Discussion:

Over the last decade, great deal of attention was directed at elucidating role of apoptosis regulators in governing survival decisions in cancer cells, particularly those of hematopoietic origin. Apoptosis regulators can be classified into two broad categories: those that regulate the activation of caspases responsible for activation and execution of the apoptotic cascade and those that modulate mitochondrial function [182]. Certain proteins (e.g., BCL-2, BCL-xL) preserve mitochondrial integrity by preventing the loss of mitochondrial membrane potential and release of pro-apoptotic proteins such as cytochrome C into the cytosol. Other proapoptotic proteins (e.g., BAX, BAK, BIM) promote to release of cytochrome C. These proteins are involved in regulation of the intrinsic, mitochondrial apoptotic pathway [183]. Other types of proteins the inhibitors of apoptosis proteins (e.g., XIAP) or FLIP block the activation of caspases, those involved in the receptor-related, extrinsic apoptotic pathway. They are frequently overexpressed in solid and haematological malignancies [184]. If these inhibitors of apoptotic proteins are targeted, that could lead for the development of novel antileukemic strategies.

So, in this study, we focused that FLIP and XIAP, inhibitor of apoptotic proteins are targeted for induction of apoptosis in imatinib resistant K562 cells.

Objective I: Identification of Inhibitor of apoptotic proteins as a target of induction of apoptosis in imatinib resistant CML cells:

In previous study, it was observed that imatinib resistant K562 cells show resistant to TRAIL induced death receptor mediated apoptosis. So, combinatory therapeutical approaches involving TRAIL along with anyagent that induces Imatinib resistant K562 cells to TRAIL induced Death receptor mediated apoptosis that could be an interesting concept in treatment of imatinib resistant CML patients.In another study, it was observed that Hydroxychavicol (a phenolic compound of Piper betel leaves) induces apoptosis of CML cells [185].In objective I, we tried to sensitize imatinib resistant K562 cell to TRAIL mediated apoptosis by using Hydroxychavicol through inhibition IAPs proteins.

In this objective I, we explore that Hydroxychavicol can sensitize imatinib resistant CML cells to TRAIL-induced cell death. It was supported by MTT assay and Annexin V and PI binding assay. Cell death is apoptotic in nature as Hydroxychavicol and TRAIL both treatments induce caspase 3, caspase 8 and PARP cleavage. Moreover, this Hydroxychavicol and TRAIL-mediated Apoptosis involved extrinsic apoptosis pathway because caspase-8 cleavage increased in the combinatorial treatment. Whereas caspase-9 cleavage did not happen at all.

DR5 antagonizing antibody significantly reversed apoptosis induced by TRAIL and Hydroxychavicol. It is known that TRAIL is involved death receptor-mediated extrinsic apoptotic pathway. We investigated whether Hydroxychavicol increases DR4 and DR5 expression. We observed that Hydroxychavicol did not upregulate DR4 and DR5 in imatinib-resistant K562 (R) cells in either dose-dependent or time-dependent manner. Decoy receptors (DcR1 and DcR2) are important in TRAIL mediated apoptosis, as they bind with TRAIL and thus decreases the free TRAIL available to bind with DR4 and DR5. Hydrochavicol did not alter the expression of DcR1 and DcR2 at protein level or mRNA. Our data also indicated that extrinsic apoptotic pathway is involved behind Hydroxychavicol-mediated TRAIL sensitization. Upregulation of death receptor expression was not reason behind enhancement of TRAIL-mediated apoptosis by Hydroxychavicol. When apoptotic inducing ligands bind with death receptor, extrinsic apoptotic pathway is activated. For example, TRAIL binds with death receptor 5 and death receptor 4, which further recruit the adaptor protein FADD (Fas-associated protein with death domain) and caspase8 binds to FADD and subsequent caspase-8 is activated. Thus, extrinsic apoptosis pathway may be regulated by inhibiting caspase cleavage at various level. IAP family member protein inhibits caspase cleavage and inhibits apoptosis. IAP family members XIAP and cIAP inhibit caspase-3 cleavage and extrinsic apoptotic pathway. Antiapoptotic protein FLIP blocks caspase-8 cleavage. Therefore, we wanted to check these protein levels. We investigated that Hydroxychavicol decreased XIAP and FLIP level at dose dependant manner. cIAP, survivin, and Bcl2 level remain unaltered after Hydroxychavicol treatment.

cIAP, survivin, and XIAP belong to the same IAP family of proteins but they are regulated and function differentially because of some structural difference. We also investigated that Bax and tBid level increased at highest dose of Hydroxychavicol. Bax and tBid level are increased due to increased level of cleaved caspase-8. Hydroxychavicol did not decrease XIAP and FLIP at mRNA level. Hydroxychavicol mediated XIAP and FLIP downregulation is proteosome dependant because proteasomal inhibitor MG132 significantly reverse Hydroxychavicol mediated XIAP and FLIP downregulation. The down-regulation of FLIP and XIAP was responsible for TRAIL-mediated apoptosis. Because knocking down XIAP and FLIP by siRNA similarly increased TRAIL-induced apoptosis.

In this study, we investigated that ROS was induced by Hydroxychavicol but not TRAIL. This ROS played role in the enhancement of apoptosis by TRAIL. Because ROS scavenger NAC reverses Hydroxychavicol and TRAIL induced apoptosis. Till now, it is confirmed that Hydroxychavicol induced TRAIL sensitization is

mediated by increased ROS and decreased levels of FLIP and XIAP. ROS is involved in Hydroxychavicol mediated XIAP and FLIP level downregulation as NAC significantly reversed Hydroxychavicol-mediated FLIP and XIAP downregulation. When exogenous ROS is applied on K562(R) cells, it decreased FLIP and XIAP levels. It confirmed that ROS act as a major player in the downregulation of FLIP and XIAP. The involvement of ROS in Hydroxychavicol-mediated TRAIL-induced apoptosis is confirmed by TRAIL-mediated caspases-3, 8 and PARP cleavage. Because NAC significantly reverses Hydroxychavicol mediated caspase 3, 8 and PARP cleavage. All results indicated that Hydroxychavicol sensitizes imatinib resistant K562 cells to TRAIL-induced apoptosis through downregulation of XIAP and FLIP by ROS generation. This could be an alternative therapeutic approach to kill imatinib-resistant CML cells.

From objective I, it is confirmed that IAPs can be targeted by Hydroxychavicol thereby TRAIL mediated apoptosis can be induced in imatinib resistant CML cell

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

Targeting inhibitor of apoptotic proteins (IAPs) XIAP and FLIP is very crucial for the treatment of cancer. Recently, in objective I, we have reported that antiapoptotic protein, FLIP and XIAP could be targeted by Piper betel leaf derived compound Hydroxychavicol improved the efficacy of TRAIL to Imatinib resistant Chronic Myeloid Leukemia cell line K562. Hydroxychavicol induced intracellular ROS which is responsible for XIAP and FLIP downregulation. In objective II, we have used external H_2O_2 as ROS for entire work. In this objective, we have mainly focused on the mechanism by which ROS downregulates XIAP and FLIP in imatinib resistant K562 cells. Major findings are followings:

H_2O_2 downregulates FLIP and XIAP at dose and time dependant manner. H_2O_2 downregulates XIAP and FLIP at protein level not RNA level. Cell death was not the reason behind this downregulation as cells remained perfectly viable at the doses of H_2O_2 which decreased FLIP and XIAP in imatinib resistant K562cells. Previous study mentioned that proteins are regulated by post translational modifications of either lysosomal or proteosomal degradation pathways. We have observed that proteasome inhibitor MG132 but not autophagosome lysosome fusion inhibitor Chloroquine reversed H_2O_2 mediated FLIP and XIAP degradation. So, it is concluded that ROS downregulates XIAP and FLIP in proteosomal mediated pathway.

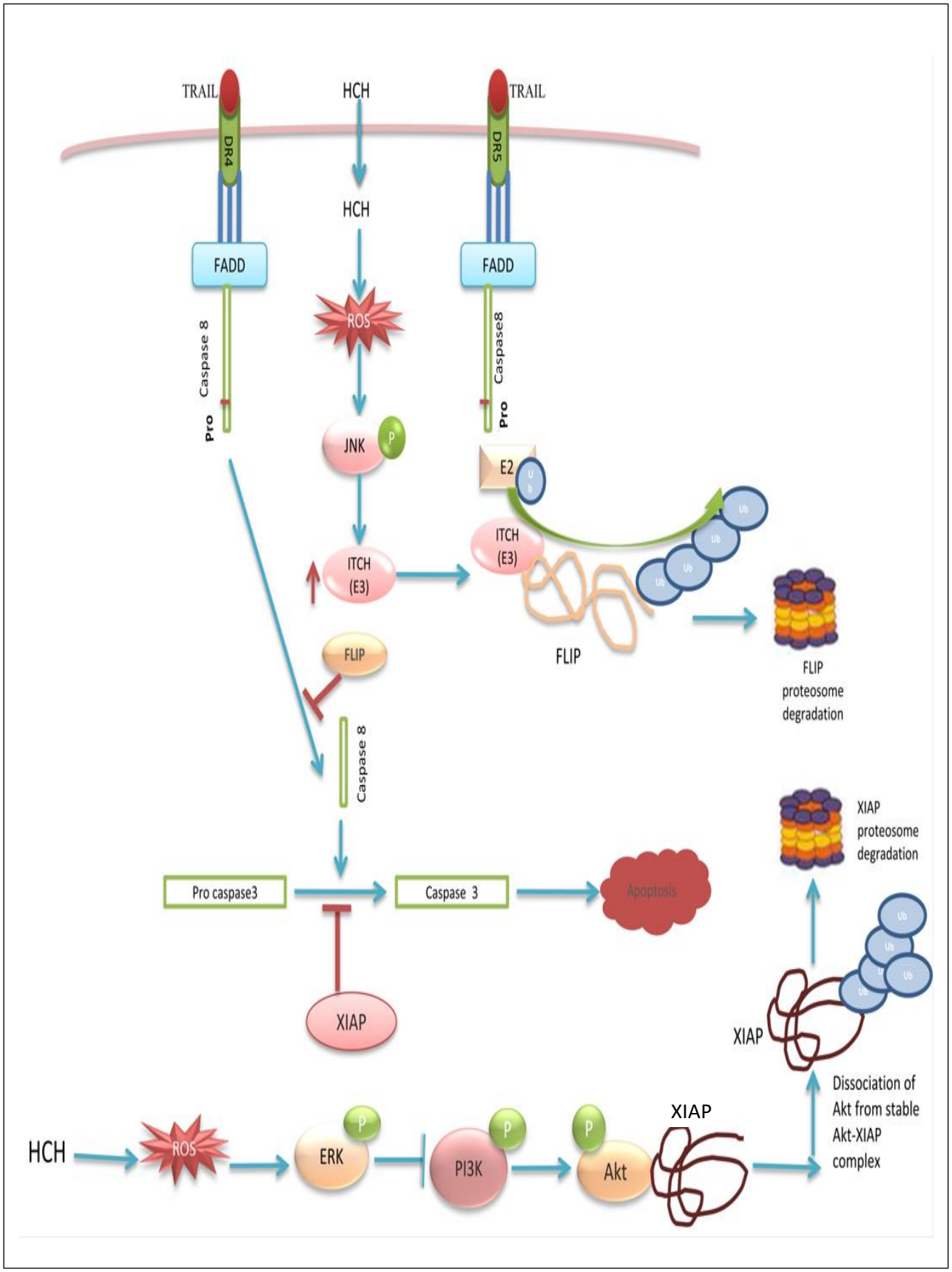
Now, we have found out that the specific ROS signaling that was involved ROS mediated XIAP and FLIP downregulation. MAPK pathway is downstream signaling pathway of ROS.ROS oxidizes Trx to dissociate from ASK-1 for its activation, resulting in the activation of JNK and p38 pathways [186]. ROS mediated XIAP and FLIP downregulationis dependent on ERK and JNK respectively as JNK inhibitor SP600125 reversed FLIP downregulation and ERK inhibitor PD98059 reversed XIAP downregulation. Although ROS also has been shown to activate p38 by others, our data didn't support its involvement in ROS mediated XIAP or FLIP regulation. Perturbation of cellular JNK level by either knockdown or overexpression changed the FLIP level but didn't show any effect on XIAP. On the other hand, alteration in the ERK at protein level by either overexpression or knockdown altered the ROS dependent downregulation of XIAP but not FLIP. We also observed that ROS increased JNK and ERK phosphorylation which preceded FLIP and XIAP degradation in their time kinetics corroborating our hypothesis that FLIP and XIAP are regulated by JNK and ERK respectively.

So, it is concluded that ERK signaling pathway is involved in ROS mediated XIAP degradation not FLIP. JNK signaling pathway is involved in ROS mediated FLIP degradation not XIAP. Interestingly, we have found that XIAP and FLIP degradation by ERK and JNK are mutually exclusive events, though both are regulated by ROS.

Now, we wanted to check the mechanism which is involved in ROS mediated activated ERK involved in XIAP downregulation. Several literatures reported that XIAP is an ubiquitin ligase with a RING domain which can modulate its own ubiquitination and degradation and Akt binds with XIAP in a phosphorylation dependent manner and give XIAP its stability by shedding the RING domain [187]. Coimmunoprecipitation data indicated that H_2O_2 markedly reduced association of Akt with XIAP. H_2O_2 markedly decreased Akt phosphorylation and also decreases PI3K phosphorylation which might be resulted in decreased Akt phosphorylation. Thereby, it was observed that reduced association between Akt and XIAP because of decrease in the phosphorylation of Akt by H_2O_2. If H_2O_2 mediated PI3K phosphorylation inhibition played a role in XIAP downregulation, then PI3K inhibition alone without the presence of H_2O_2 should have similar effects on XIAP. We found exactly the similar results when PI3K inhibitor wortmanin dose dependently decreased XIAP level not FLIP level. Wortmannin enhanced the effect of H_2O_2 mediated decrease in XIAP level in cells which further supported our hypothesis. ERK inhibitor PD98059 reversed the H_2O_2 mediated decrease in Akt phosphorylation. So, it is concluded that ROS decreased the association between Akt and XIAP by decreasing Akt phosphorylation and thus increased the auto-ubiquitination of XIAP.

On the other side, ITCH, E3 ubiquitin ligase is involved in ubiquitination and degradation of FLIP by JNK [188]. Now, we wanted to check JNK has role in FLIP degradation in imatinib resistant K562 cells. Coimmunoprecipitation data indicated that ITCH might bind to FLIP in presence of H_2O_2.Furthermore, for first time, we have observed that ROS dose dependently increased ITCH at protein level and also RNA level. Previous data indicated that JNK activation decreased FLIP. Coimmunoprecipitation data indicated that reduced association between JNK and FLIP in presence of JNK inhibitor SP. It was due to JNK inhibitor, SP decreased cellular level of ITCH by inhibiting ITCH expression. So, it is confirmed that H_2O_2 increased expression of ITCH via JNK activation andthis ITCH, as ubiquitin ligase, decreased FLIP level via ubiquitin proteasome pathway.

From objective II, it is concluded that 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 degradesit by ubiquitin-proteasome pathway.


TRAIL
HCH
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DR4
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ROS
Caspase 8
Pro
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Caspase 8
XIAP
proteosome
degradation
Pro caspase3
Caspase 3
Apoptosis
XIAP
XIAP
HCH
ROS
ERK
P
PI3K
P
Akt
P
XIAP
Dissociation of
Akt from stable
Akt-XIAP
complex

Figure 1

Figure 1: Apoptosis regulators (antiapoptotic protein XIAP, FLIP) are targeted by Hydroxychavicol (HCH) via intracellular ROS generation to induce apoptosis in imatinib resistant CML cell: In picture, HCH induces ROS inside cells specially H_2O_2.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 XIAP 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. In this way, ROS downregulates XIAP and FLIP. So, caspase8 and caspase3 cleavage is possible. TRAIL mediated apoptosis is occurred. In this signaling pathway, HCH sensitize imatinib-resistant CML cells to TRAIL-induced apoptosis through downregulation of XIAP and FLIP via ROS generation.

Objective III: To check the key findings in imatinib resistant cells from objective I and objective II in imatinib sensitive cells.

We have found key findings in K562(S) cells from objective I, II in K562(R) cells in followings

In imatinib sensitive and Imatinib resistant K562 both cell lines, Hydroxychavicol sensitizes CML cells to TRAIL mediated apoptosis. Because K562(S) and K562(R) cells shows TRAIL resistant. In both cell lines, Hydroxychavicol-mediated TRAIL sensitization is ROS dependant because ROS scavenger NAC significantly reverses Hydroxychavicol and TRAIL mediated apoptosis. In previous objective, we have observed that ROS mediated activated ERK/JNK signaling pathway involved in antiapoptotic protein FLIP, XIAP downregulation in K562(R). We also have known that XIAP and FLIP have been responsible for TRAIL resistance. So, we have targeted antiapoptotic proteins XIAP and FLIP. Hydroxychavicol downregulated XIAP and FLIP in K562 (S) cells that was similar in case of K562(R) cells. In previous study, we knew that Hydroxychavicol induces intracellular ROS in CML cells. In this study, Hydroxychavicol degrades XIAP and FLIP in ROS dependant manner. Because externally ROS treatment degrades XIAP and FLIP in imatinib sensitive and imatinib resistant K562 cells. In imatinib sensitive K562 cells, ROS degrades XIAP and FLIP in lysosomal dependant pathway. In imatinib resistant K562 cells, ROS degrades XIAP and FLIP in proteosomal dependant pathway. This is main difference between K562(S) and K562(R) in ROS mediated FLIP and XIAP downregulation.

Otherside it was also observed that JNK signaling pathway is involved in Hydrochavicol and TRAIL mediated apoptosis because JNK inhibitor, SP reverses Hydroxychavicol and TRAIL mediated apoptosis in imatinib sensitive K562 (S) cells. ERK signaling pathway has inhibitory role in Hydroxychavicol mediated TRAIL sensitization because ERK inhibitor PD do not reverse in Hydroxychavicol mediated TRAIL induced apoptosis in imatinib sensitive K562(S) cells.

Conclusion:

From thesis, it is concluded that XIAP and FLIP could be targeted to induce apoptosis in Imatinib sensitive and Imatinib resistant CML cell by TRAIL. Hydroxychavicol downregulates XIAP and FLIP in a ROS dependent manner.

In Imatinib resistant CML cell, ROS decreases anti-apoptotic protein XIAP and FLIP by activation of ubiquitin proteasomal pathway. However, It is the Lysosomal degradation pathway that plays key role in ROS-dependent XIAP and FLIP downregulation. 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. Thus, we have, for the first time, shown that ERK and PI3K/Akt pathways crosstalk in the regulation of XIAP which could be further another target for CML therapy. We have also shown for the first time that ROS increases expression of ITCH which, by its association with FLIP, degrades it via proteasome pathway. So, In Imatinib resistant CML cell, two component of MAPK pathway i.e JNK and ERK had stimulatory role in ROS mediated FLIP and XIAP proteosomal mediated downregulation therby stimulates TRAIL mediated apoptosis. However, ROS activated JNK signaling pathway has a role in TRAIL mediated apoptosis of Imatinib-sensitive CML cells although ERK signaling pathway has been observed to have no role in TRAIL mediated apoptosis in Imatinib-sensitive CML cells. This finding may be further investigated for a specific targeted therapy for Imatinib-resistant CML cells.

Therapeutic implications of this study:

Resistance to chemotherapy, antibody-based therapy or TRAIL therapy has been fast emerging in various types of cancer including CML where imatinib (1st generation TKI against Bcr-Abl kinase) resistance is extremely prevalent. Inhibitor of apoptotic proteins like XIAP and FLIP have been shown to play a role in promoting tumorigenesis in ovarian and breast cancer [189]. In the study, shRNA-mediated knockdown of XIAP even sensitized the tumor cells to cisplatin therapy [190]. Most of the anti-cancer drugs ultimately try to upregulate apoptosis in cancer cells and hence modulating IAPs in combination with existing anti-cancer drugs like cisplatin or imatinib or pemetrexed could prove to be fruitful in bypassing the drug resistance. Modulating IAPs is also advantageous as it overcomes the need to target upstream signaling molecules of the apoptotic pathways that might have a role in other important signaling cascades.

Main therapeutic implication from thesis is that downregulation of XIAP and FLIP leads to TRAIL induced apoptosis in imatinib resistant K562 cells. We also have targeted PI3K/Akt for antileukemic treatment in imatinib resistant K562 cells. For first time, our data indicated that ROS downregulates FLIP and XIAP (IAPs) via ITCH and ERK in K562-imatinib resistant cells. Hence, using these insights, XIAP and FLIP can be targeted as a monotherapy or in combination with other therapies to sensitize CML and other related leukemia patients.

Future Direction:

We studied that ROS regulates MAPK kinase signaling pathway in Imatinib sensitive and resistant K562 cells. We studied that the mechanisms by which ROS mediated MAPK kinase signaling pathway regulate antiapoptotic proteins FLIP, XIAP expression. In future, we will find out several signaling pathways other than MAPK pathway that will be regulated by ROS. Then we will study the mechanisms by which these ROS mediated signalling pathways will regulate several proapoptotic proteins and antiapoptotic proteins. We will study the mechanism of differential control of XIAP and FLIP by ROS between Imatinib resistant and sensitive CML cells.