	100ml	20% v/v

Composition of TBS buffer (10X): for 500ml

NaCL	22g
1M Tris (ph 8)	25 ml
D_2H_2O	200 ml

Composition of TBST buffer (1X) :

1X TBS Buffer	100ml
0.05% Tween-20	50µl

- **Blocking and antibody incubation**

The blocking process was performed by immeresed the membrane with 5%skim milk in TBST buffer for 1-2hrs. Then it was washed for 3 times in 1X TBST. After that the primary antibody [anti rabbit p-p38 Ab (1.5 micro lit Ab+1500 micro lit. TBST)] was added and sealed properly and incubated for overnight at 4°c. Then the next day the membrane was washed 3 times with TBST and added secondary antibody [anti rabbit 2°(0.5 micro lit + 2500 micro lit. TBST + 5% milk)].Then washed with TBST 2 times and TBS for 2 times.

Composition of blocking buffer:

1X TBST buffer	20 ml
5% non – fat dry milk	1 gm

● **Developing the membrane**

Developer composition: (450 ml) (KODAK)
Packet A : 6.5 gm in 225 ml water ,then add
Packet B: 40.5 gm
Then volume make up to 450 ml with distilled water.
Fixer (Kodak): 120.5g in 450 ml double distilled water.
The kodak film was cut in dark room and kept inside the cassette and the saram wrap was cut and placed on a paper. The luminol reagent was prepared in dark –

100mM TrisHcl (ph 8.3)	5 ml
Hydrogen peroxide	**1.4 micro lit.**
Cumaric acid	12.5 micro lit.
Luminal	2.5 micro lit

The TBS buffer was discarded and the luminol reagent was added to the membrane. Then the membrane was wrapped on saram wrap with forceps. The X ray film was placed in the cassette. The film was immersed in Developer (10-15min) and the 2nd film was placed in the membrane. The whole film was placed in fixed (30sec -1min) and washed with water and finally air dried.

3. Result:

Result of IL -1β PCR:

For analysis of the mRNA level of pro inflammatory cytokine IL-1β and TNF-α, real time PCR was performed.

Data from real time PCR of pro inflammatory cytokine IL-1β showed that a 24 hours exposure of HaCaT cells to a particular concentration of TNF- α solution caused a significant increase in IL-1β level comparing to untreated cell. ΔCt value is decreased in TNF α treated cell but increased when the cell was treated with TNF α and SAC. The value of fold difference which indicates IL-1β level, was increased in case of TNF alpha treated cell compared with untreated cell and decreased in TNF-α and SAC treated cell.

Sample	**IL-1β Avg Ct**	**β-actin Avg Ct**	**ΔCt**	**ΔΔCt**	**Fold difference**
Untreated	22.83	13.69	9.14	0.00	1.00
TNF-α 100ng/ml	21.02	12.89	8.13	-1.01	2.01
TNF-α 100 ng/ml+ SAC 70μM	22.59	13.96	8.63	-0.50	1.42

Fold difference = $2^{-\Delta\Delta CT}$

The following graph shows that the cells treated with TNF-α (ng/ml) caused a significant increase in IL-1β mRNA level but when treated with SAC (70Mm) and TNF-α (100ng/ml) caused a significant reduction in IL-1β level compared to only TNF α treated cell. So, it is clear that SAC downregulate TNF-α level.

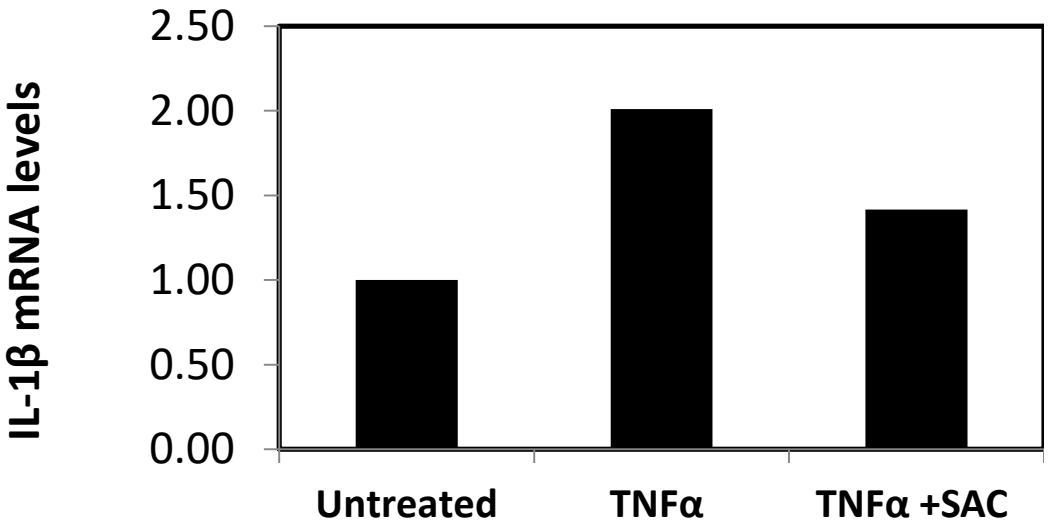


Fig. 1

Result of TNF α PCR:

For unveiling that how SAC inhibit TNF-α induced inflammation, real time PCR of pro inflammatory cytokine TNF-α is necessary. From this data it was showed that Ct value of TNF-α (100ng/ml) treated cell was lower than untreated cell whether Ct value of TNF-α alpha with SAC (70µM) treated cell was higher. The value of Fold difference which indicates the level of TNF-α alpha in cell was higher in TNF-α treated cell comparing untreated and in case of TNF-α with SAC treatment, was lower. In this experiment actin was used for normalisation of Ct value with TNF-α.

From the following graph plot we observed that SAC significantly downregulates the level of TNF-α comparing with only TNF-α treated HaCaT cell.

Sample	TNF-α Avg Ct	β-Actin Avg Ct	ΔCt	ΔΔCt	Fold difference
Untreated	**22.57**	**13.69**	**8.88**	**0.00**	1.00
TNF-α 100ng/ml	**18.56**	**12.89**	**5.67**	**-3.21**	**9.23**
TNF-α 100ng/ml +SAC 70µM	**21.96**	**13.96**	**8.01**	**-0.87**	**1.83**

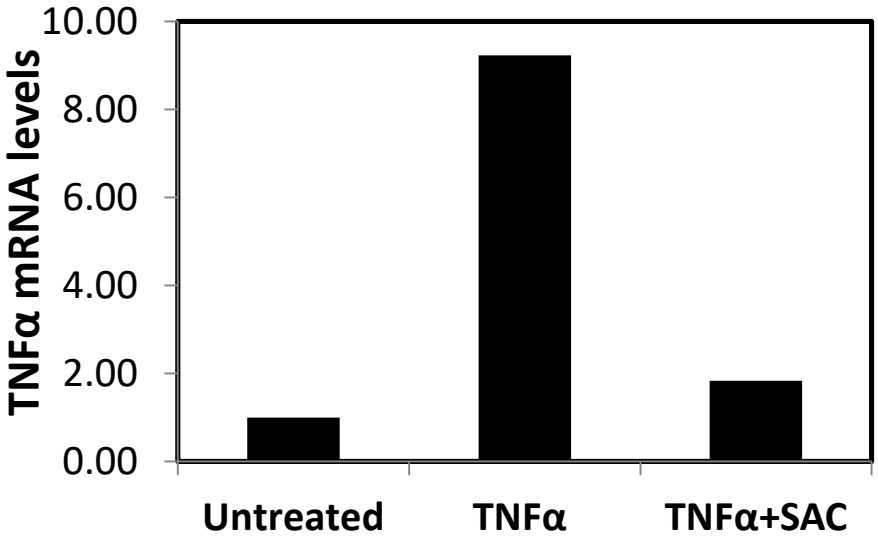


Fig.2

Result of western blot:

In order to detect the effects of drug SAC on TNF α induced p38 and JNK phosphorylation in HaCaT cells, we performed western blot analysis.

Figure 3 suggests that the level of p38 phosphorylation was increased when the cell was treated with TNF-α and SAC treatment reversed this effect via inhibiting p38 phosphorylation.

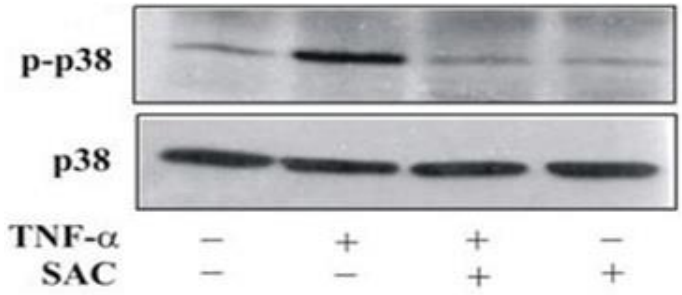


FIG. 3

Figure 4 suggests that the level of JNK phosphorylation was increased when the cell was treated with TNF α and SAC treatment reversed this effect via inhibiting JNK phosphorylation.

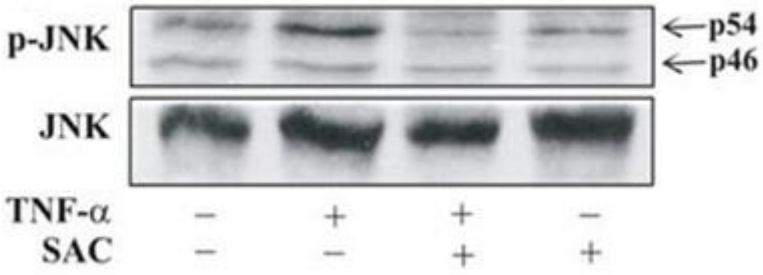


FIG. 4

4. Conclusion:

It is clear from the above results that SAC effectively inhibits TNF-α induced inflammation via down-regulating p38 and JNK MAP kinase activation.

So, from our study we are able to analyze that –

1. SAC inhibits inflammation in HaCaT cell by reducing TNF-α and IL-1β cytokine level.
2. SAC inhibits TNF-α induced inflammation by down regulating the phosphorylation of p38 and JNK MAP kinases.

5. Future objectives:

1. Assessment of the effect of SAC on other inflammatory pathways including ERK MAP kinase and NF-κB pathway.
2. Investigation of the efficacy of this compound on *in vivo* system.

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