Chapter 1
Objective 1
Identification of Inhibitor of apoptotic protein as a target of induction of apoptosis in imatinib resistant CML cells

Introduction: Imatinib is widely accepted drug for the treatment of CML. Development of resistance of this drug is major problem for the treatment of CML. Alternative therapeutic approach might prove effective treatment for the imatinib resistance patients with CML. TRAIL is the tumor necrosis factor-related apoptosis-inducing ligand. TRAIL is member of TNF superfamily. It induces only apoptosis of cancer cell only [150]. There are five receptors of TRAIL. Among them TRAIL-R1 (Death receptor 4 or DR4) and TRAIL-R2 (Death receptor 5 or DR5) [151]. TRAIL binds to its receptor and induces apoptosis via death receptor's cytoplasmic death domain. TRAIL leads to oligomerization of intracellular death domains of the DR4 or DR5 and it recruits the adaptor molecule Fas-associated death domain. After FADD recruitment, Caspase8 is activated then after caspase 3 is activated. DcR1 and DcR2 are two receptors for TRAIL. But they do not induce apoptosis due to lack of intracellular death domain [152]. TRAIL has little cytotoxicity toward normal cells but has cytotoxity toward cancer cells [153]. So, TRAIL is a promising anticancer drug for various cancers.

CML (Chronic Myeloid Leukemia) is a disease of hematopoietic origin which result from reciprocal translocation between chromosome 9 and chromosome 22. Thus, constitutively active Philadelphia chromosome is formed. In Philadelphia chromosome, fused BCR-ABL gene encodes constitutively active tyrosine kinase protein, BCR-ABL [154]. Imatinib mesylate (Gleevec) is a potent inhibitor of the tyrosine kinases ABL. Imatinib is successful in the treatment of patients with CML [155].Generation of resistance against imatinib is occurred due to BCR/ABL amplification and over-expression, BCR/ABL kinase domain mutations and clonal evolution with activation of additional oncogenic pathways. However, generation of resistance against imatinib leads to successive generation of tyrosine kinase inhibitors like nilotinib and dasatinib. So, an alternative therapeutic approach other than tyrosine kinase inhibitor might be important in treatment of patient with Imatinib resistant CML patients.

IAP (Inhibitor of Apoptotic Protein) contains a family of antiapoptotic proteins that provides prosurvival signals pathways by interfering with the activation of caspases and inhibits apoptosis. There are eight IAP proteins in humans: neuronal apoptosis inhibitory protein (also known as BIRC1), cellular IAP1 (cIAP1, also known as BIRC2), cellular IAP2 (c-IAP2, also

known as BIRC3), X-chromosome-linked IAP (XIAP, also known as BIRC4), survivin (also known as BIRC5), ubiquitin-conjugating BIR domain enzyme apollon (alsoknown as BIRC6), melanoma IAP (ML-IAP, also known as BIRC7), and IAP-like protein 2 (ILP2, also known as BIRC8). IAP proteins are frequently overexpressed in various human cancers. So, IAPs are emerged as a promising targetfor therapeutic intervention [156].

Hydroxychavicol (HCH), a phenolic compound of Piper betel leaves. It has antimutagenic and anticarcinogenic activity. HCH induces ROS behind their anticarcinogenic activity [157].

In previous study, it was reported that Hydroxychavicol sensitizes K562(S) cells to drug induced apoptosis. We use Hydroxychavicol to extend that work into resistant cells. TRAIL has been shown to induce Extrinsic apoptotic pathway [158]. However, K562(R) cells were not sensitive to TRAIL. In obejective I, IAPs can be targeted by Hydroxychavicol via ROS generation thereby TRAIL mediated apoptosis can be induced in imatinib resistant CML cell

Materials:

Imatinib was bought from Sigma-Aldrich as imatinib mesylate, and DR5 blocking chimera rhTRAIL was bought from R&D Systems Inc. (Minneapolis, Minnesota, USA). MTT and NAC (N-acetyl-L-cysteine) were bought from Calbiochem. Hydrogen peroxide used in this study was purchased from Merck. iScript Reverse Transcription Supermix and Sso-Fast Evagreen Supermix were purchased from BIORAD. FITCAnnexin V was taken from BD Pharmingen. Propidium iodide was brought from Sigma-Aldrich. 2′,7′- Dichlorofluorescin diacetate was used to estimate ROS and was brought from Calbiochem. The antibodies against caspase-8, caspase-9, caspase-3, PARP, DR5, XIAP, c-FLIP, Bcl2, survivin, CIAP, BAX, tBID, anti-mouse IgG HRP-linked antibody and anti-Rabbit IgG HRP-linked antibody were bought from Cell Signaling Technology (Danver, Massachusetts, USA). DR4 antibody was bought from Santa-Cruz Biotechnology (SantaCruz, California, USA). Anti-γ-actin antibody used as a AQ5 control was bought from Biobharati LifeScience (India).

Methods:

Cell lines and culture condition: K562 cell line was a kind gift from Dr Santu Bandyopadhyay of Indian Institute of Chemical Biology. Cells were maintained in RPMI medium supplemented with 10% fetal bovine serum and 0.1% ampicillin and 1% penicillin-streptomycin at 37°C in a humidified atmosphere with continuous flow of 5% carbon dioxide (CO2).

Development of imatinib-resistant K562 cells: 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% cells were made viable. These cells were designated as K562(R) and henceforth used for all the studies here.

Real-time PCR: mRNA from samples was extracted using Trizol as per manufacturer's instructions. Isolated mRNA was converted to cDNA using iScriptTM Reverse Transcription Supermix for RT-qPCR (BIORAD). For evaluation of the level of gene expression, real-time PCR with SYBR green dye SsoFastTM Evagreen Supermix was used in BIORAD CFX96TM Real-Time System C1000 TouchTM Thermal Cycler machine. The real-time PCR mixture of 10 µl contained 5 µl Evagreen Supermix, 100 nmol/l of each primer (below table containing list of primer used) and 500 ng of cDNA. All samples were run in duplicate and each experiment was repeated at least three times independently.

Knock down assay: Cells in the exponential phase of growth were plated in six-well plates at 1 × 106 cells/well, and then transfected with siRNA oligo using Lipofectamine2000 (Invitrogen) and RPMI (without serum and antibiotic) medium, according to the manufacturer's protocol. The concentrations of siRNAs were chosen based on dose–response studies. Silencing was examined 48 h after transfection.

Western blot analysis: Cells were washed in ice-cold PBS and extracted for 15 min with a buffer containing 50 mmol/l Tris/HCl (pH: 7.5), 140 mmol/l NaCl, 5 mmol/l EDTA, 5 mmol/l NaN3, 1% (v/v) Triton X-100, 1% (v/v) Nonidet P40, 1 mmol/l EGTA and protease inhibitor cocktail. Lysates were cleared by centrifugation at 15 000 rpm for 15 min in an Eppendorf centrifuge, and protein concentrations were determined using Lowry protein assay. Proteins were denatured in 1%SDS-containing reducing sample buffer and the same total protein amount was transferred on to an Immun-Blot PVDF Membrane (BIORAD). The membranes were probed with specific antibodies. Immunocomplexes were detected using horseradish peroxidase-conjugated secondary antibodies followed by Clarity Western ECL Substrate detection (BIORAD) using BIORAD ChemiDoc MP Imaging System.

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:

Imatinib-resistant CML cell lines show resistance against TRAIL-induced apoptosis

We generated imatinib-resistant K562 cells as described in materials and method section. To check resistance of this K562 cells to imatinib, K562 cells were treated 1.5 μg/ml of Imatinib. Annexin V/PI binding assay showed that imatinib induced almost 37% cell death in normal untreated K562 cells whereas only 10% cell death was observed in case of the imatinib-resistant K562(R) cell (Fig1A). The normal K562 cells without any treatment were used as control. These imatinib-resistant K562 cells were used in all our subsequent experiments. To check the cytotoxicity of TRAIL to K562 CML cell lines (both imatinib resistant and imatinib sensitive), two cell lines were incubated with various doses of TRAIL. Annexin V/PI binding assay indicated that little or no cell death in K562(R) cells by TRAIL (Fig 1B). So, Imatinib resistant K562 cells show resistance against TRAIL induced apoptosis.

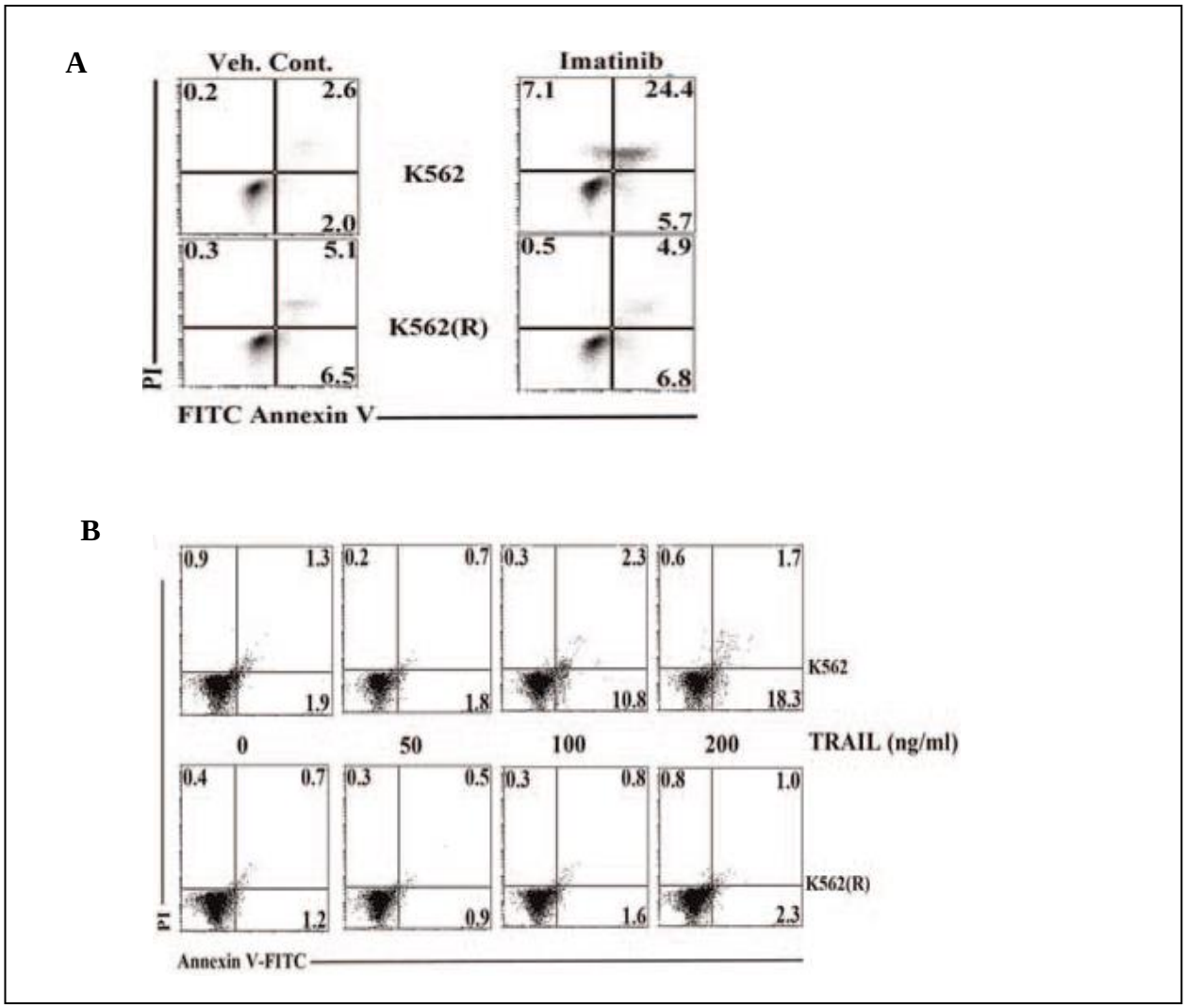


Figure 1

Figure 1: Imatinib resistant K562 cells show resistance against TRAIL induced apoptosis: A) imatinib-sensitive K562 cells and Imatinib-resistant K562(R) were treated with 1.5 µ g/ml of imatinib. After 24 h, cells were subjected to annexin V/PI binding assay by flowcytometer. B) K562(R) and sensitive K562 cells were treated with indicated doses of TRAIL, and annexin V/PI binding assay was performed in flow cytometer.

Hydroxychavicol sensitizes imatinib-resistant CML cell lines to TRAIL-induced apoptosis

To check the effect of Hydroxychavicol on TRAILinduced cytotoxicity in Imatinib resistant K562 cells (K562(R)), K562(R) cells were treated with either 4 µM of Hydroxychavicolalone

or in combination with 200 ng/ml of TRAIL. MTT assay at various time point indicated that Hydroxychavicol and TRAIL alone induced only 5–10% cell death at 24 h [Fig 2A].To confirm the role of Hydroxychavicol on TRAIL-induced cell death, cells were treated with Hydroxychavicol for 6 h and then incubated with 200 ng/ml of TRAIL for 18 h. Apoptosis assay (annexin V/PI binding assay) indicated that Hydroxychavicol alone induced apoptosis of around 41% but increased up to 81% when TRAIL was applied along with Hydroxychavicol [Fig 2B]. These data indicated that Hydroxychavicol facilitates TRAIL-induced apoptosis inK562(R) cell

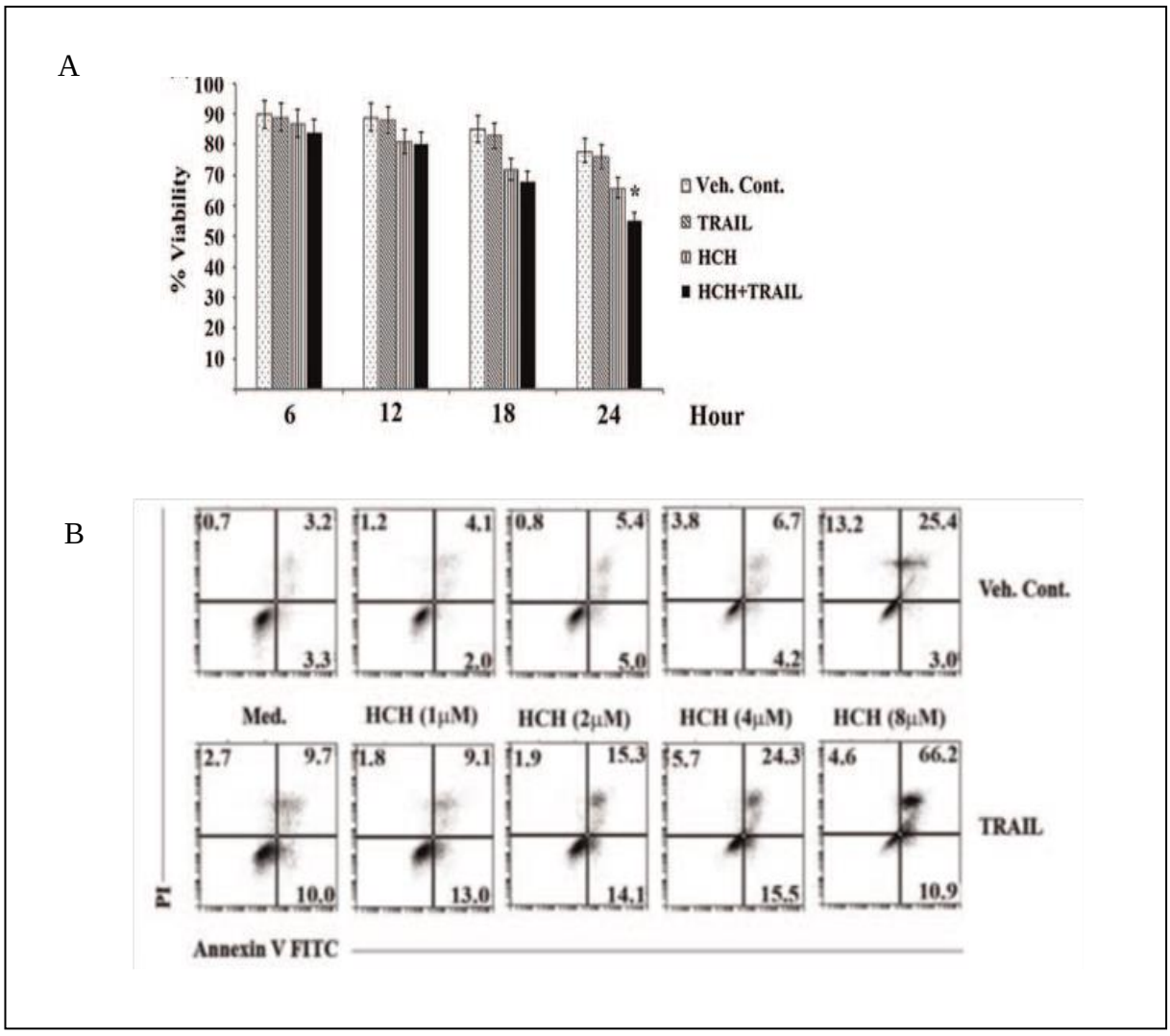


Figure 2

Figure 2: Hydroxychavicol sensitizes imatinib-resistant CML cell lines to TRAIL-induced apoptosis: A) K562(R) cells were treated with 6μmol/l of HCH and 200 ng/ml of TRAIL simultaneously. After 24 h of incubation, cell viability was assessed by MTT assay. Data represents mean ±SD of three independent experiments (*P<0.05). B) K562(R) cells were treated with indicated doses of HCH and 200 ng/ml of TRAIL. Annexin V/PIbinding assay was performed in flowcytometer. The flowcytometer data are a representative dot plot of three independent experiments.

To confirm TRAIL induced apoptosis enhancement by Hydroxychavicol (HCH), caspase cleavage was analyzed by Western blot. Imatinib resistant cells were treated with 4 μM and 6 μM of HCH alone or in combination of 200 ng/ml TRAIL for 18 h. Cell lysates were subjected to Western blot analysis. Result indicated that TRAIL and Hydroxychavicol enhanced cleavage of caspases-3, 8 and PARP significantly (Fig 3A).

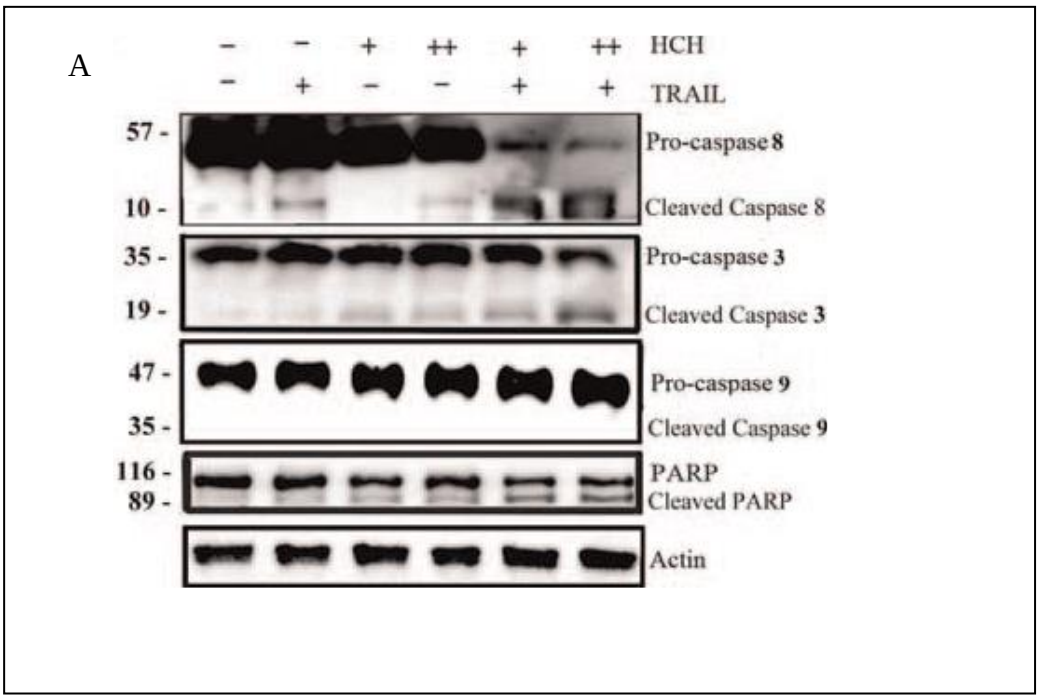


Figure 3

Figure 3: Hydroxychavicol and TRAIL combinatory treatment enhanced cleavage of Pro caspase 8, pro caspase 3 and PARP: HCH and TRAIL-treated K562(R) cells were lysed and proteins were used for immunoblotting analysis using antibody against pro caspases-8, 3, 9 and PARP, and actin was used as a loading control.

Involvement of TRAIL mediated extrinsic apoptotic pathway in Hydroxychavicol (HCH) and TRAIL combinatory treatment in K562(R) cells

As TRAIL binds to its receptors DR4 and DR5 and induces apoptosis which involves caspase-8 cleavage, we wanted to confirm the involvement of TRAIL-apoptosis pathway by HCH and TRAIL. To do that, DR5 blocking chimera antibody has been used. This antibody antagonizes TRAIL binding and prevents TRAIL action. Cells were preincubated with DR5 blocking chimera antibody for 2 h and then treated the cells with HCH and TRAIL either alone or in combination. The flowcytometer analysis with annexin V/PI showed thatDR5 blocking chimera antibody significantly reversed TRAIL-mediated apoptosis [Fig 4a]. This indicated that TRAIL mediated extrinsic apoptotic pathway was involved in above combinatory effect.

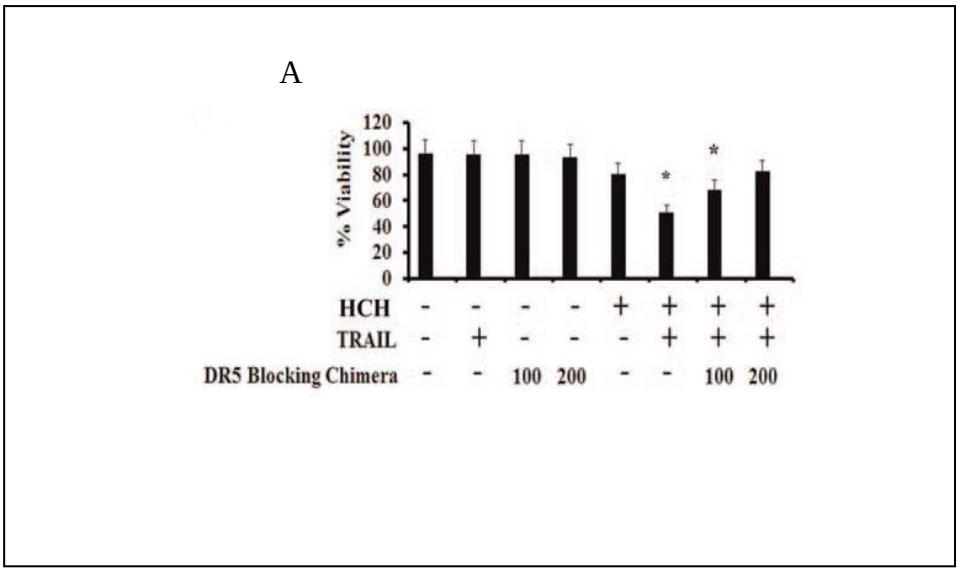


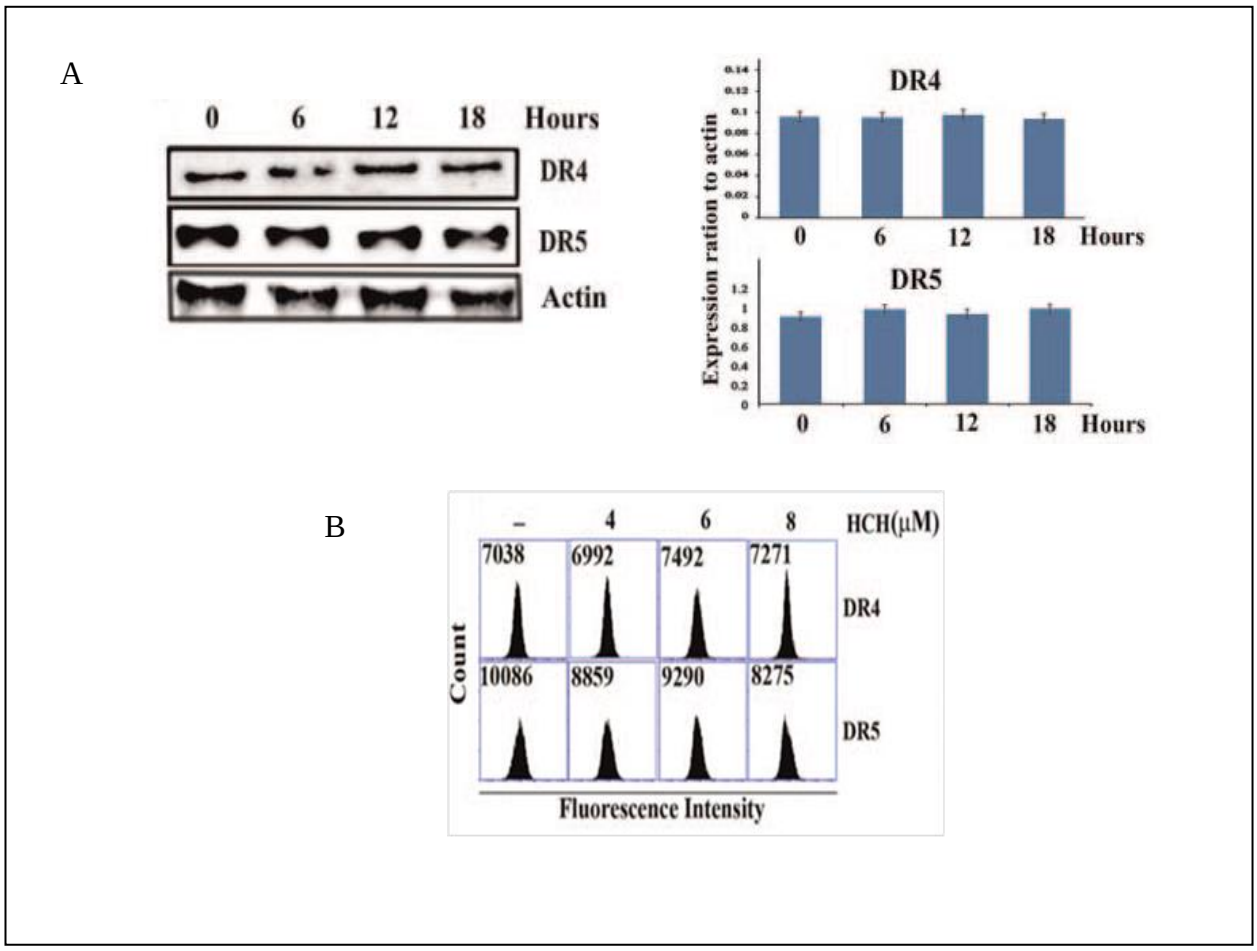
Figure 4A

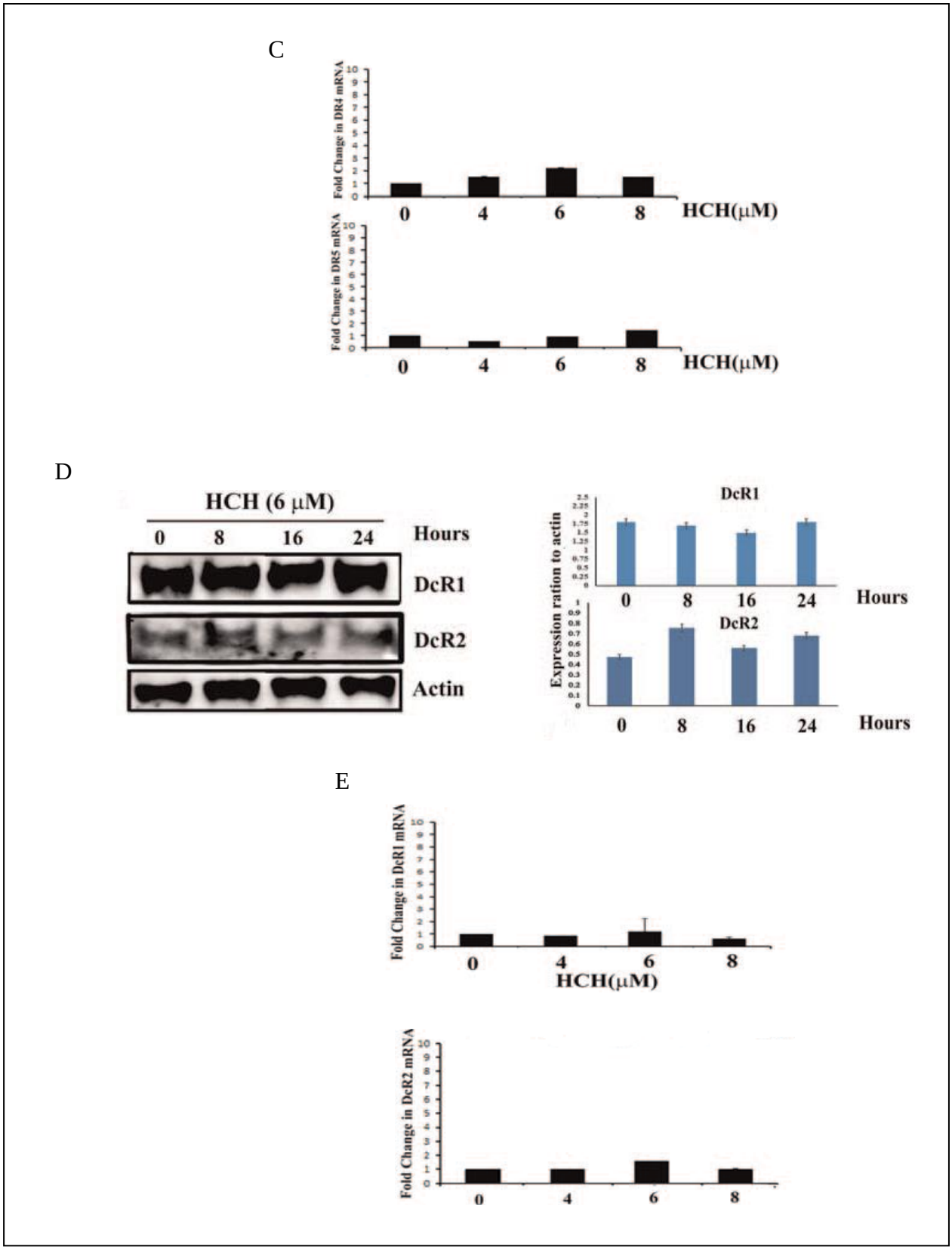
Fig 4: Involvement of TRAIL mediated extrinsic apoptotic pathway in Hydroxychavicol and TRAIL combinatory treatment in K562(R) cells. A) K562(R) cells were pretreated with DR5 blocking chimera antibody and then treated with the combination of HCH and TRAIL. Cell viability assay was performed by MTT assay. Data represents mean ±SD of three independent experiments (*P< 0.05).

Hydroxychavicol did not alter DR4 and DR5 expression and DCR1 and DCR2 expression at RNA level and Protein level

One possibility might be that whether Hydroxychavicol increased DR4 and DR5 expression. Cells were treated with Hydroxychavicol at various time points. Whole cell lysates were subjected to western blot analysis. Western blot analysis data indicated that no increase in either

DR4 or DR5 at the protein level after 18 h of Hydroxychavicol treatment [Fig 5A]. 18 hrs time point was considered because apoptosis was detected at 24 hrs. The flow cytometry data revealed that DR4 and DR5 were unaltered at various doses of Hydroxychavicol [Fig 5B]. K562(R) cells were treated with different doses of Hydroxychavicol. Real time PCR data revealed that there is no change at DR4 and DR5 at the mRNA level [Fig 5C]. DcR1 and DcR2 are two important TRAIL receptors. They have binding affinity for TRAIL.They cannot induce apoptosis due to lack of intracellular death domain which is important for activating downstream signaling [12]. K562(R) cells have been treated with Hydroxychavicol at various time point. Whole cell lysates were subjected to western blot analysis. Western blot analysis indicated that no significant changes in DcR1and DcR2 at the protein level [Fig:5D]. Real time PCR data indicated that no significant changes in DcR1and DcR2 at the mRNA level [Fig:5E].




C
Fold Change in DR4 mRNA
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4
6
8
HCH(μM)
Fold Change in DR5 mRNA
0
4
6
8
HCH(μM)
D
HCH (6 μM)
0
8
16
24
Hours
DcR1
DcR2
Actin
DcR1
Expression ration to actin
0
8
16
24
Hours
DcR2
0
8
16
24
Hours
E
Fold Change in DcR1 mRNA
0
4
6
8
HCH(μM)
Fold Change in DcR2 mRNA
0
4
6
8

Figure 5

Fig 5: Hydroxychavicol did not alter DR4 and DR5 and DCR1, DCR2 expression at RNA level and Protein level A) K562(R) cells were treated with various doses of Hydroxychavicol. Whole cell lysate was subjected to western blot analysis for checking DR4/DR5 at protein level. β-actin acts as a loading control. B) K562 cells were treated with various doses of Hydroxychavicol for 14 hrs. DR4 and DR5 were checked in flowcytometer by labelling them with DR4/DR5 antibody and fluorescent conjugated secondary antibody. Data shown here area representative histogram of three independent experiments. C) Cells were treated with various doses of Hydroxychavicol. DR4 and DR5 expression were checked by real-time PCR (n =3: data as mean ±SEM). D) K562 (R) cells were treated Hydroxychavicol for different time point. DcR1 and DcR2 surface protein levels were checked by Western blot analysis. Here β-actin acts as a loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. E) K562(R) cells were treated with increasing doses of Hydroxychavicol. DcR1 and DcR2 mRNA expressions were checked by real time PCR (n=3, mean ± SD).

From above data it is concluded that Hydroxychavicol involved death receptor-mediated signaling pathway for induction TRAIL mediated apoptosis, although death receptor expression remains unaltered.

Hydroxychavicol decreased XIAP and FLIP at the protein level

Downregulation of proapoptotic genes and inhibition of death receptor signaling because of death receptor's low expression are possible mechanisms that leads to increased resistance to apoptosis. The overexpression of antiapoptotic genes is most common mechanism of apoptosis inhibition. There are three antiapoptotic family of proteins, including Bcl2, inhibitors of apoptosis proteins (IAPs) and FLICE-inhibitory proteins (FLIPs). They regulate caspase-dependent apoptotic pathways. So, we wanted to check whether any change of these anti apoptotic proteins could be responsible here. K562(R) cells were treated with Hydroxychavicol at different doses for 20 hrs. Whole cell lysates were subjected to western blot analysis. Western Blot analysis data indicated that FLIP and XIAP were decreased significantly when treated with increasing concentration of Hydroxychavicol. However, cIAP and Bcl2 levels remains unaltered with increasing doses of Hydroxychavicol [Fig:6a]. When we have checked whether there is any alteration of these proteins at mRNA level, cells were treated with

increasing concentration of Hydroxychavicol. Real time PCR data indicated that no significant increase in XIAP, FLIP and cIAP at mRNA level [Fig:6b].

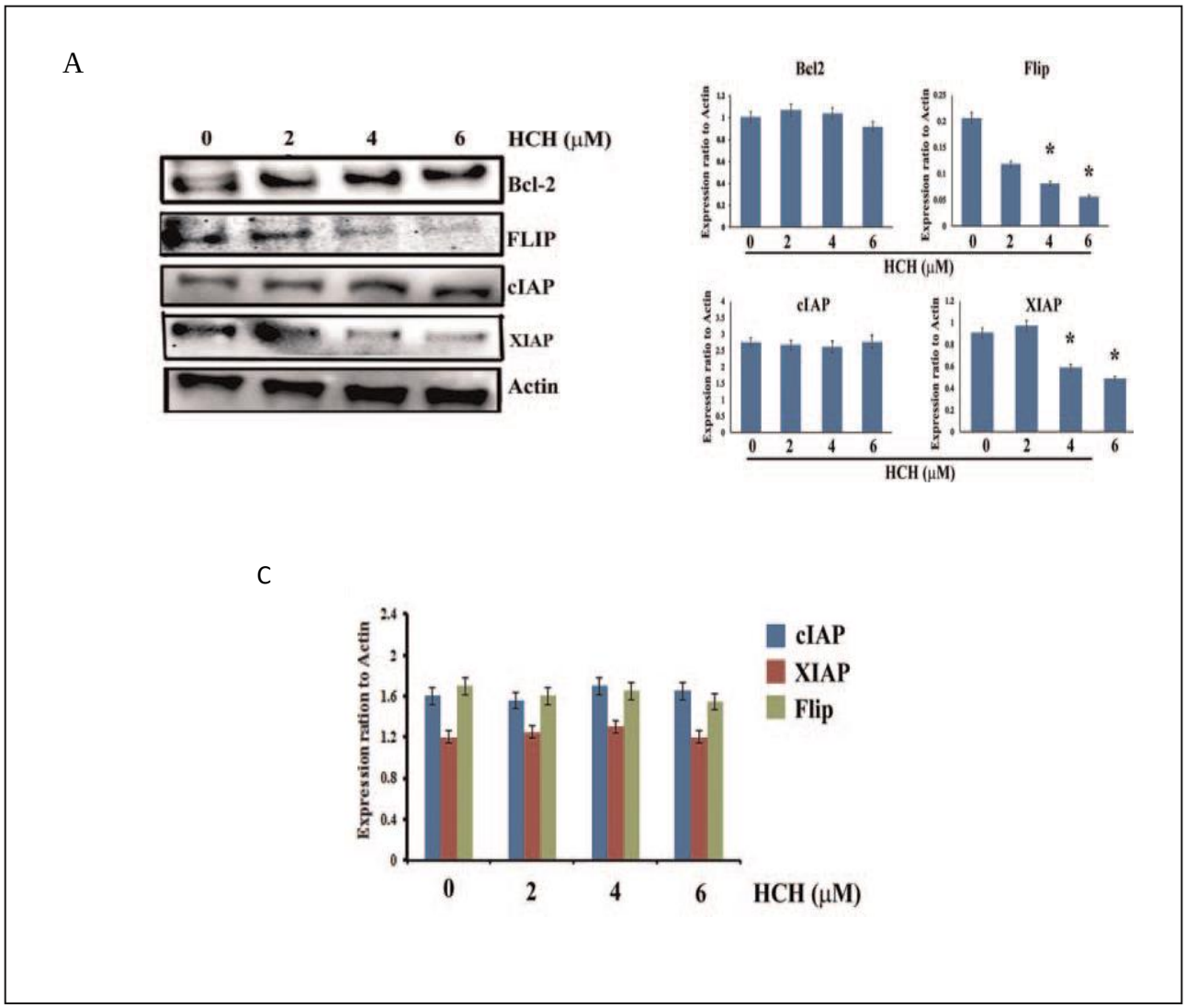


Figure 6

.**Fig 6**: Hydroxychavicol decreased XIAP and FLIP at the protein level**: A)** K562 (R) cells were treated with 6 μmol/l Hydroxychavicol (HCH)for 18 h and whole cell lysates were subjected to Western blot analysis. Data shown here is a representative Western blot of three independent experiments. On the right, data were also presented graphically as ratio to actin after densitometry analysis and indicate mean ±SD of three independent experiments (*P< 0.05). **B)** Levels of endogenous c-FLIP, cIAP, and XIAP mRNA were assessed by real-time PCR after treatment with increasing doses of Hydroxychavicol for 14 hrs. and graphically represented as expression ratio to actin as mean ±SD of three independent experiments.

Hydroxychavicol mediated FLIP and XIAP is occurred via proteosomal mediated pathway:

To understand the molecular mechanisms behind Hydroxychavicol mediated XIAP and FLIP downregulation at the protein level, K562(R) cells were treated with MG132, proteasome inhibitor then treated with Hydroxychavicol. Whole cell lysate was subjected to western blot analysis. Western blot analysis data indicated that significant inhibition of both FLIP and XIAP downregulation by MG132 [Fig 7A].

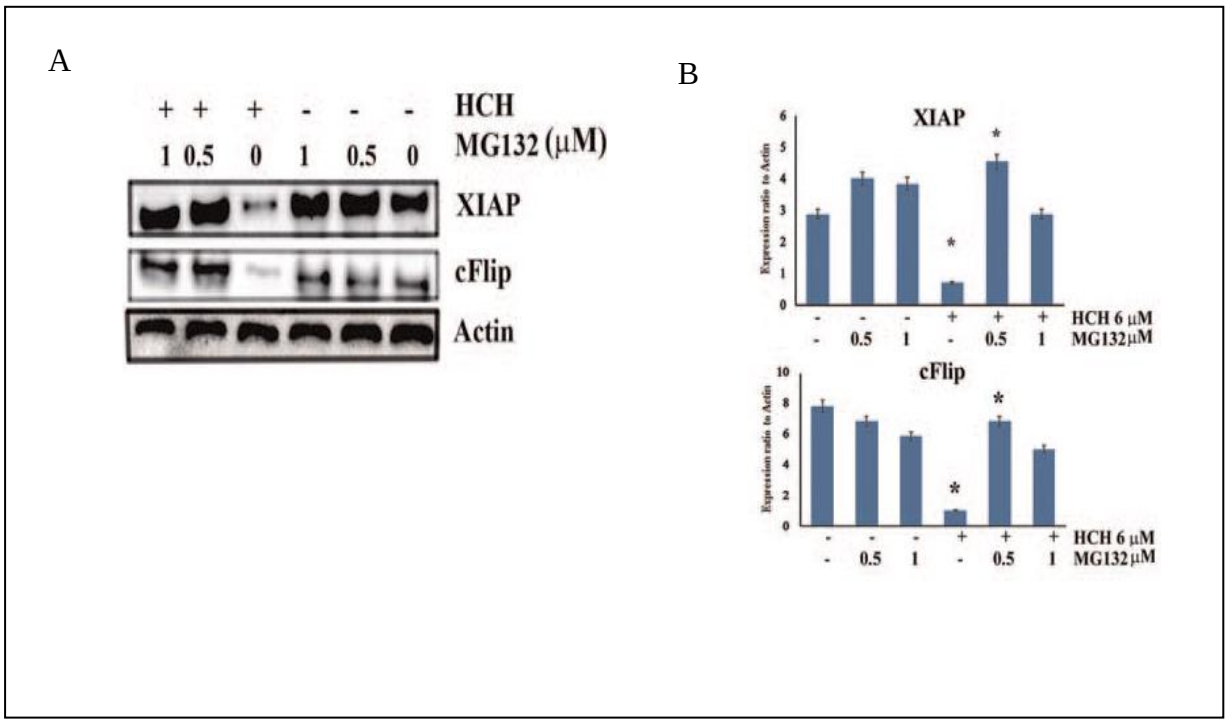


Figure 7 A

Figure 7: Hydroxychavicol mediated FLIP and XIAP is occurred via proteosomal mediated pathway: **A)** K562(R) cells were preincubated with MG132 and then treated with Hydroxychavicol for 16 hrs. Whole cell lysate were subjected to western blot. The Western blot data are a representative one from three independent experiments. Data were also presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments (*P<0.05).

This data suggests that proteasomal degradation might played role in Hydroxychavicol mediated XIAP and FLIP downregulation.

Knockdown of XIAP and FLIP involved in TRAIL mediated apoptosis:

Now we checked whether downregulation of XIAP and FLIP play role in TRAIL induced apoptosis. FLIP and XIAP were knocked down in K562(R) cells by siRNA then incubated with TRAIL. Annexin V/PI binding assay data indicated that knocking down FLIP and XIAP either alone or in combination by FLIP and XIAP siRNA significantly enhanced TRAIL induced apoptosis compared with the control siRNA (Fig 8A).

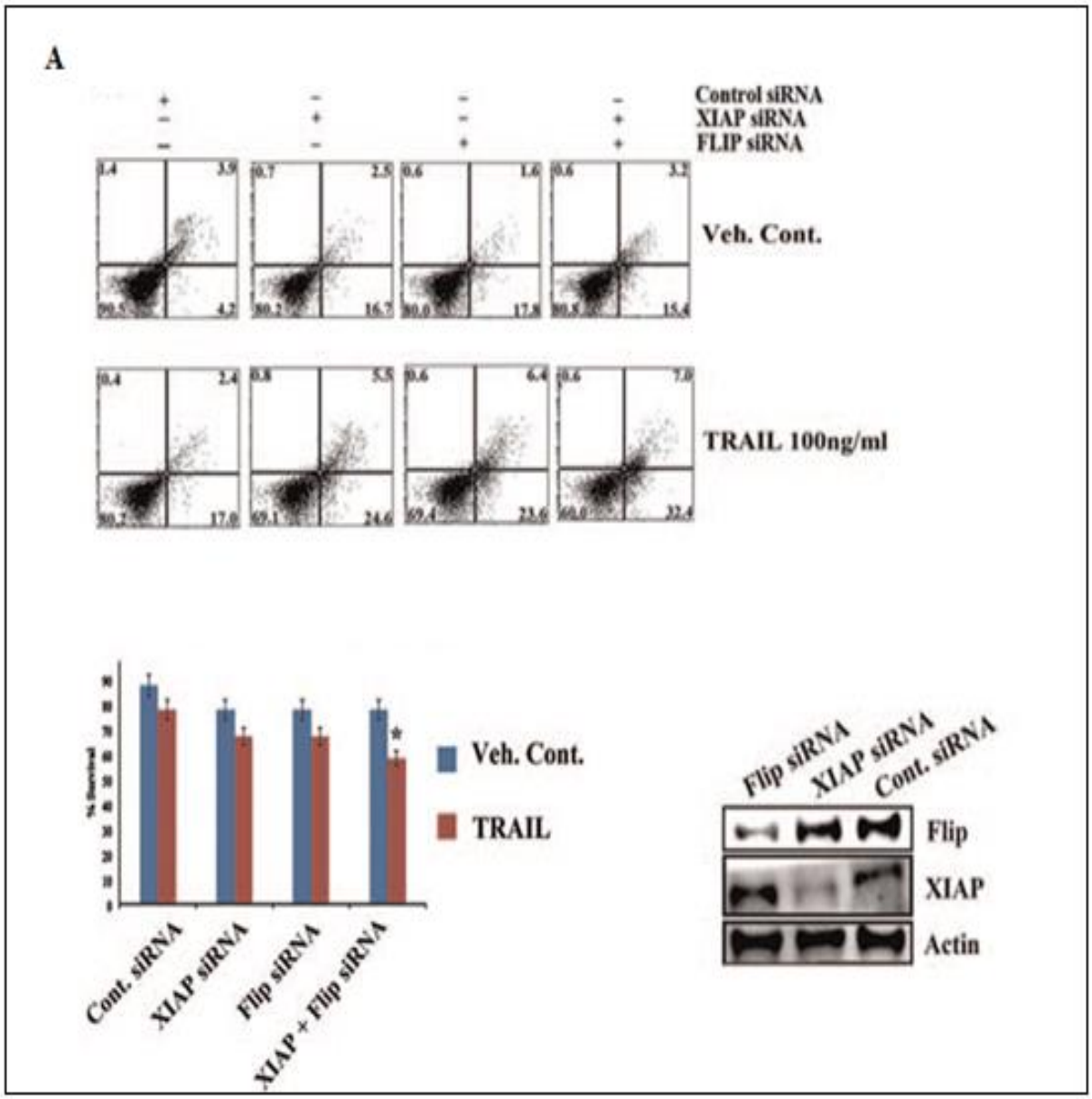


Figure 8 A

Figure 8: Knockdown of XIAP and FLIP involved in TRAIL mediated apoptosis: A) K562(R) cells were transfected with control siRNA, FLIPsi RNA, XIAPsi RNA for 48 h and then treated with 200 ng/ml TRAIL. Apoptosis assay was done by annexin V/PI binding assay by flowcytometer. Data represents mean ±SD of three independent experiments (*P<0.05). At bottom panel, XIAP and FLIP level was checked after knockdown of cell with XIAP siRNA and FLIP siRNA respectively

So, It is concluded that the down-regulation of XIAP and FLIP is responsible for TRAIL mediated apoptosis.

ROS played a major role in Hydroxychavicol -mediated TRAIL sensitization

In previous study, it was reported that Hydroxychavicol increases the intracellular ROS level [159]. Therefore, it must be possible that here ROS has played some role to play insensitizing imatinib resistant K562 cells to TRAIL-mediated apoptosis. K562(R) cells were preincubated with ROS scavenger NAC and then treated with HCH and TRAIL. AnnexinV/PI binding assay showed that NACreversed TRAIL-mediated enhancement of apoptosis. It indicated that the involvement of ROS in this process [Fig 8].

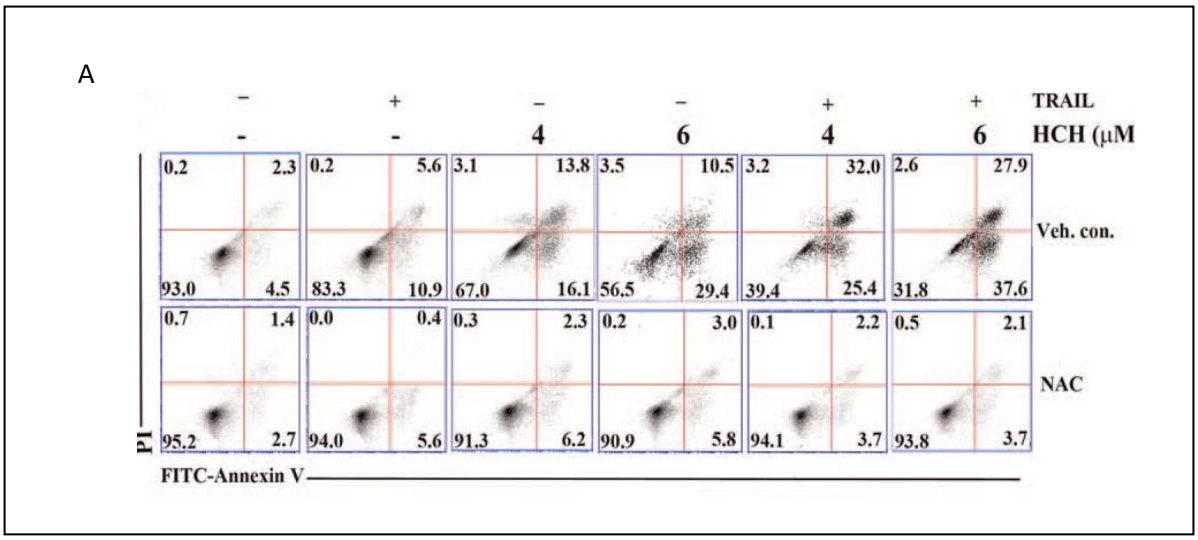


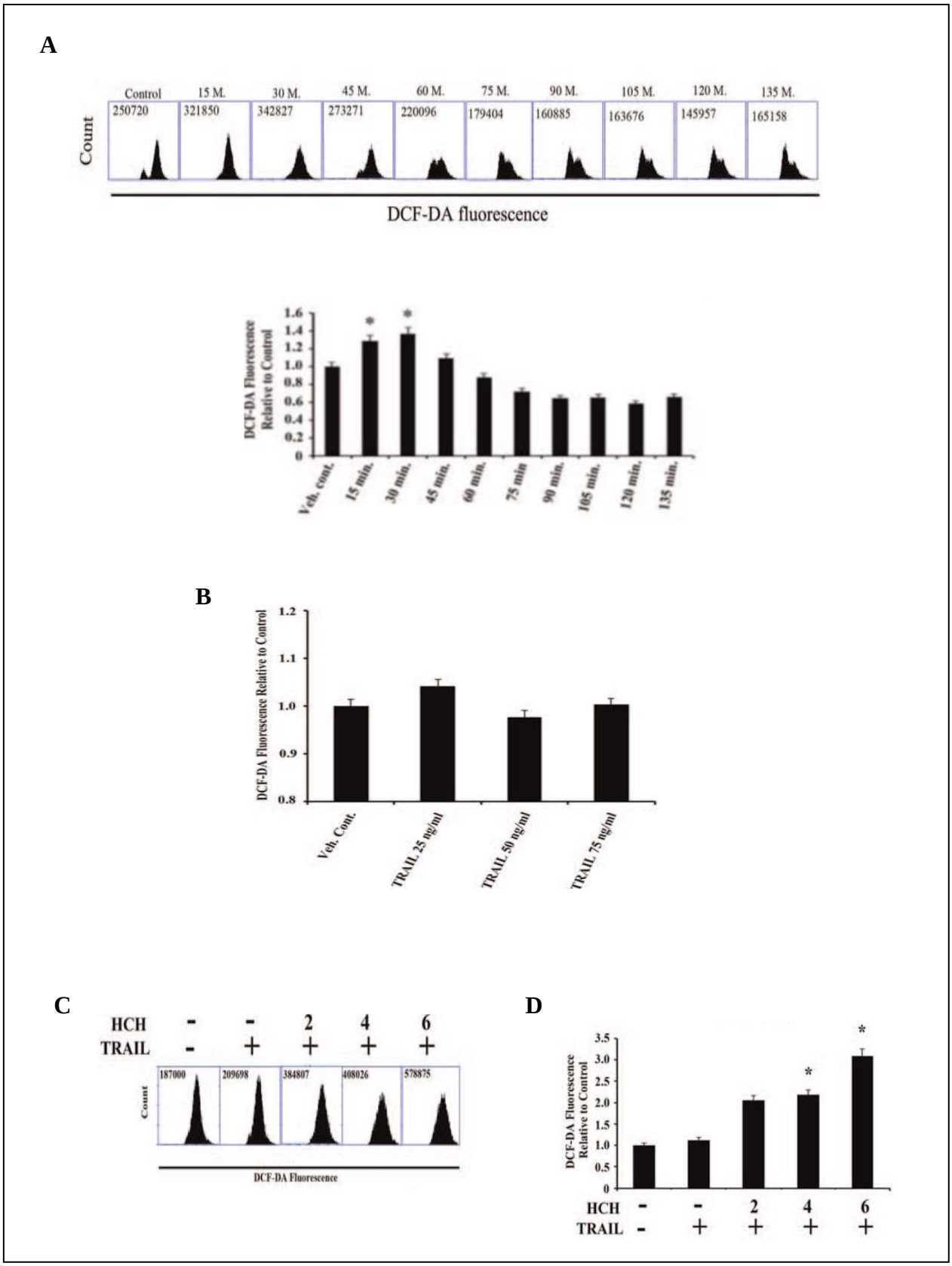
Figure 9

Fig 9: Hydroxychavicol (HCH)-mediated TRAIL sensitization of K562(R) cell is ROS dependent: K562(R) cells were treated with HCH and TRAIL in presence and absence of 1 mmol/l NAC for 24 h and Annexin V/PI binding assay was performed in flowcytometer. Results were representative of three independent experiments.

From above experiment it is concluded that ROS played major role in Hydroxychavicol mediated TRAIL induced apoptosis.

Hydroxychavicol only increased intracellular ROS level in Imatinib resistant K562 cells:

Next, we wanted to check whether Hydroxychavicol increases intracellular ROS directly. So, K562(R) cellswere incubated with 6 μM of Hydroxychavicol for various time interval. ROS generation was measured with DCFDA by flowcytometer. This data indicated that ROS level started to increase from 15 min and reached at its peak at around 30 min [Fig 10A]. Next, we checked whether TRAIL has any role to play in the generation of ROS which might have additive effect in apoptosis. If that so, then increasing TRAIL concentration, either alone or in combination with different doses of Hydroxychavicol, would result in increased amount of ROS. So, K562(R) cells were preincubated with TRAIL at different concentration and then ROS was measured by DCFDA fluorescence in flowcytometer. Results indicated that TRAIL did not change ROS level when incubated at various concentration alone [Fig 10B]. On the other side, ROS was measured at various concentrations of HCH, keeping TRAIL concentration fixed, ROS level was increased dose dependently [Fig 10c].


A
Control
15 M.
30 M.
45 M.
60 M.
75 M.
90 M.
105 M.
120 M.
135 M.
250720
321850
342827
273271
220096
179404
160885
163676
145957
165158
Count
DCF-DA fluorescence
DCF-DA Fluorescence Relative to Control
Veh. cont.
15 min.
30 min.
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60 min.
75 min
90 min.
105 min.
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B
DCF-DA Fluorescence Relative to Control
Veh. Cont.
TRAIL 25 ng/ml
TRAIL 50 ng/ml
TRAIL 75 ng/ml
C
HCH
TRAIL
187000
209698
384807
408026
578875
Count
DCF-DA Fluorescence
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DCF-DA Fluorescence Relative to Control
HCH
TRAIL

Figure 10

Fig 10: Hydroxychavicol induces intracellular ROS level K562(R) cells: **A**) K562(R) cell line was treated with HCH (6 μmol/l), and then cells were stained with DCFDA for measuring ROS level in Flow cytometry. The histograms are representative data of three independent experiments. Below is the bar diagram of mean ± SD of three independent experiments. **B**) K562(R) cell line was treated with various doses of TRAIL and ROS level was measured in flowcytometer after incubation with DCFDA. The bar diagram represents mean ±SD of three independent experiments. **C)** K562(R) cells were incubated with 200 ng/ml of TRAIL, and different doses of Hydroxychavicol, and DCFDA fluorescence was measured in flowcytometer. Bar diagram of mean ±SD of three independent experiments.

From above data, It is concluded that Hydroxychavicol increases intracellular ROS in K562 (R) cells. TRAIL have no any additive effect in the generation of ROS.

Hydroxychavicol downregulates XIAP and FLIP in ROS dependant manner:

It is observed from previous experiment, Hydroxychavicol plays a role in TRAIL sensitization of K562 (R) cells by downregulating XIAP and FLIP. So, we wanted to check whether Hydroxychavicol-mediated XIAP and FLIP downregulation is ROS mediated or not. K562(R) cells were preincubated with ROS scavenger, NAC and treated with Hydroxychavicol. Whole cell lysates were subjected to western blot analysis. Western blot analysis data indicated that NAC treatment reversed Hydroxychavicol mediated XIAP and FLIP downregulation [Fig11A]. This indicated that ROS was involved in the downregulation of XIAP and FLIP. If ROS played role in the downregulation of XIAP and FLIP, then adding ROS externally should show similar effect. When, K562(R) cells were incubated with various doses of H_2O_2, western blot analysis showed that H_2O_2 dose dependently decreased both XIAP and FLIP [Fig 11 B].

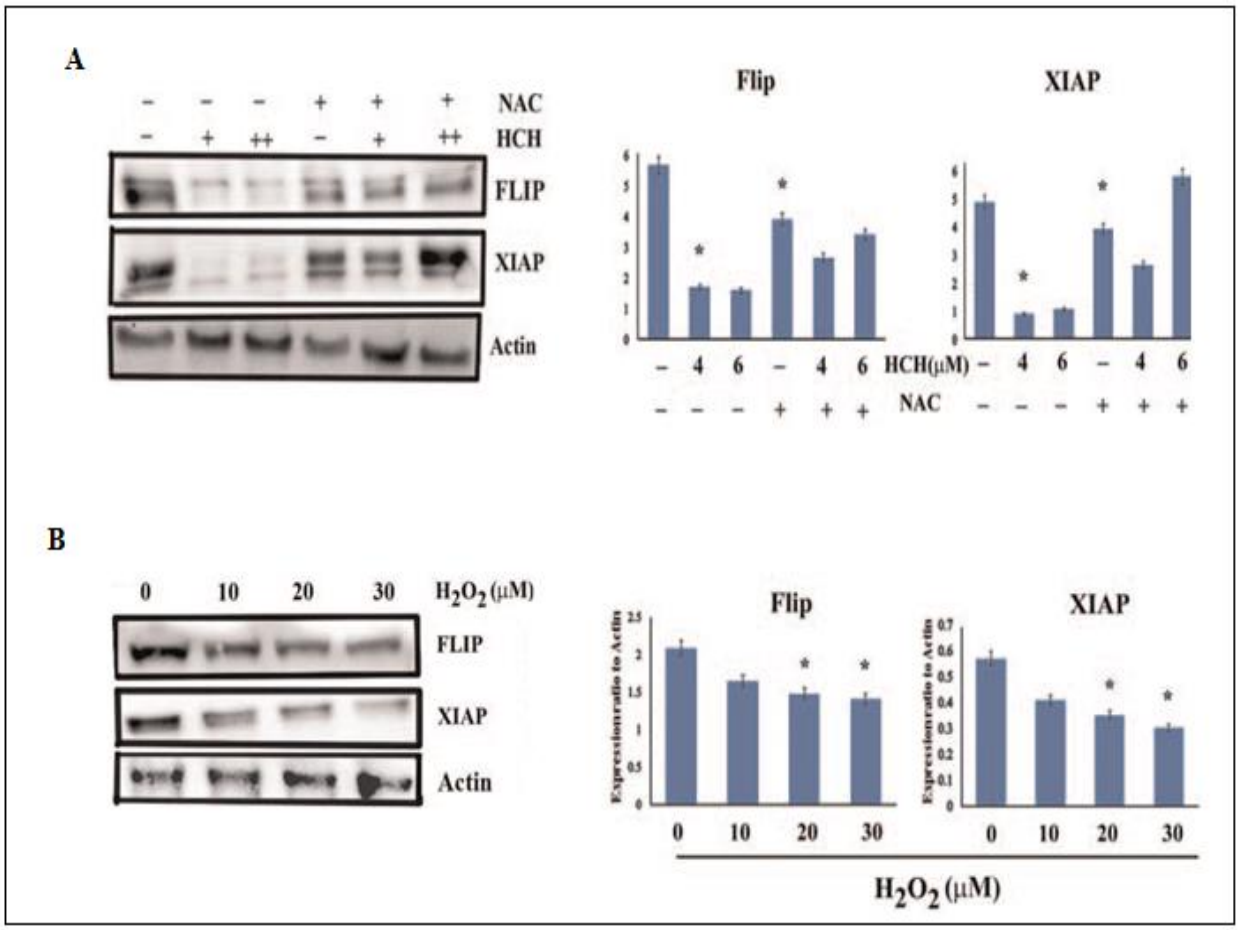

Figure 11

Figure 11: ROS plays a key role in HCH-mediated XIAP and FLIP downregulation (a) K562(R) cells were treated with HCH either in presence or in absence of NAC, and Western blot analysis was done with the whole cell lysates to measure FLIP and XIAP protein level. Data were also presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments (*P<0.05). (b) FLIP and XIAP protein level were checked by Western blot in the indicated doses of H_2O_2-treated K562 (R) cells after 16 h of incubation. Data were also presented graphically as ratio to actin after densitometry analysis and indicate mean ±SD of three independent experiments (*P<0.05).

From above data, it is concluded that Hydroxychavicol downregulates XIAP and FLIP in ROS dependant manner.

ROS is involved in Hydroxychavicol mediated TRAIL induced apoptosis

Till now, we observed that downregulation of XIAP and FLIP is ROS dependant manner. FLIP and XIAP downregulation play role in TRAIL sensitization of K562(R) cells. So, if ROS is responsible for FLIP and XIAP downregulation, then ROS itself should sensitize imatinib resistant K562 (R) cells to TRAIL mediated apoptosis. Now, TRAIL was applied on H_2O_2 pre-treated K562(R) cells and analyzed in flowcytometer for determination of apoptosis. Annexin V/PI binding assay indicated that apoptosis is enhanced by TRAIL in H_2O_2-treated cells [Fig 12a]. Involvement of ROS in Hydroxychavicol mediated TRAIL sensitization was also confirmed when caspases-8 and 3 cleavage was observed. The western blot data indicated that caspases-8 and 3 cleavage was significantly reversed in the presence of ROS scavenger NAC [Fig12b].

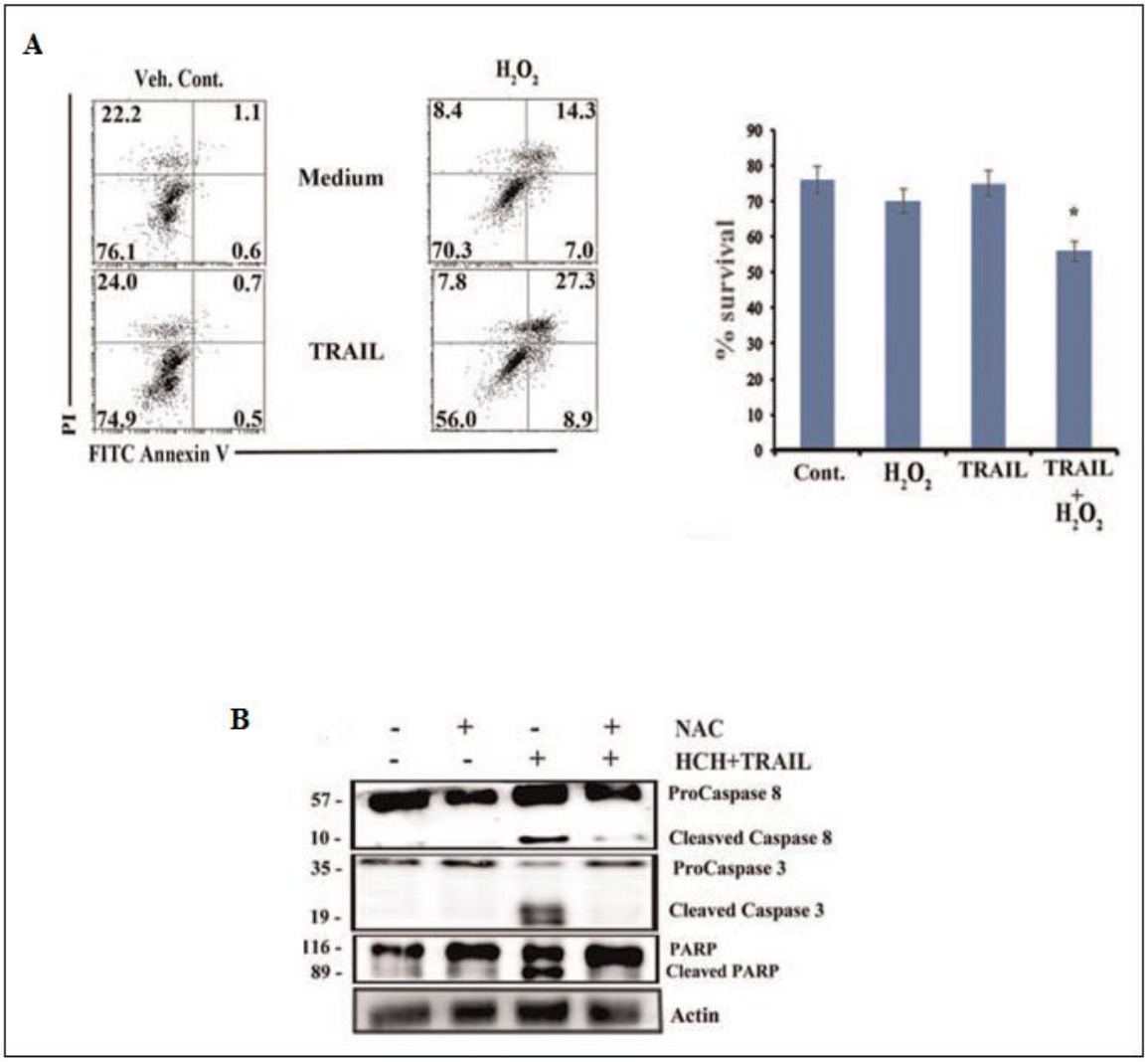

Figure 12

Figure 12: ROS is involved in Hydroxychavicol mediated TRAIL induced apoptosis. **A)** K562 (R) cells were treated with $H_2O_2$and TRAIL and apoptosis assay was performed by annexin V/PI binding assay. Data are a representative dot plot of three independent experiments. The data are also represented as Bar graph which denotes mean ±SD of viable cells [100– (upper left upper right lower right quadrant percent)] from three independent experiments (*P< 0.05). **B)** K562(R) cells were treated with HCH either in presence or in absence of NAC, and Western blot analysis was performed with the whole cell lysates for pro caspase-8, 3, and PARP. Data are a representative blot of three independent experiment.

So, it is concluded that Hydroxychavicol induces intracellular ROS. This ROS decreases XIAP and FLIP. Thereby TRAIL mediated extrinsic apoptosis is occurred.

Discussion:

Imatinib mesylate is widely accepted drug for the treatment of patients with CML. However, development of resistance to imatinib is major drawback for Gleevec treatment. So, any alternative treatment strategy might prove effective treatment choice for imatinib-resistant patients with CML.TRAIL could be one of the alternative therapeutic choices for the treatment of patients with CML [160,161]. TRAIL is Tumour Necrosis Factor Related Apoptosis Inducing Ligand. TRAIL has been considered promising anticancer drug because it has ability to induce apoptosis specifically in malignant cells. But TRAIL has no cytotoxicity toward normal cells [162]. However, some cancer cells show resistant to TRAIL-mediated apoptosis. Chronic Myeloid Leukaemia K562 is one of them. Combination therapeutic approach involving TRAIL, along with any agents that may sensitize CML cells to TRAIL mediated apoptosis, could be an attractive concept in the treatment of patients with CML. It was reported that Hydroxychavicol has promising cytotoxicity toward leukemic cells In this present study, we wanted to explore whether Hydroxychavicol is able to enhance the cytotoxic activity of TRAIL to imatinib-resistant CML cells. Major findings from this study are followings:

TRAIL treatment alone does not induce apoptosis in imatinib resistant K562 cells. But combinatorial treatment of TRAIL and Hydroxychavicol (HCH) induce imatinib resistant K562 cells to death receptor mediated apoptosis. This combinatory treatment induces cleavage of procaspase 8. So, it suggested that extrinsic pathway is involved in Hydroxychavicol-mediated TRAIL-induced apoptosis. Although TRAIL was involved death receptor-mediated extrinsic apoptotic pathway but hydroxychavicol did not upregulates Death receptor 4 and 5 expression in imatinib resistant K562 cells. Since IAPs inhibit apoptosis by blocking cleavage of caspases, we checked whether hydroxychavicol targeted IAPs. Hydroxychavicol targets and downregulates XIAP and FLIP antiapoptotic proteins at protein level not m RNA level in imatinib resistant K562 cells. cIAP, survivin, and Bcl2 level remain unaltered after Hydroxychavicol. Interestingly cIAP, survivin, and XIAP belong to the same IAP family of proteins but they are regulated and function differentially because of some structural difference. For example, both cIAP and XIAP contain BIR domains. The BIR domains 2 and 3 of the cIAPs are able to bind caspases-7 and 9. However, neither of these BIR domains is able to inhibit caspases because of critical substitutions in the regions that target

caspase inhibition in the XIAP, a tight binding caspase inhibitor. We also observed that Bax and tBid level increased at the highest dose of Hydroxychavicol which might be because of increased level of cleaved caspase-8 that generates truncated Bid.

Hydroxychavicol downregulated XIAP and FLIP via proteosomal mediated degradation pathway. Knocking down FLIP and XIAP, either alone or in combination, by FLIP and XIAP siRNA significantly enhanced TRAIL mediated apoptosis compared with the control siRNA. So, it is concluded that XIAP and FLIP play inhibitory role in apoptosis. In this study, we have observed that Hydroxychavicol increases intracellular ROS level in imatinib resistant cells. This intracellular ROS downregulates XIAP and FLIP thereby enhancement of apoptosis by TRAIL.

So, from objective I, it is confirmed that IAPs can targeted by hydroxychavicol in ROS specially H_2O_2 dependant manner to sensitize imatinib resistant K562 cells to TRAIL mediates apoptosis.

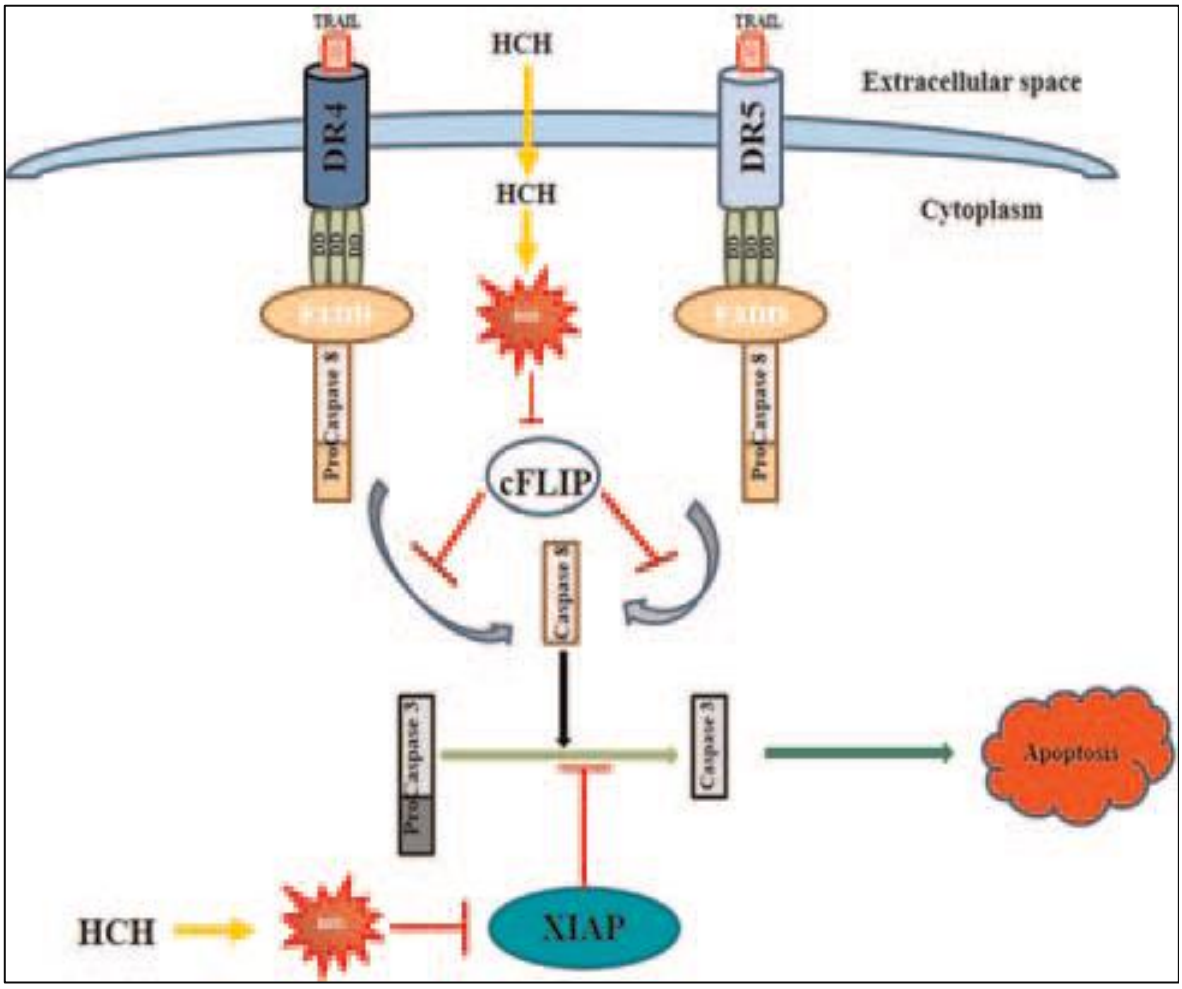

Figure 13

Fig 13: Pictorial representation of the mechanism of action of Hydroxychavicol (HCH) in thesensitization of TRAIL in K562(R) cells. Hydroxychavicol induces intracellular ROS. This ROS decrease XIAP and FLIP. Thereby TRAIL mediated extrinsic apoptosis is occurred.