Chapter 2
Objective II

Objective II: To identify the modifications of IAPs and signalling mechanisms involved in the modification

Introduction: Since XIAP (X-linked Inhibitor of Apoptosis Protein) and FLIP (Flice like Inhibitor of Apoptosis Protein) both are anti-apoptotic proteins, downregulation of these proteins could lead to the apoptosis of the leukemic cells. Apoptosis (both Intrinsic and Extrinsic pathway) is regulated in various level. FLIP and XIAP are both well-established regulators of apoptosis.

FLIP is a pro-caspase 8 mimetic. It contains two DEDs like the initiator caspase. It is anti-apoptotic protein due to the absence of caspase like activity, due to the lack of the catalytic cysteine residue that is present in pro-caspase 8. FLIP competes with caspase-8 to prevent BID activation and inhibits intrinsic apoptotic pathway. FLIP upregulation also blocks TRAIL mediated apoptosis because the recruitment of FLIP to the DISC instead of procaspase-8 or -10 can block caspase activation [163].

XIAP belongs to the Inhibitor of Apoptosis Protein (IAP) family. It contains 1-3 baculovirus IAP repeat (BIR) domains, a carboxyterminal RING (really interesting new gene) zinc-finger domain which possess E3 ubiquitin ligase activity [164]. It inhibits active caspase 9 through its BIR3 domain and inhibits active caspase 3,7 through its linker BIR2 domain. XIAP has ubiquitin ligase activity, ubiquitinate the caspase 3 and degrade by proteosome [165].

XIAP and FLIP regulate apoptosis at various level. Apoptosis is a genetically programmed cellular mechanism(s) to commit suicide. Apoptosis is critically important for survival of multicellular organisms by elimination of damaged cells that may interfere with normal function [166-169]. When these normal processes of cell death go uncontrolled, it leads to some of leading causes of death and disability worldwide, including neurodegenerative, cardiovascular, autoimmune and infectious disease [170]. Despite the undeniable role of apoptosis in normal homeostasis, our understanding of cell death processes and their regulation is still nascent. Recent literatures demonstrate that dynamic nature of cell death regulation during development, ageing and disease. Different types caspases are involved in apoptosis. BIR domain of XIAP inhibits the activity of different caspase through physical interaction and also inhibits apoptosis [171]. Dysregulation (mostly upregulation) of XIAP can lead to promote tumour metastasis, anti-cancer therapy, tumorigenesis [172]. Other side, c-FLIP binds to FADD and/or caspase 8 and caspase 10 and TRAIL receptor 5 (DR5). In turn, this interaction

prevents Death-Inducing Signaling Complex (DISC) formation and activation of caspase cascade [173]. C-FLIP are known to have multiple roles in various signaling pathways and upregulating several prosurvival signaling proteins including Akt, Nf-kb. c-FLIP upregulation promotes defects of DR-mediated apoptosis and resistance toseveral anti-cancer drugs.

XIAP and FLIP are promising targets for therapeutic involution because these proteins are overexpressed in many cancer cells including haematological malignancy and linked with treatment failure [174]. Elevated expression of these antiapoptotic proteins resists agonist antibodies that stimulate Death Receptor (DR) mediated apoptosis [175]. Overexpression of XIAP, FLIP activates different type of signal transduction pathways that enhance cell proliferation, promote cell survival [176]. These two actions promote unrestrained tumour growth, resilience that make XIAP, FLIP, promising target for killing cancer cells.

In objective I, we have reported that downregulation of XIAP, FLIP become a strong strategy for TRAIL mediated apoptosis in imatinib resistant CML. Hydroxychavicol downregulates XIAP, FLIP in ROS dependant manner, specifically H_2O_2 dependant manner. In this objective II, we have examined that the signalling mechanism that is involved in H_2O_2 dependant XIAP, FLIP downregulation.

Materials&Methods

Materials: Hydrogen peroxide used in this study was purchased from Merck Life Science Pvt. Ltd (India). iScript™ Reverse Transcription Supermix for RT-qPCR and SsoFast™ Evagreen® Supermix were purchased from BIO RAD (USA). FITC Annexin V was taken from BD Pharmingen™. Propidium iodide was bought from Sigma-Aldrich. The antibodies against XIAP, c-FLIP, Akt, pAkt, PI3K, pPI3K, ERK and JNK, pJNK and pERK1/2, anti-mouse IgG HRP-linked antibody and anti-Rabbit IgG HRP-linked antibody, Control siRNA, ERK1/2 siRNA and SAPK/JNK siRNA were bought from Cell Signaling Technology (Denver, Massachusetts, USA). Anti-γ-actin antibody used as a control was bought from Biobharati LifeScience (India). pcDNA3 Flag JNK2a1 was a gift from Roget Davis (Addgene plasmid # 13754; http://n2t.net/addgene:13754; RRID: Addgene_13754) and pcDNA3-HA-ERK2 WT was a gift from John Blenis (Addgene plasmid # 8974; http://n2t.net/addgene:8974; RRID: Addgene_8974). SureBeads™ Protein G Magnetic Beads was bought from BIO RAD (USA). JNK inhibitor SP600125, ERK inhibitor PD98059, p38 inhibitor SB203580 were purchased from Abcam (Cambridge, MA, USA).Fetal Bovine Serum (FBS) Standard (origin Brazil) was purchased from Gibco®.Life Technologies, USA.

Methods:

Cell Line and culture condition: Imatinib-resistant K562 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and 0.1% gentamycin and 1.5 μg/ml Imatinib at37°C in humidified atmosphere with continuous flow of 5% carbon dioxide (CO2). Imatinib resistant K562 cells were prepared as described in objective 1. In short, Imatinib was added to the cell culture medium in a graded increasing dose and the viable cells were selected and placed in the medium with next higher doses of imatinib. In this way final imatinib concentration was reached at 1.5 μg/ml where 100 percent cells were made viable.

Annexin V/PI binding assay: 1.0 × 105 cells were seeded in 24 well plates and the treatments were done. Cells were washed with 1X PBS two times. Cells were washed with 1X Annexin V/ PI binding buffer and annexin V/FITC was added according to the manufacturer's protocol. Next 50 μg/ ml PI was added and flow cytometry was done mixing the cells with 400μl of 1X Annexin V binding buffer. The instrument, used was BD Accurieand analysis was done with BD C6 software.

Western blot analysis: Cells from treatment as indicated in the figure legends were washed with 1X PBS two times. Cells were lysed with 1X RIPA (CST, Cat no 9806S) and protease inhibitor cocktails. Lysate were collected by centrifugation at 15000 rpm for 15 min. Protein concentration was estimated by Lowry (Folin-Ciocalteau) method. Proteins samples were prepared for SDS PAGE by boiling them with Laemmli buffer at 95 ℃ for three minutes and subjected to electrophoresis. Proteins were transferred to PVDF membrane for subsequent steps. Then the membranes were blocked with 5% BSA (for phospho-epitope) or 5% non-fat dried milk diluted in TBST for 1 hr. proteins were detected using the respective antibodies corresponding HRP linked secondary antibodies were used for detection. Bands were developed using BIO RAD Clarity™ Western ECL substrate. Images were obtained using BIO RAD ChemiDoc™ MP Imaging System and ImageLab 5.0 software.

Knock down assay: 2 × 106 cells/well were plated in six-well plates and kept overnight without serum and antibiotics. Next day, transfection was performed with siRNA oligo using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) and RPMI (without serum and antibiotic) medium, according to the manufacturer's protocol. The concentrations of siRNAs were chosen based on dose–response studies. Western blot wasperformed to confirm the knock down of the respective proteins, 48 h after transfection.

Gene Overexpression: To ectopically overexpress JNK and ERK gene, cells were transfected with pcDNA3Flag JNK2a1 (Addgene plasmid # 13754) and pcDNA3-HA-ERK2 WT (Addgene plasmid # 8974) using Lipofectamine 2000 according to the manufacturer's protocol. Cells were treated with H2O2 24 hr post transfection and overexpression was checked by western blot.

Real time PCR:

Using Trizol reagent the total RNA was isolated from appropriately treated cells according to the manufacturer's instructions. RNA quality was checked by agarose gel electrophoresis and concentration was determined using Synergy H1 Microplate Reader. cDNA was prepared from isolated total RNA using iScript TM Reverse Transcription Supermix for RT-qPCR (BIO RAD). Real-time PCR was done with SsoFastTM Evagreen Supermix (BIO RAD), in CFX96 Touch™ Real-Time PCR Detection System (BIO RAD). 40 cycles were run for each round of PCR for total saturation and the Cq values were considered for further analysis. All RT-qPCR setup were done in duplicate sets and the complete experiments were independently repeated at least three times. For graphical representation of the gene expression data, Actin was used as house-keeping gene and the mRNA levels were normalized accordingly.

The primers used were:

CFLIP

Forward: 5'-GGC CGA GGC AAG ATA AGC AA-3'

Reverse: 5'-TTG TCC CTG CTC CTT GAA CA-3'

XIAP

Forward :5'-GGA CCC TCC CCT TGG AC-3'

Reverse: 5'-TGA TGT CTG CAG GTA CAC AAG TT-3'

Actin

Forward: 5'-GCA CCG TCA AGG CTG AGA AC-3'

Reverse: 5'-TGG TGA AGA CGC CAG TYGG-3'

Coimmunoprecipitation:

Approximately 3.5 to 4 million cells from the treatment were taken and pellet down at 5000 rpm for 3 minutes, and washed with 1x PBS. Then the pellet was dissolved in 200 – 250 µl of 2x RIPA and vortex was done gently for 30 to 40 minutes by keeping the sample alternatively on ice. The solution was then centrifuge at 15000 rpm for 15 minutes at 4 ºC and supernatant was collected in fresh 1.5 ml tube. 5µl of each sample was taken out for measuring protein concentration. 5% of lysates were boiled with 2x Laemmli buffer and used as input. Lysates were precleared with control antibody plus Protein G magnetics beads (BIORAD) for 1hr at 4 degrees Celsius. Protein of interest was captured by rotating remaining lysate with 1µg specific antibody overnight at 4 degree Celsius. Immuno complexes were attached with magnetic beads, pelleted and washed with 1Xpbs for 2 times. Input lysate and IP complexes were boiled with Laemmli buffer and subjected to SDS-PAGE, transferred to PVDF membrane for western blot analysis, which was described above.

Statistical analysis: Data were analyzed as mean ± SD of at least three independent experiments and statistically significant differences between mean values (from three independent experiment at least) were determined using Student's t-test.

Result:

In this objective, we wanted to explore the mechanisms that is involved in H_2O_2 mediated XIAP, FLIP downregulation in imatinib resistant chronic myelogenous leukemia cell line K562(R).

Hydrogen Peroxide Down-regulated FLIP and XIAP

To check the effect of ROS on XIAP and FLIP, K562(R) cells were treated overnight with different doses of H_2O_2.The whole cell lysate was subjected to western blot analysis with XIAP and FLIP antibody. The result indicated that H_2O_2 dose dependently decreased XIAP and FLIP at the protein level (Fig 1A). K562(R) cells were treated with 30 µM H_2O_2 for various time points. The whole cell lysate was subjected to western blot analysis with XIAP and FLIP antibody. Result indicated that FLIP level decreased after 12 hours while XIAP level decreased after 16 hours of H_2O_2 treatment (Fig. 1B).

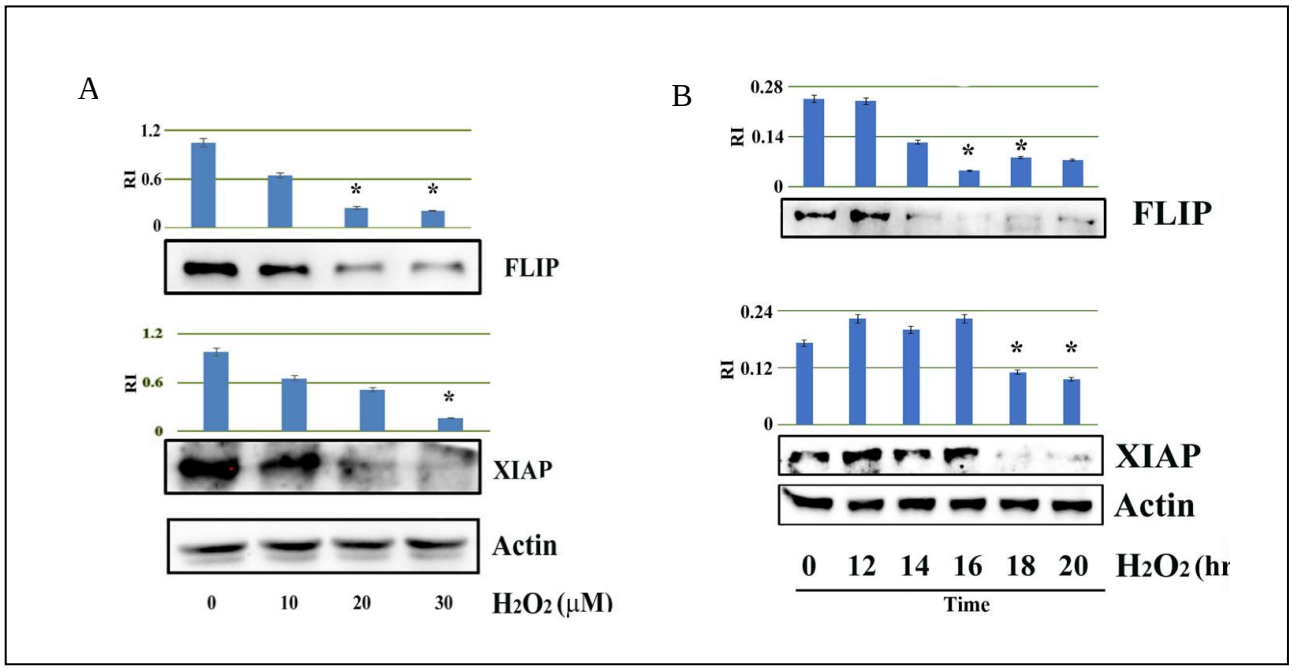


Figure 1

Fig 1: ROS downregulates XIAP and FLIP at protein level in dose and time dependant manner. **A)** Imatinib resistant K562 cells were treated with indicated doses of H_2O_2 for overnight. Western blot analysis of FLIP and XIAP was performed from whole cells. YActin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean± SD of three independent experiments. *$p < 0.05$ **B)** Immunoblot analysis of FLIP and XIAP was performed from imatinib resistant K562 cells after treating the cells with 30μM H_2O_2 for the indicated time points. YActinwas used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.

H_2O_2 did not cause cell cytotoxicity on K562 (R) cells:

As cells were treated with H_2O_2, there might be possible that induction of apoptosis, reduction in cellular protein level nonspecifically. K562(R) cells, treated with different doses of H_2O_2 for 24 hours. Result showed that there was no cytotoxicity of these doses of H_2O_2 on K562(R) cell after 24 hr (Fig 2A).

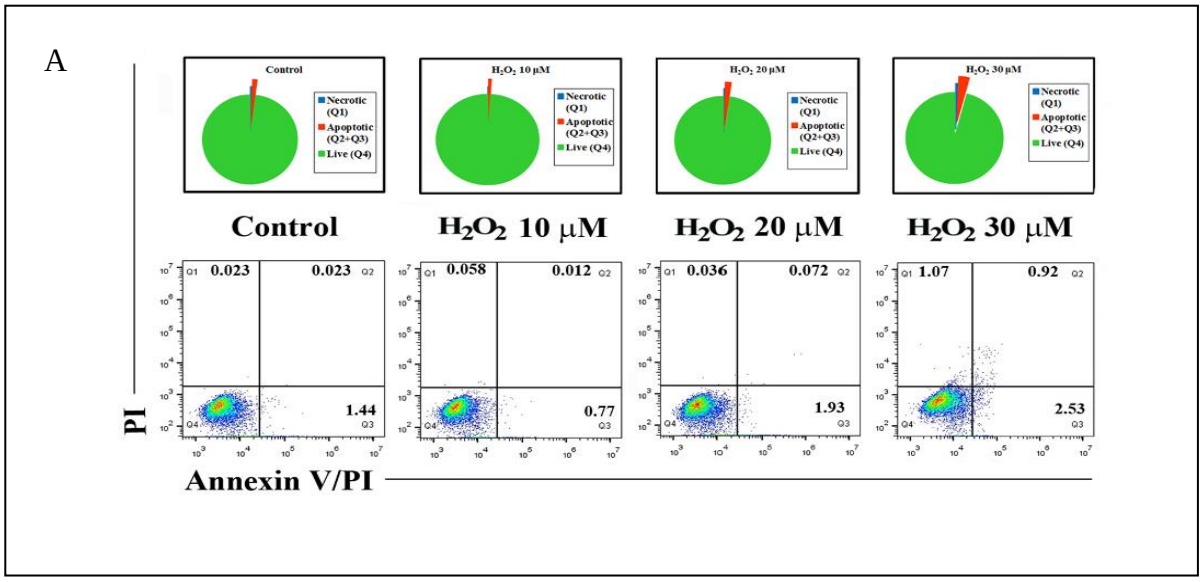


Figure 2

Fig 2:H_2O_2 did not cause cell cytotoxicity: A) Imatinib resistant K562 were treated with indicated doses of H_2O_2. After 18 hrs, cells were subjected to Annexin V/ PI binding assay by flow cytometry. Upper panel is the graphical representation of various population in pie chart and lower panel represents representative dot plots.

H_2O_2 did not downregulate XIAP and FLIP at RNA level

Now**,** we checked whether ROS mediated downregulation of XIAP and FLIP is due to reduced expression at RNA level. FLIP and XIAP at RNA levels were checked. Total RNA was isolated from H_2O_2 treated K562(R) cell. Real time PCR data showed that there was no significant increase of either FLIP or XIAP at RNA level (Fig 3A).

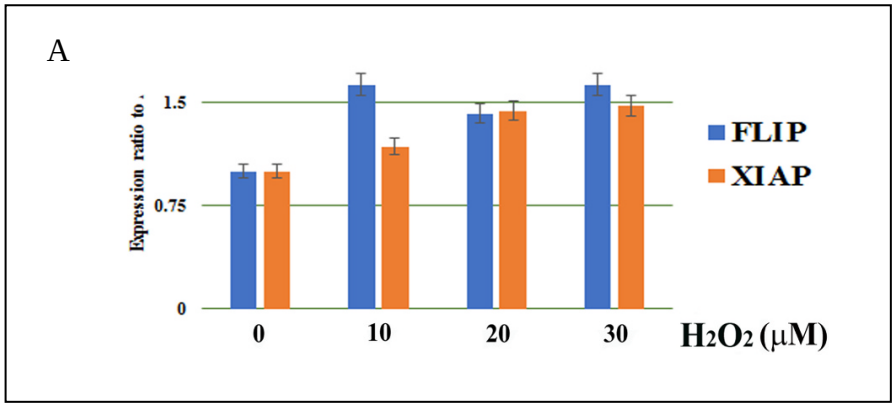


Figure 3

Fig 3: H_2O_2 do not downregulate XIAP and FLIP at RNA level. A) Imatinib resistant K562 were treated with increasing concentration of H_2O_2 for hrs and then total RNA was extracted from cells. Relative levels of endogenous FLIP and XIAP mRNA were assessed by Real time PCR and graphically represented as fold change. Data represents mean ± SD of three independent experiments

H_2O_2 was solely involved in XIAP and FLIP downregulation:

To check whether H_2O_2 is solely involved in XIAP and FLIP downregulation, cells were pretreated with ROS scavenger NAC then treated with H_2O_2, NAC significantly reversed H_2O_2 mediated XIAP, FLIP downregulation.

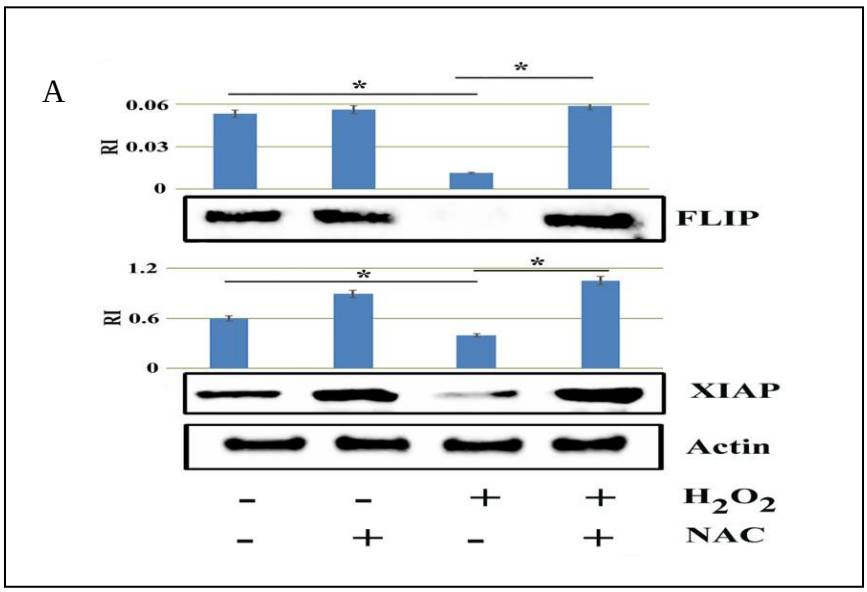


Figure 4

Fig4: H_2O_2 solely involved in XIAP and FLIP downregulation: A) K562 cells were pre-treated with 1mM NAC for 1 hr and then 30μM H2O2 was added and incubated for overnight. Whole cell lysates were extracted and Immunoblot analysis of FLIP and XIAP was performed. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.

This overall data confirmed that H_2O_2 is involved in down-regulation of XIAP and FLIP at protein level. This result also indicated that FLIP and XIAP might be getting altered by posttranslational modification.

XIAP and cFLIP degradation was proteasome-dependent

Cellular proteins level gets reduced by Lysosomal or Proteosomal degradation pathway. Therefore, we wanted to check involvement of any of these two pathways was responsible for H_2O_2 mediated XIAP and FLIP downregulation.

Now, K562(R) cells were pretreated with 1.5 μM of Proteasome inhibitor, MG132 and then treated with 30 μM H_2O_2.Whole cell lysates were taken after 18 hours then subjected to western blot analysis. The result indicated that proteasome inhibitor MG132 significantly reversed H_2O_2 mediated FLIP and XIAP downregulation (Fig 3A and 3B upper panel). Cells are preincubated with MG132 then treated with H_2O_2, cell lysate were taken after 12hrs and 16hrs, subjected with western blot analysis by anti-ubiquitin antibody after immunoprecipitation with FLIP, XIAP antibody. The result showed that MG132 increased XIAP, FLIP ubiquitination by H_2O_2 (Fig 5A,5B). So, It is concluded that H_2O_2 mediated XIAP and FLIP degradation is proteosomal dependant.

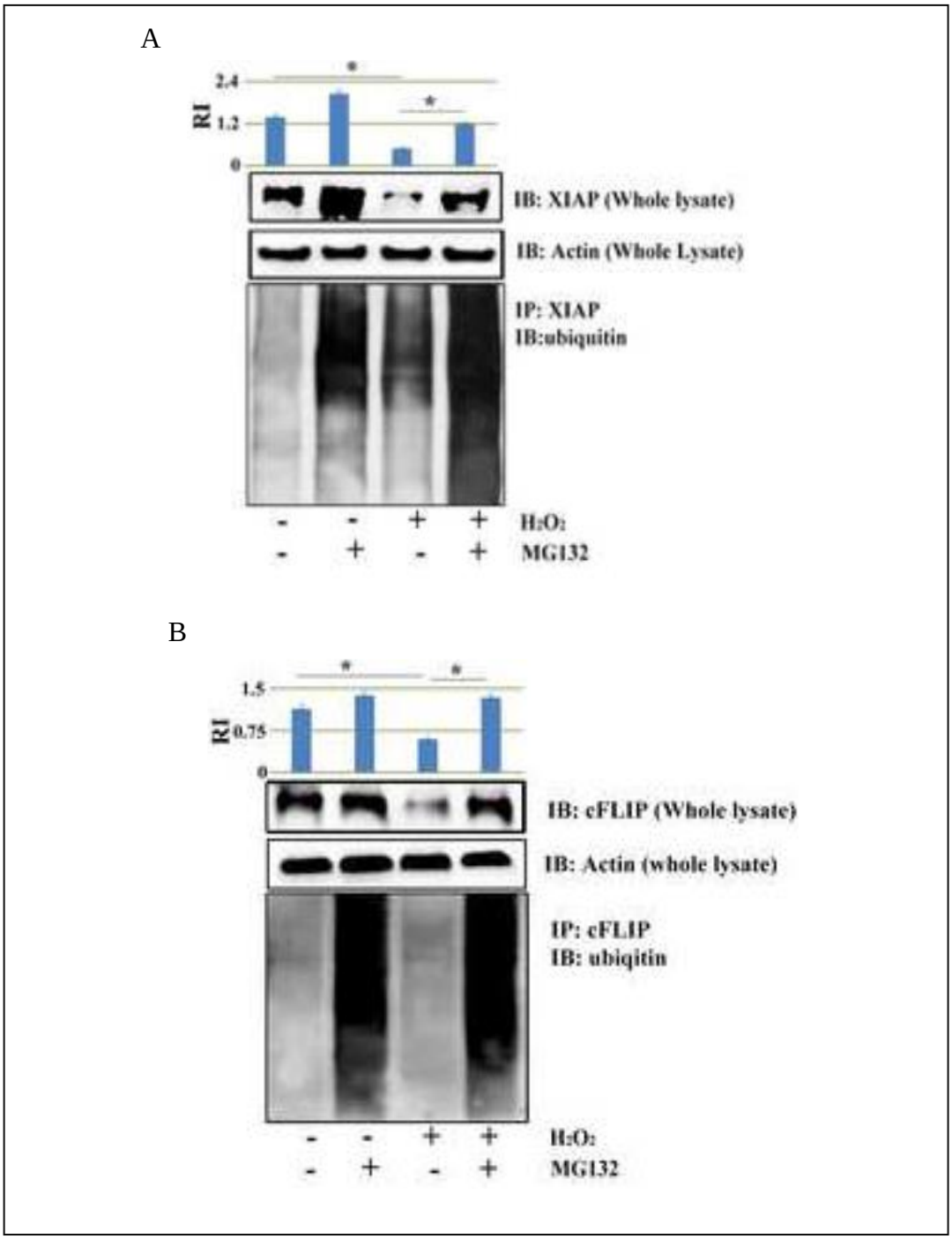

A
RI
2.4
1.2
0
IB: XIAP (Whole lysate)
IB: Actin (Whole Lysate)
IP: XIAP
IB:ubiquitin
- - + + H2O2
- + - + MG132
B
RI
1.5
0.75
0
IB: cFLIP (Whole lysate)
IB: Actin (whole lysate)
IP: cFLIP
IB: ubiqitin
- - + + H2O2
- + - + MG132

Figure 5

Fig 5: XIAP and cFLIP degradation is proteasomal dependent. A) K562 (R) cells were preincubated with 2 μM of MG132 and then 30μM H_2O_2. Whole cell lysates were subjected to co-Immunoprecipitation with XIAP antibody and then western blot analysis was performed with anti-ubiquitin antibody. The upper panel shows the whole cell lysates and lower panel show the immunoprecipitates. Polyubiquitination of XIAP was happened in lower panel. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *p < 0.05. B) K562 (R) cells were preincubated with 2 μM of MG132 and then 30μM H_2O_2. Whole cell lysates were subjected to co-Immunoprecipitation with FLIP antibody then western blot analysis was performed with anti-ubiquitin antibody. The upper panel shows the whole cell lysates and lower panel show the immunoprecipitates. Polyubiquitination of FLIP was happened in lower panel. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *p < 0.05

XIAP and cFLIP degradation was not lysosomal dependent

To check the involvement of Lysosomal pathway, K562(R) cells were pre-treated with 20 μM of Chloroquine, an inhibitor of autophagy pathway by impairing autophagosome fusion with lysosomes then cells were incubated with 30 μM H_2O_2 for 18 hours. Whole cell lysates were then subjected to western blot analysis with anti-XIAP and anti-FLIP antibody. Result indicated that Chloroquine didn't reverse H_2O_2 mediated XIAP and FLIP downregulation. So, it is confirmed that H_2O_2 mediated XIAP and FLIP downregulation is not lysosomal dependant [6A].

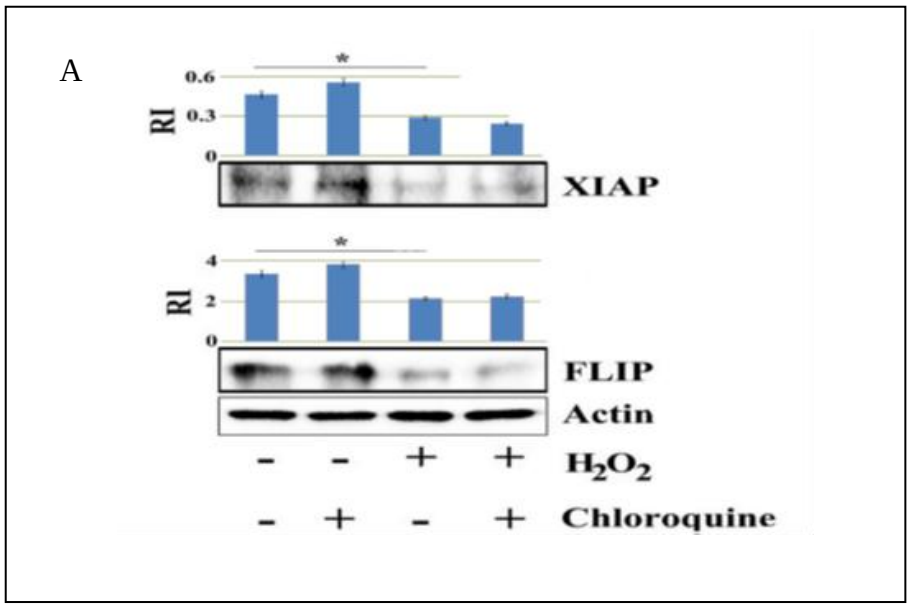


Figure 6 A

Fig 6: XIAP and cFLIP degradation is not lysosomal dependent. A) K562(R) cells were preincubated with 20 μM Chloroquine and then treated with 30 μM H_2O_2. Whole cell lysates were subjected to western blot analysis with anti-FLIP and anti-XIAP antibodies. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.

So, it is proved that H_2O_2 mediated XIAP and FLIP downregulation is proteosomal dependant not lysosomal dependant.

Activation of ERK and JNK played a role in XIAP, FLIP downregulation respectively:

MAP kinase pathways are one of the major stress signaling pathways [177]. Since XIAP and FLIP have been down-regulated by ROS, the involvement of MAPK pathways in this down-regulation were checked. K562(R) cells were incubated with ERK signaling inhibitor PD98059 and then treated with 30 μMH_2O_2.The whole cell lysate was subjected to western blot analysis with XIAP and FLIP antibody after 18 hours. Result indicated that PD98059 markedly reversed H_2O_2 mediated XIAP down-regulation. However, FLIP level has not been reversed by PD98059 [Fig 7A]. To check involvement of JNK pathway in FLIP and XIAP downregulation, K562(R) cells were incubated with JNK signaling inhibitor SP and then treated with 30 μM H_2O_2. The whole cell lysate was subjected to western blot analysis with XIAP and FLIP antibody after 18 hours. SP reversed H_2O_2 mediated FLIP down-regulation but not XIAP [Fig 7B]. However, when the same experiment was performed with P38 inhibitor SB202190, it didn't reverse either XIAP or FLIP [Fig 7C]. Result indicated that ERK and JNK played a role in the H_2O_2 mediated downregulation of XIAP and FLIP respectively.

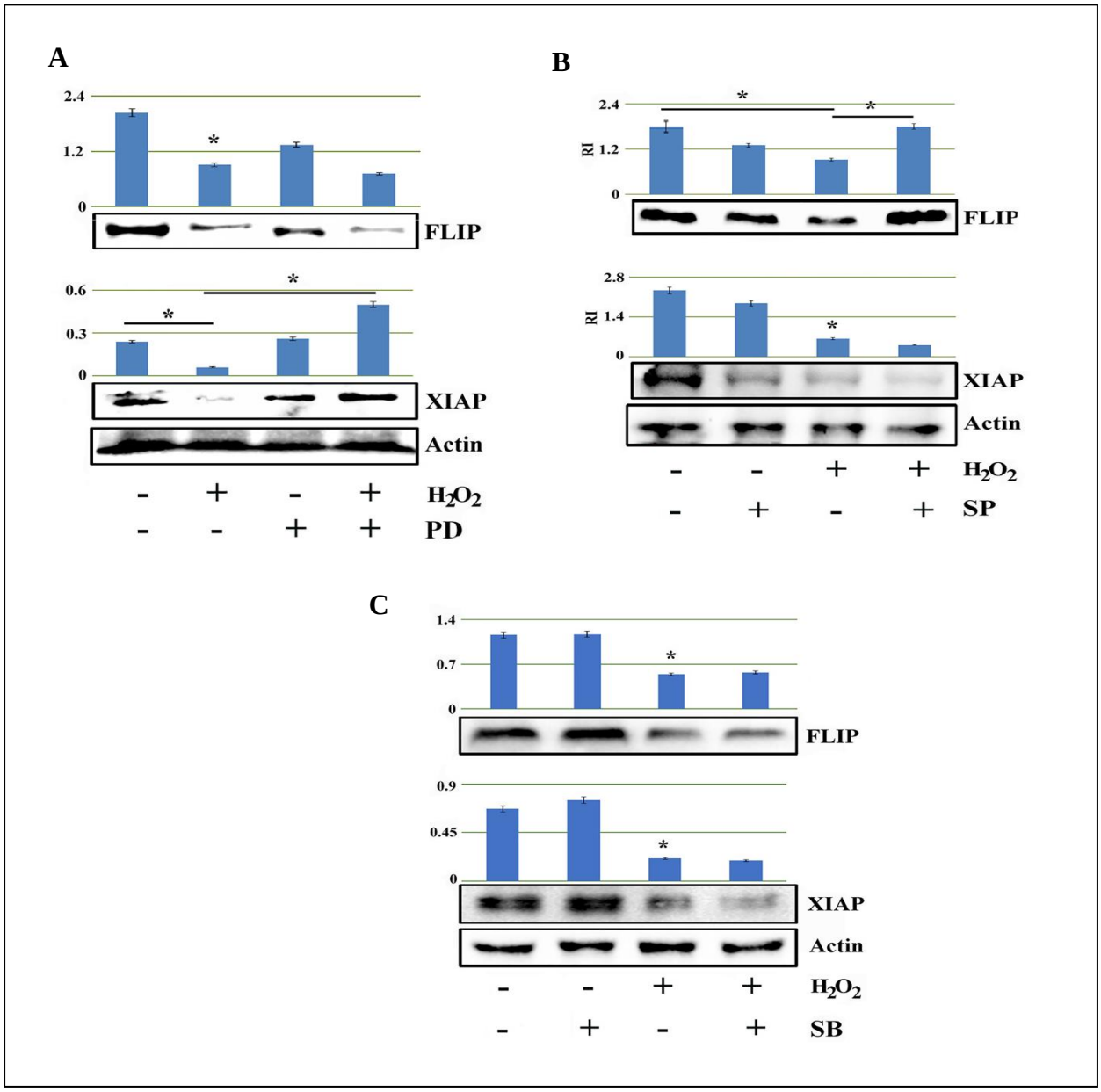


Figure 7

Fig 7: Involvement of JNK and ERK in the regulation of FLIP and XIAP respectively in H_2O_2 treated K562(R) cells. (A) XIAP and FLIP protein levels were checked by immune-blot analysis in whole cell lysates from K562(R) cells in presence or absence of 60 μM PD treatment for 18 hours. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *p < 0.05. **(B)** XIAP and FLIP protein levels were checked by western blot analysis in whole cell lysates from K562(R) cells in presence or absence of 40 μM SP treatment for 18 hours. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry

analysis and indicate mean ± SD of three independent experiments. *p < 0.05. **(C)** XIAP and FLIP protein levels were checked by immune-blot analysis in whole cell lysates from K562(R) cells in presence or absence of 30 μMSB203580 treatment for 18 hours. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments.

H_2O_2 increased JNK and ERK activation by phosphorylation dependant manner

JNK and ERK shows its activity by phosphorylation mediated activation [178]. K562(R) cells were treated with 30 μM H_2O_2 for various time points starting from 4 hours upto 20 hours. The whole cell lysate was subjected to western blot analysis with phospho-JNK and phospho-ERK specific antibodies. Results showed that JNK phosphorylation started to increase after 4 hours of incubation with H_2O_2 and reached its peak around 16 hours [Fig 8A,8B]. On other side, ERK phosphorylation started to increase around 8 hours and after 16 hours it decreased to its basal level [Fig 8A,8B]. K562(R) cells have been treated with different doses of H_2O_2 for 14 hours. The whole cell lysates have been subjected to western blot analysis. The results showed that H_2O_2 dose dependently increased the phosphorylation of JNK and ERK [Fig 8C]. However, there were no any alteration in JNK and ERK at protein level [Fig 8C].

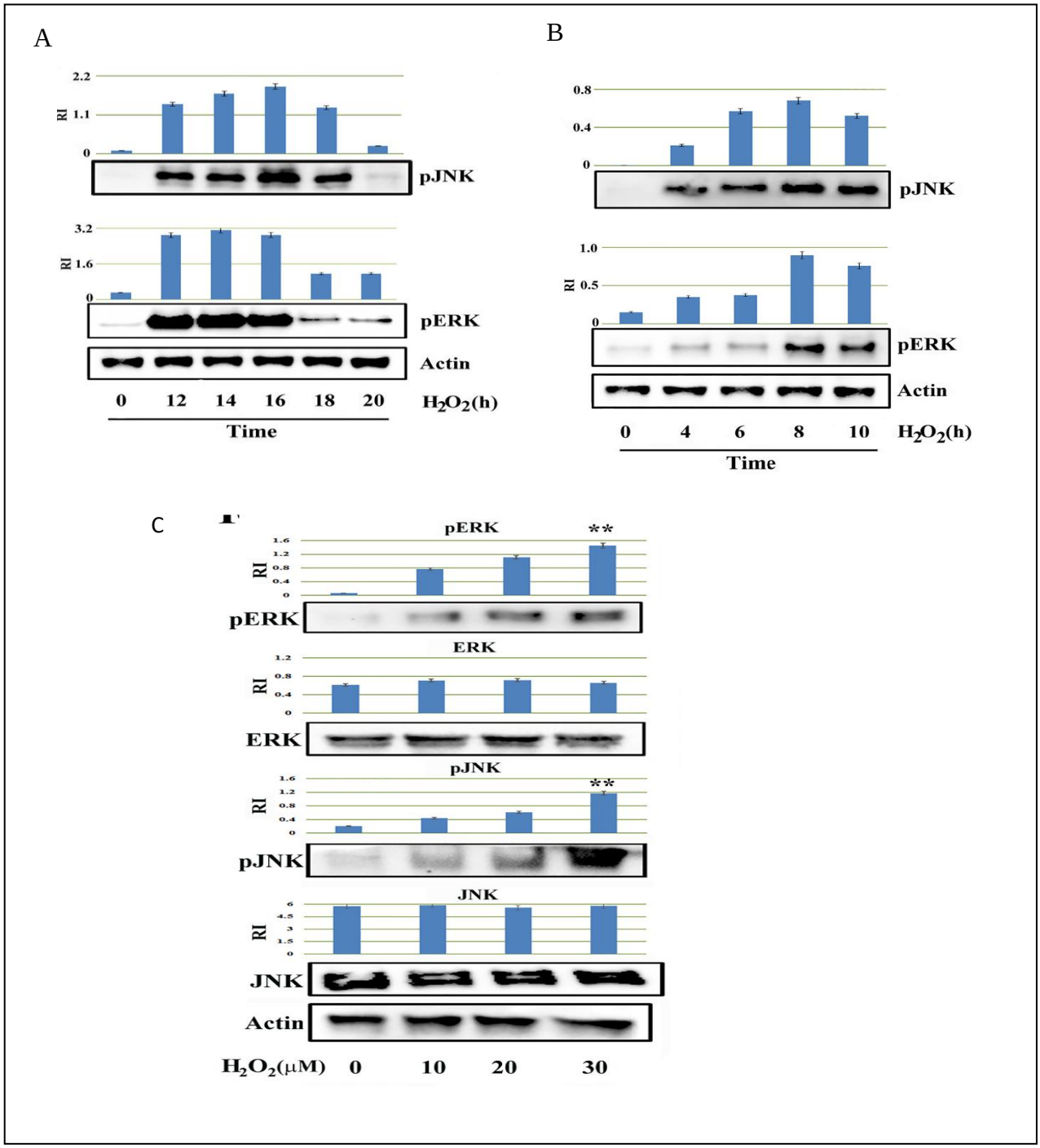


Figure 8

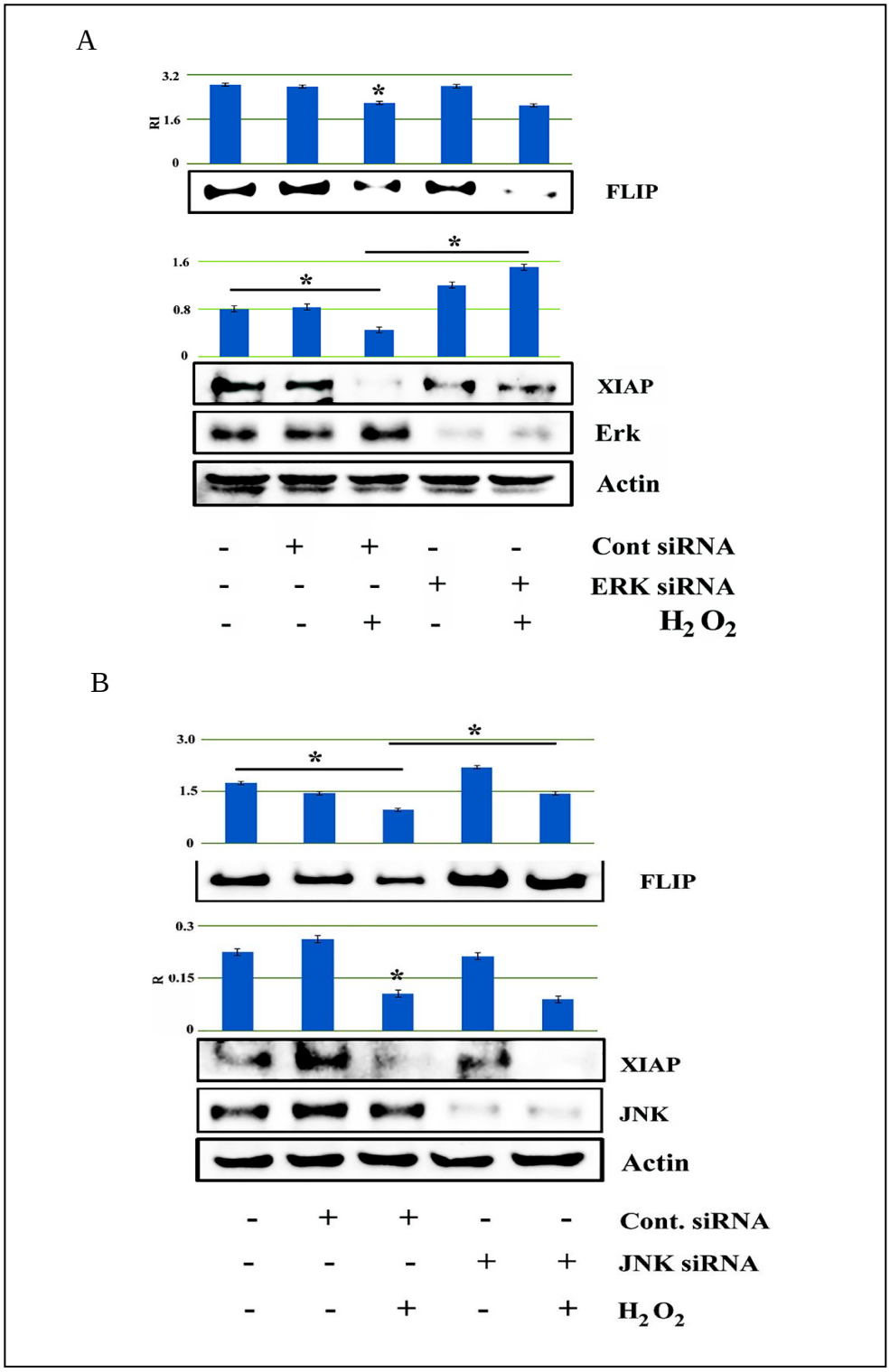
Fig 8: H_2O_2 increases JNK and ERK activation by phosphorylation dependant manner: A) K562(R) cells were treated with 30 μM H_2O_2 for indicated times and pJNK and pERK were checked by immune-blot analysis with the whole cell lysates. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. B**)** K562(R) cells were treated with indicated doses of H2O2 for 16 hours and the whole cell lysates were subjected to immuneblot

analysis with ERK, JNK, pERK, pJNK antibodies. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. **$p < 0.01$.

It is concluded that H_2O_2 phosphorylated and activated ERK and JNK and ROS mediated activation JNK and ERK played a role in the downregulation of FLIP and XIAP respectively.

JNK, ERK played differential role in downregulation in cellular level of FLIP, XIAP respectively:

To confirm role of ERK, JNK in XIAP, FLIP downregulation respectively, K562(R) cells have been treated with ERKsiRNA to knock down ERK then treated with H_2O_2.Scrambled siRNA was used as negative control. Whole cell lysate was subjected to western blot analysis with XIAP and FLIP antibody. Western blot data indicated that H_2O_2 mediated XIAP downregulation partially reversed by ERKsiRNA treatment, but FLIP remains unchanged. ERK protein was decreased by ERKsiRNA [Fig 9A]. K562(R) cells have been treated with JNK siRNA to knock down JNK then treated with H_2O_2. Scrambled siRNA was used as negative control. The whole cell lysate was subjected to western blot analysis with XIAP and FLIP antibody. Result indicated that H_2O_2 mediated FLIP downregulation partially reversed by JNK siRNA treatment, but FLIP remains unchanged. JNK protein was decreased by JNK siRNA [Fig 9B].


A
3.2
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FLIP
1.6
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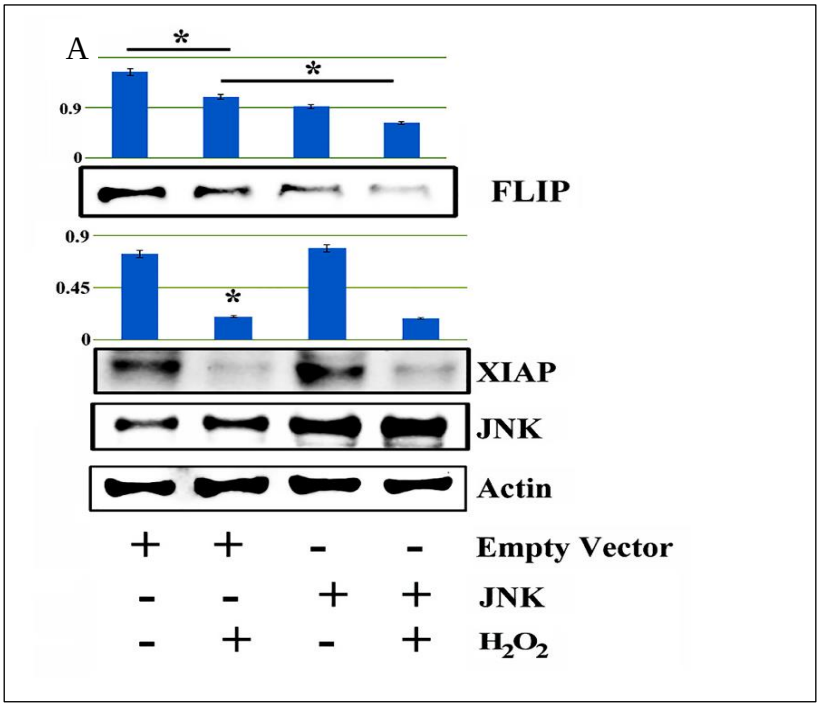
XIAP
Erk
Actin
- + + - - Cont siRNA
- - - + + ERK siRNA
- - + - + H2O2
B
3.0
1.5
0
FLIP
0.3
R 0.15
0
XIAP
JNK
Actin
- + + - - Cont. siRNA
- - - + + JNK siRNA
- - + - + H2O2

Figure 9

Fig 9: Inhibition of ERK and JNK by siRNA reverses degradation of XIAP and FLIP respectively **(A)** ERK was knocked down by siRNA in K562(R) cells and immunoblot analysis of FLIP, XIAP and ERK were performed with the whole cell lysates taken after 18 hours of 30 µM H_2O_2 treatment. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *p < 0.05 **(B)** JNK was knocked down by siRNA in K562(R) cells and immunoblot analysis of FLIP, XIAP and JNK were performed with the whole cell lysates taken after 18 hours of 30 µM H_2O_2 treatment.

To confirm role of JNK in FLIP downregulation, K562(R) cells were transfected with pcDNA3 Flag JNK2a1 plasmid, followed by treatment with 30 µM H_2O_2.Western blot analysis data indicated that JNK overexpression down-regulated FLIP more in the pcDNA3 Flag JNK2a1 containing cells than the cells where empty vector was transfected. JNK overexpression did not downregulate XIAP by H_2O_2 [Fig 10A]. To confirm role of ERK in XIAP downregulation, K562(R) cells were transfected with pcDNA3-HA-ERK2WT plasmid, followed by treatment with 30 µM H_2O_2. Result indicated that ERK overexpression down-regulated XIAP more in the pcDNA3-HA-ERK2WT containing cells than the cells where empty vector was transfected. ERK overexpression did not downregulate FLIP by H_2O_2 [Fig 10B].



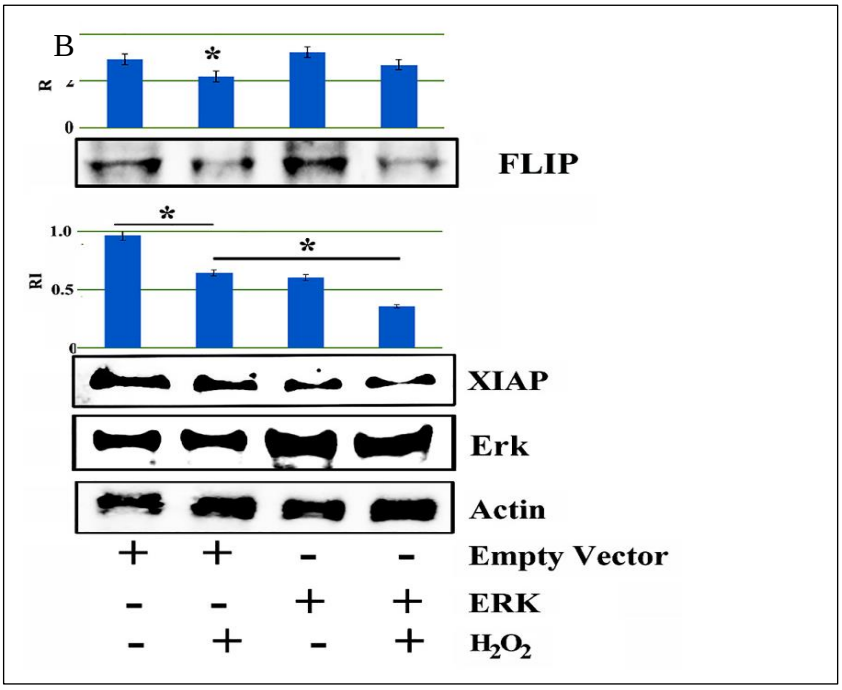


Figure 10

Fig 10: Degradation of FLIP and XIAP is JNK and ERK mediated respectively. **(A)** K562(R) cells were transfected with either pcDNA3 Flag JNK2a1 or empty vector and Immunoblot analysis of FLIP, XIAP and JNK were performed after 30 μM of H_2O_2 treatment for 18 hours. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.**(b)** K562(R) cells were transfected with either pcDNA3-HA-ERK2 or empty vector and Immunoblot analysis of FLIP, XIAP and ERK were performed after 30 μM of H_2O_2 treatment for 18 hours. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ±SD of three independent experiments. *$p < 0.05$.

It is concluded that JNK and ERK differentially regulate FLIP and XIAP level where JNK regulates H_2O_2 mediated FLIP down-regulation, ERK regulates H2O2 mediated XIAP down-regulation.

ERK mediated PI3K and Akt dephosphorylation involved in XIAP ubiquitination and degradation

Recent literature showed that PI3K and Akt play role in the ubiquitination of XIAP and its downregulation [179]. K562 (R) cells were treated with PD98059, either alone or with H_2O_2. After 14 hrs, whole cell lysate was coimmunoprecipited with XIAP antibody, western blot done with Akt antibody. Result indicated that amount of Akt decreased in presence of H_2O_2 alone, Akt level is reversed to level, as in control level, in presence of PD98059[Fig 11A]. This result indicated that Akt and XIAP might interact which get disrupted in presence of H_2O_2 and this is mediated by ERK. So, we checked Akt and its upstream activator PI3K. As PI3K, Akt were activated by phosphorylation. K562(R) cells were incubated with various doses of H_2O_2. The whole cell lysate was subjected to western blot analysis. PI3K, Akt phosphorylation were decreased with increasing concentration of H_2O_2 [Fig 11B]. Both PI3K and Akt remained unaltered at the protein level. To check role of PI3K, Akt in XIAP downregulation, cells were treated with PI3K inhibitor, wortmanin for 18 hrs. The whole cell lysate was subjected to western blot analysis. Result indicated that Wortmanin dose dependently decreased XIAP level which became lowest at 30 µM of H_2O_2, FLIP remained unchanged [Fig 11C]. To check role of PI3K and Akt in XIAP downregulation more clearly, cells were pre-incubated with wortmanin followed by H_2O_2 treatment. Whole cell lysates were subjected to western blot analysis. Western blot analysis data indicated that XIAP level decreased further than H_2O_2.

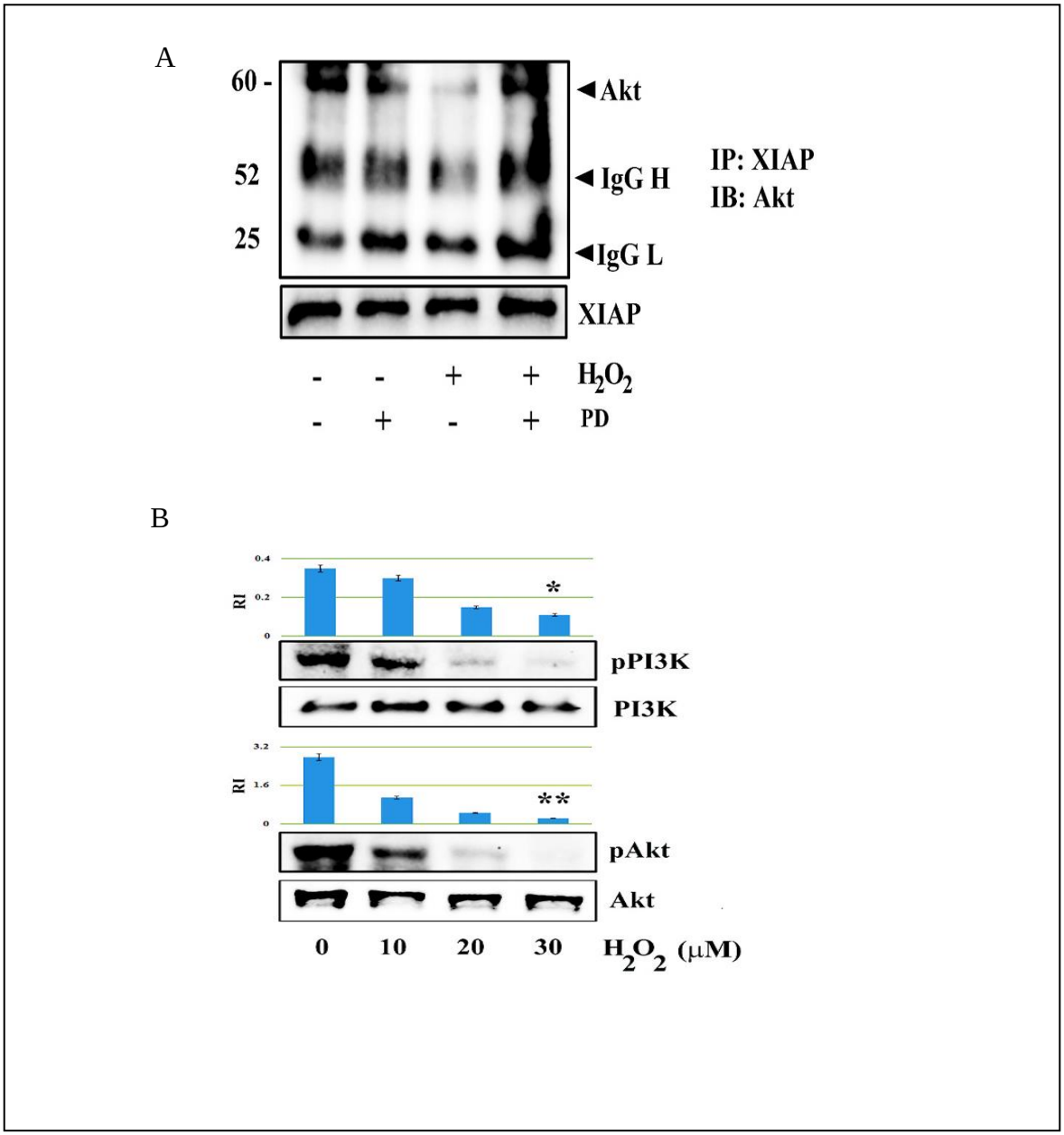

A
60 -
52
25
◄Akt
◄IgG H
◄IgG L
IP: XIAP
IB: Akt
XIAP
- - + + H2O2
- + - + PD
B
RI
0.4
0.2
0
*
pPI3K
PI3K
RI
3.2
1.6
0
**
pAkt
Akt
0 10 20 30 H2O2 (μM)

Figure 11

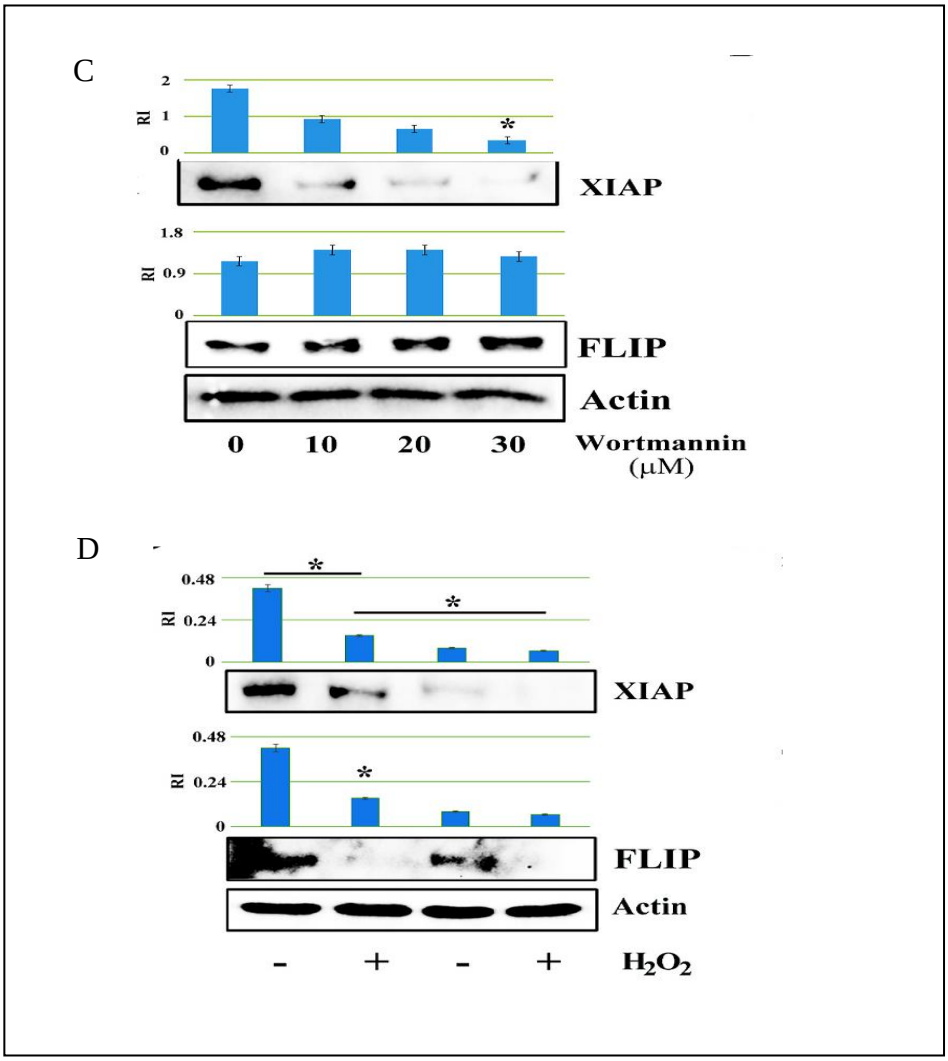


Figure 11

Figure 11: ERK mediated XIAP regulation in presence of H_2O_2 in K562(R) cells, involves pAkt and pPI3K:(A) K562 (R) cells were treated with either 30μM H_2O_2 alone or in combination of PD98059 for 14 hours and the whole cell lysates were subjected to co-immunoprecipitation with XIAP antibody and then western blot analysis were performed with anti-Akt antibody. The lower panel shows the whole cell lysates and upper panel, the immunoprecipitates. (B) K562(R) cells were treated with 30 μM H2O2 for 14 hours and then whole cell lysates were subjected to immune-blot analysis with PI3K, pPI3K, Akt, pAkt antibodies. Phospho-proteins were presented graphically as ratio to corresponding protein level after densitometry analysis and error bar indicate mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$. (C) K562(R) cells were treated with indicated doses of Wortmannin for18 hours and then whole cell lysates were taken and subjected to western blot analysis for FLIP

and XIAP expression. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *p < 0.05(D) K562(R) cells were incubated with or without 10 μM wortmannin and 30 μM H_2O_2 for 18 hours, then cell lysates were subjected to western blot and XIAP and FLIP protein levels were checked. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ±SD of three independent experiments. *p < 0.05.

Role of ERK in PI3K and Akt dephosphorylation

To check the role of ERK in PI3K and Akt phosphorylation, K562(R) cells were treated with ERK inhibitorPD98059 followed by treated with H_2O_2 for 14 hours. The whole cell lysate is subjected to western blot analysis with pAkt and p PI3K antibody. Westen data indicated that PD significantly reversed the H_2O_2-mediated dephosphorylation of PI3K and Akt. To confirm effect of ERK in PI3K and Akt dephosphorylation, K562(R) cells were transfected with either pcDNA3-HAERK2WT plasmid or its empty backbone followed by treated with H_2O_2. Result indicated that H_2O_2 increased PI3K, Akt dephosphorylation in presence of ERK overexpression than H_2O_2 alone.

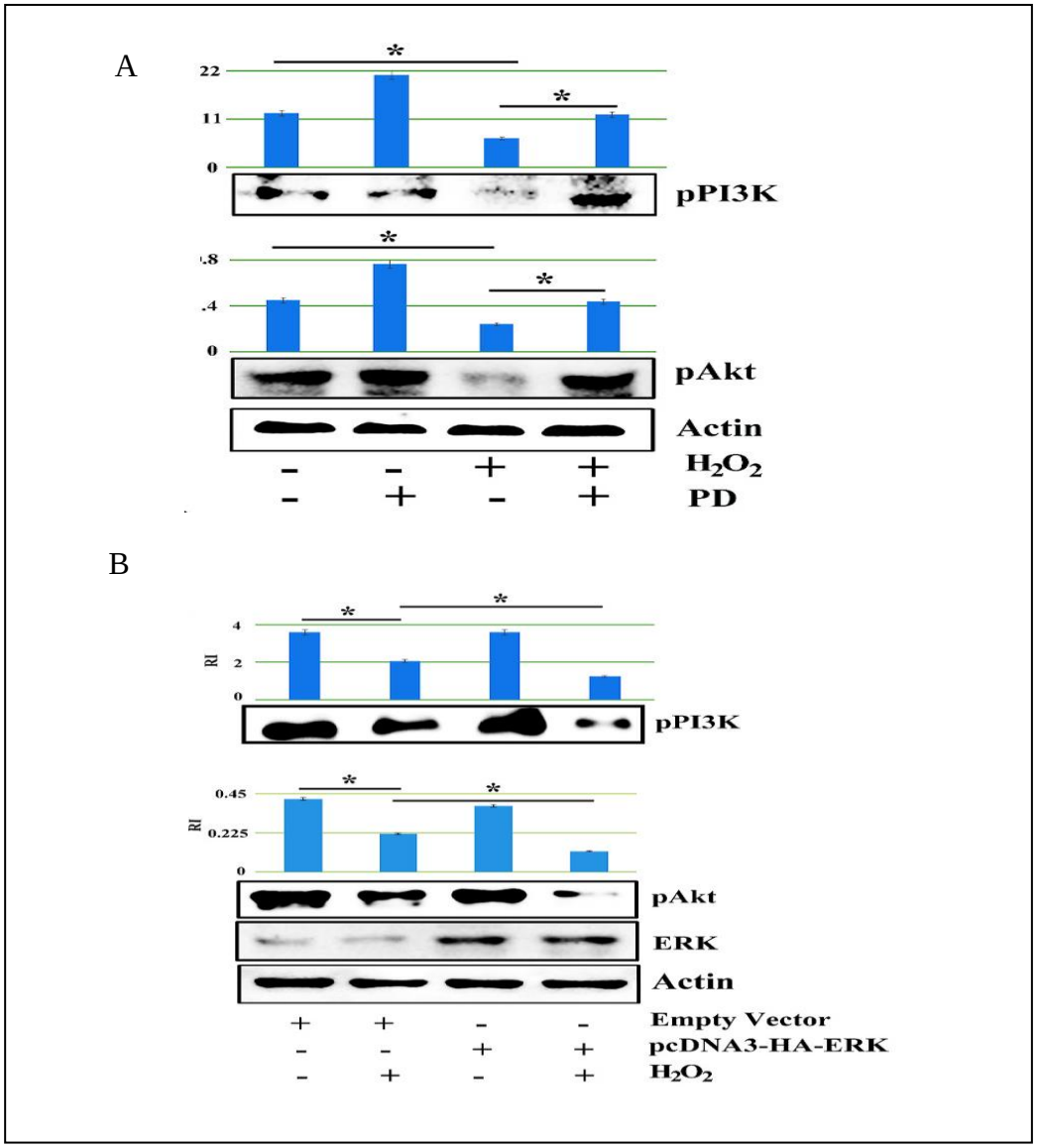


Figure 12

Figure 12: Role of ERK in PI3K and Akt phosphorylation. (a) K562(R) cells treated with 30 µM H_2O_2 either in presence or absence of 30 µM PD and western blot analysis was done for checking pPI3K and pAKT. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. (b) K562(R) cells were transfected with either pcDNA3-HA-ERK2 or empty vector and then treated with 30µM H_2O_2 for 18 hrs. Western blot analysis was done for checking pPI3K, pAkt and ERK level. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.

JNK was not involved in Akt and PI3K dephosphorylation

To check the role of JNK in PI3K and Akt phosphorylation, K562(R) cells have been treated with JNK inhibitor SP followed by treated with H_2O_2 for 14 hours. The whole cell lysate is subjected to western blot analysis with pAkt and pPI3K antibody. Western blot data indicated that SP did not reverse the H_2O_2-mediated dephosphorylation of PI3K and Akt [Fig 13]. So, JNK signaling pathway is not involved in pAKT and p PI3K dephosphorylation.

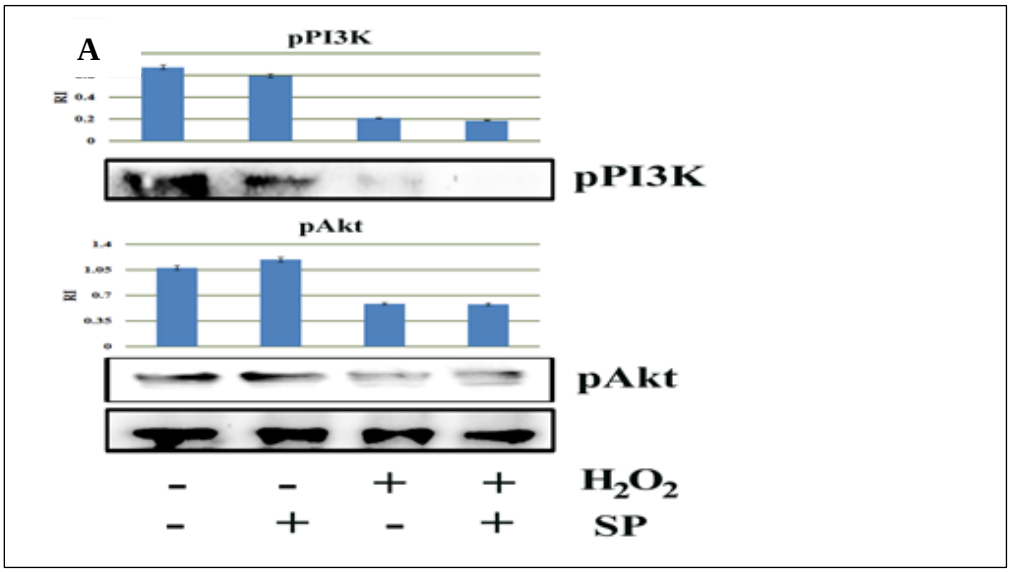


Figure 13

Fig 13: JNK signaling pathway is not involved in pAkt and p PI3K dephosphorylation: A) K562(R) cells treated with 30 µM H_2O_2 either in presence or absence of 40µM SP and western blot analysis was done for checking pPI3K and pAKT. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.

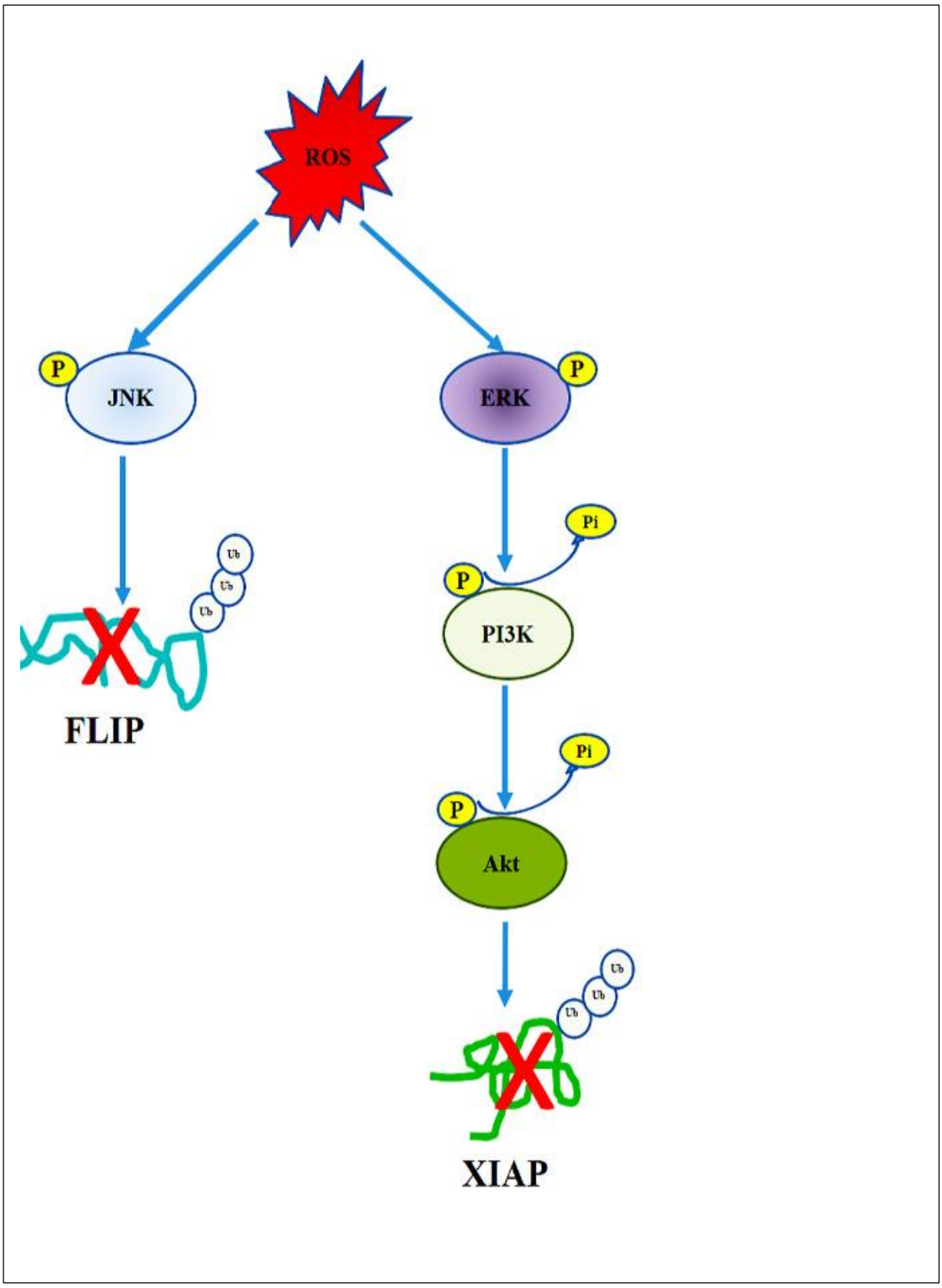


Figure 14

Fig 14 : H_2O_2 activated ERK, which inhibited PI3K,Akt by dephosphorylation, thus binding of Akt to XIAP was inhibited, thus degradation and ubiquitination of XIAP was occurred.

ITCH is responsible for FLIP ubiquitination and degradation

In previous study, it is also known that ITCH, an ubiquitin ligase binds with FLIP and mediates ubiquitination and degradation of FLIP [180]. To check whether ITCH has a role in FLIP degradation, K562(R) cells have been treated with H_2O_2 for 10hrs, cell-lysate has been coimmunoprecipitated with FLIP-antibody followed by western blot with ITCH antibody. Coimmunoprecipitation data indicated that ITCH level increased in presence of H_2O_2 [Fig 15A]. This result indicated that FLIP binds with ITCH in presence ofH_2O_2.To check effect of ROS on ITCH, K562(R) cells have been treated with H_2O_2 for 10 hours. Western blot analysis data indicated that ITCH has been increased at protein level in presence of increasing doses of H_2O_2 [Fig 15B]. To check RNA level of ITCH after H_2O_2 treatment, K562(R) cells have been treated with different concentrations H_2O_2. The total RNA is isolated from different doses of H_2O_2-tretaed K562(R) cells. Realtime qRTPCR analysis data indicated that ITCH expression has been increased at the mRNA level in presence of ROS [Fig 15C].

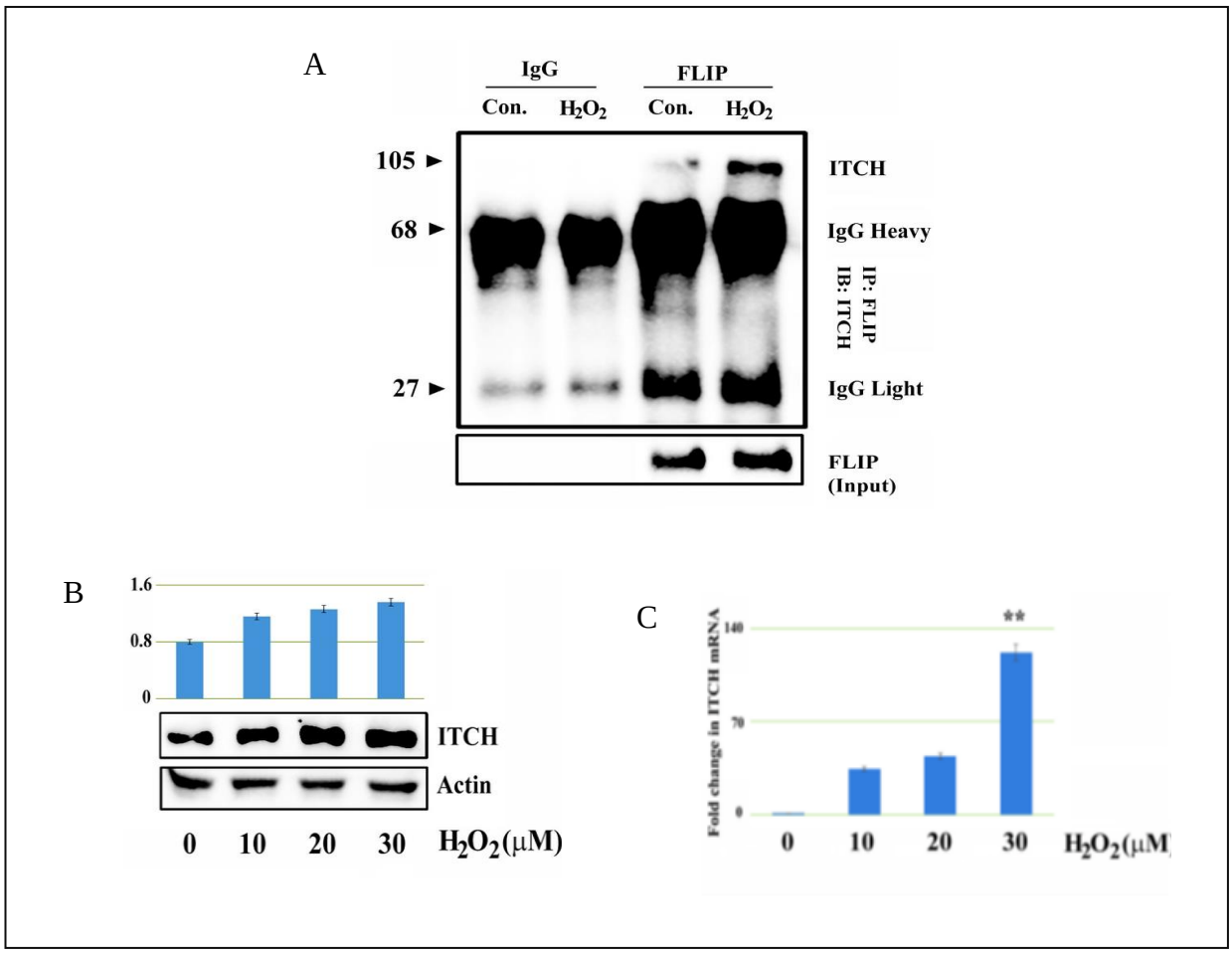


Figure 15

Figure 15: Degradation of FLIP is mediated by increased expression of ITCH :**(A)** K562 (R) cells were treated with 30μM H_2O_2 for 11 hours and the whole cell lysates were subjected to co-Immunoprecipitation with FLIP antibody and control IgG and then western blot analysis were performed with anti-ITCH antibody. The lower panel shows the whole cell lysates and upper panel, the immunoprecipitates. **(B)** Immunobloting of ITCH was performed using whole cell lysate of K562 (R) cells under indicated concentration of H_2O_2 treatment for 11hours. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. **(C)** K562(R) cells were treated with indicated doses of H_2O_2 and Real time qRTPCR was performed with the isolated total RNA using ITCH primer. Data was normalized with actin as housekeeping gene and presented graphically as fold change relative to control. Data indicates mean ± SD of three independent experiments. **$p < 0.01$.

JNK was involved in ITCH mediated FLIP ubiquitination and downregulation

Till now, we have observed that H_2O_2 activated JNK by phosphorylation. Activated JNK down-regulated FLIP levels in K562(R) cell. In previous data, it also indicated that ITCH, a ubiquitin ligase binds with FLIP and mediates ubiquitination and degradation of FLIP. Now, we wanted to check whether JNK has any role in ITCH mediated FLIP ubiquitination and downregulation. So, K562(R) cells have been treated with H_2O_2 alone or in combination with JNK inhibitor SP600125.ITCH was coimmunoprecipitated with FLIP antibody. Coimmunoprecipitation data indicated that amount of ITCH decreased in presence of JNK inhibitor SP600125 than H_2O_2 alone [Fig 16A]. Next, we checked the effect of JNK on ITCH.K562(R) cells have been treated with either H_2O_2 alone or in presence of JNK inhibitor, SP600125. Western blot analysis data indicated thatSP600125 reversed H_2O_2 mediated increase of ITCH [Fig 16B]. However, ERK didn't play any role in ITCH upregulation, K562(R) cells were treated withH_2O_2 either alone or in combination with ERK inhibitor PD98059. Western blot analysis with the whole cell lysates showed that ITCH did not change in presence of ERK inhibitor PD than H_2O_2 alone [Fig 16C].

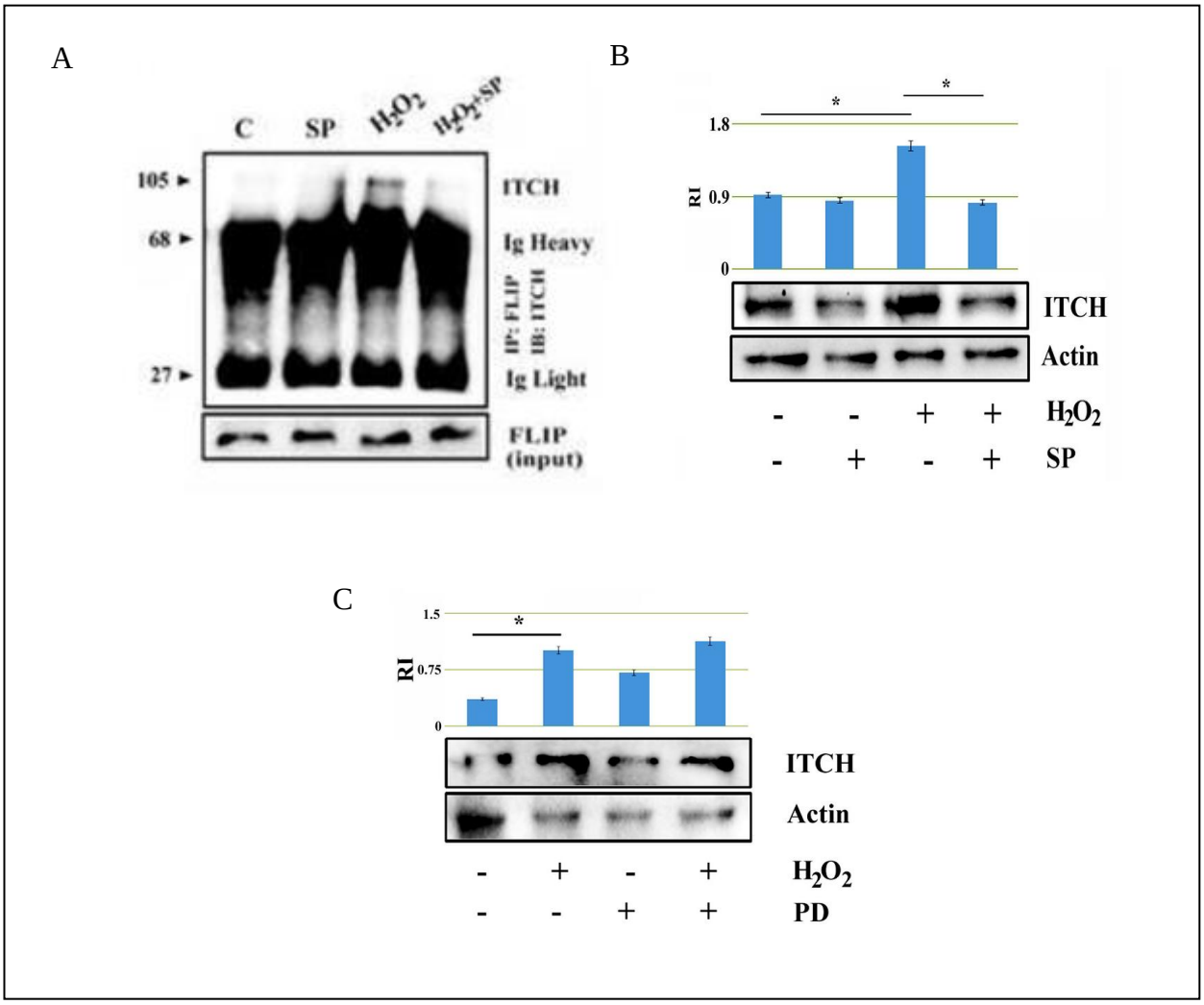


Figure 16

Fig 16: ITCH is responsible for JNK mediated FLIP ubiquitination and degradation: **(A)** K562 (R) cells were treated with either 30μM H_2O_2 alone or in combination of SP600125 for 11 hours and the whole cell lysates were subjected to co-Immunoprecipitation with FLIP antibody and then western blot analysis were performed with anti-ITCH antibody. The lower panel shows the whole cell lysates and upper panel, the immunoprecipitates. **(B)** Immunoblot analysis of ITCH from whole cell lysate of K562 (R) cells under SP600125 treatment. Actin wasused as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. **(C)** K562 (R) cells were treated with PD98059 alone or in combination of H_2O_2. Western blot analysis was done for checking the level of ITCH at protein level. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.

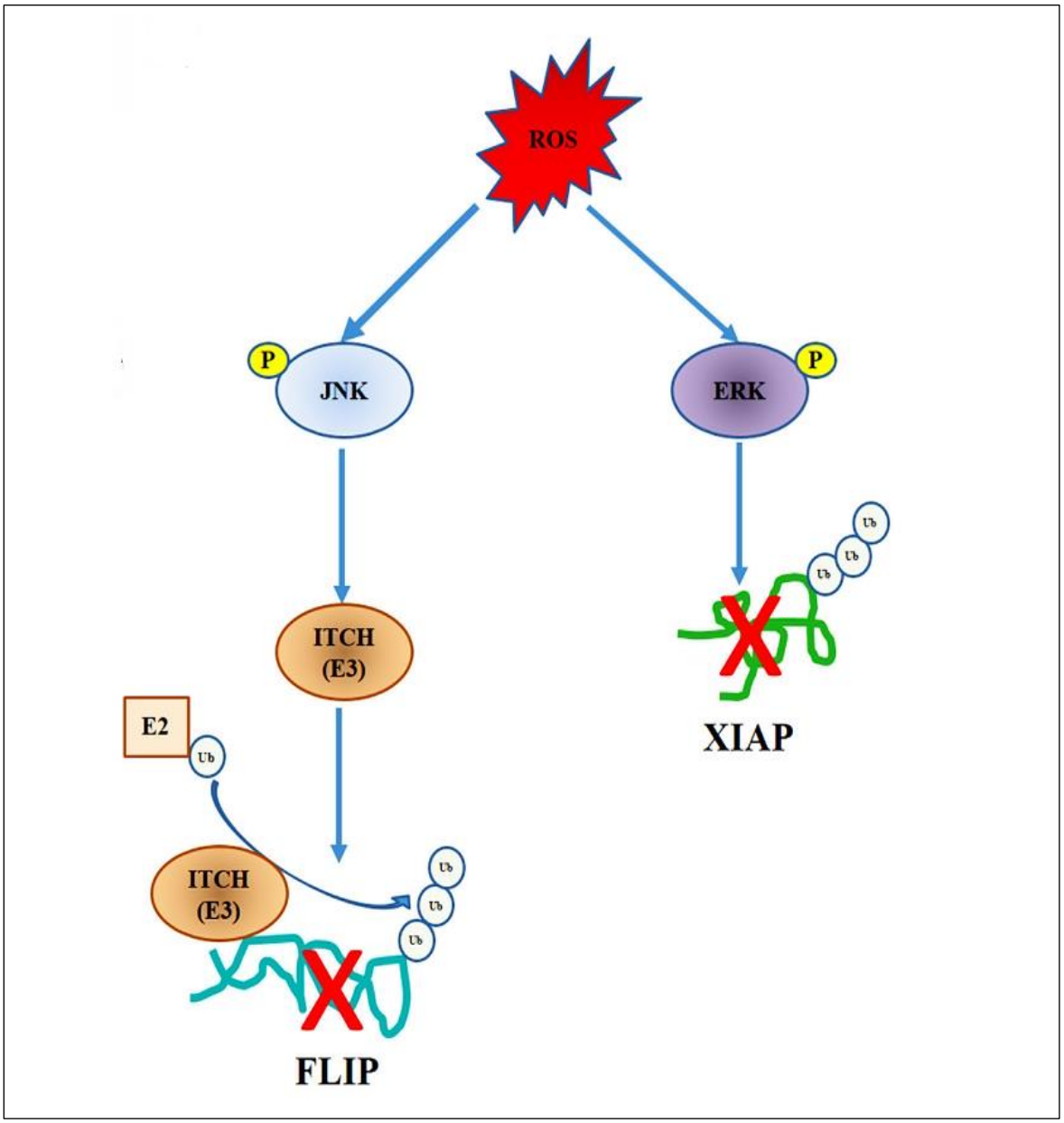


Figure 17

Figure 17: ROS increases ITCH at protein level by activation of JNK. ITCH, being an E3 ubiquitin ligase, associates with FLIP and degrades it by ubiquitin-proteasome pathway. Other side, ERK is involved in XIAP ubiquitination.

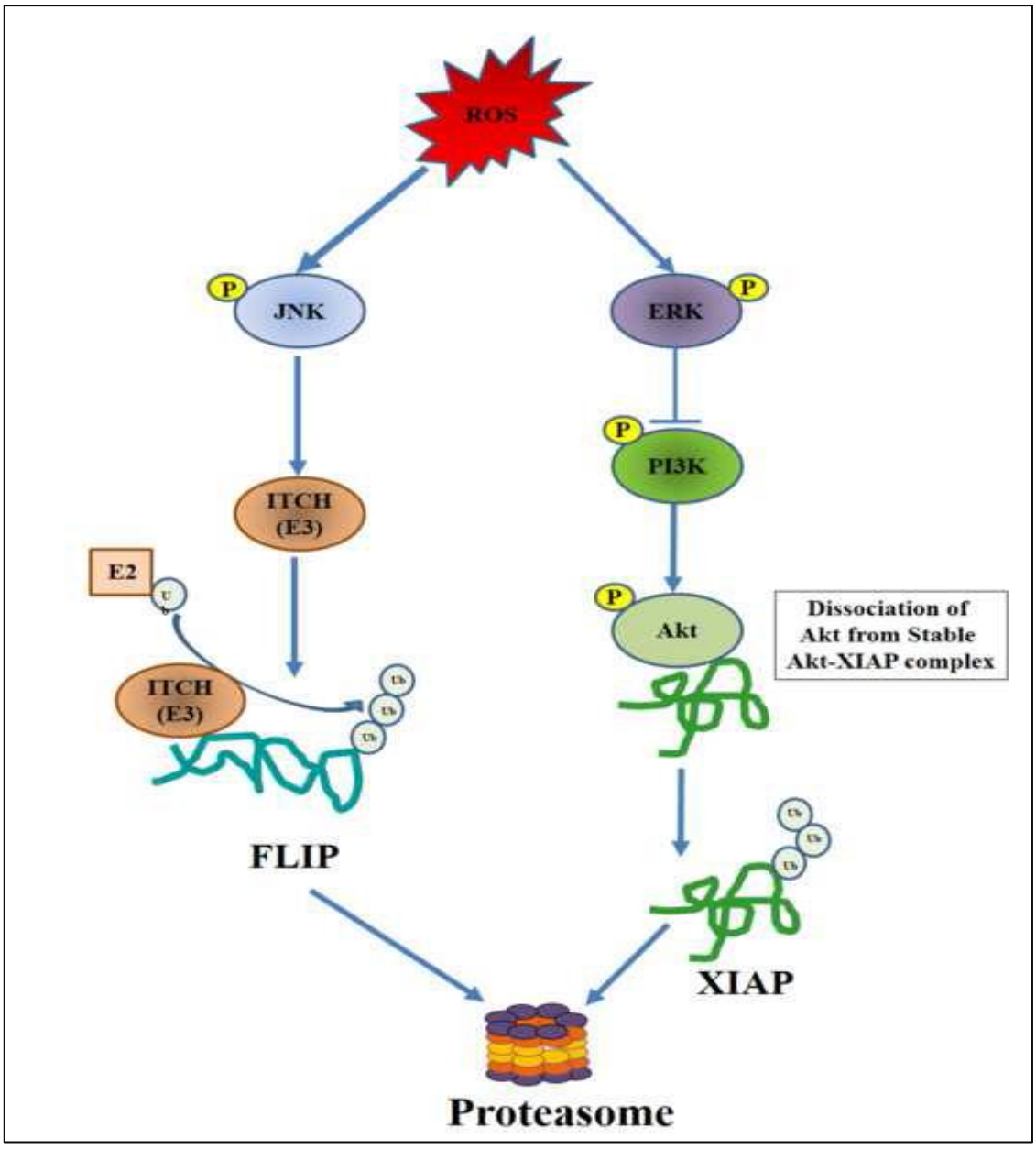


Figure 18: ROS decreases anti-apoptotic protein XIAP and FLIP by activation of ubiquitin proteasomal pathway. ROS-activated ERK subsequently decreases Akt phosphorylation which inhibits the binding of Akt to the XIAP and increases its ubiquitin mediated degradation. On the other hand, ROS increases ITCH at protein level by activation of JNK. ITCH, being an E3 ubiquitin ligase, associates with FLIP and degrades it by ubiquitin-proteasome pathway.

Discussion:

Pro apoptotic signaling is inhibited due to overexpression of antiapoptotic proteins. So, Previous literature showed that down-regulation of XIAP and FLIP could be an interesting strategy to specifically kill cancer cells [181]. In objective I, we have shown that the anti-apoptotic proteins XIAP and FLIP can be downregulated by Hydroxychavicol to improve the efficacy of TRAIL to Imatinib resistant CML cell line K562. We have also seen that Hydroxychavicol decreases FLIP and XIAP via ROS generation. In this objective II, the signaling mechanism by which ROS downregulated XIAP and FLIP has been explored in details in the imatinib-resistant K562 cells.

Targeting IAPs protein XIAP, FLIP could be a viable strategy for the treatment of cancer. In this objective, it is observed that ROS decreased XIAP and FLIP by activation of ubiquitin-proteasomal pathway in imatinib resistant K562 cells. ROS activated ERK and JNK, played a crucial role in XIAP and FLIP degradation respectively because ectopic expression or knock down of ERK and JNK changed the ROS mediated down-regulation of XIAP and FLIP respectively. We also observed that ERKand JNK differentially regulates XIAP and FLIP respectively. Furthermore, our data suggested that ROS activated ERK decreased Akt phosphorylation which inhibited the binding of Akt to the XIAP and increased its ubiquitin mediated degradation. On the other hands, ROS increased ITCH at protein level by activation of JNK. ITCH, being an E3 ubiquitin ligase, associates with FLIP and degrades it by ubiquitin-proteasome pathway.

For first time, we have observed that ROS mediated ERK-Akt crosstalk regulates XIAP and causes XIAP autoubiquitination. For the first time, we have also observed that ROS increases expression of ITCH which, by its association with FLIP, degrades FLIP through proteasome pathway.