SUMMARY AND CONCLUSION

Cancer cases throughout the globe are on the rise and are estimated to reach 25 million in next two decades. Cancer is now the major cause of mortality with 8.2 million deaths worldwide (Cancer Facts and Figure, 2014). Since each cancer has a different etiology, signalling profile and mutational background, therefore a single drug cannot be used to treat all the cancers. Anticancer agents from natural sources have been considered as an effective alternative for the treatment of cancer. Majority of the drugs currently used for the treatment of cancer are derived natural sources (Bhanot et al., 2011). Gambogic acid (GA) is a recently discovered natural anticancer agent derived from *Garcinia hanubari*. GA has been shown effective against glioblastoma, leukemia, breast cancer, gastric cancer etc (Wang et al., 2002; Felth et al., 2013; Wang et al., 2015). Interestingly, GA exerts its cytotoxic potential by diverse mechanisms in different cancer types like by inhibition of Hsp90, proteasomal function, NF-κB, and antagonizing Bcl-2 family members. GA has been also shown to induce apoptosis by generation of reactive oxygen species (ROS). Most of the anticancer agents initiate apoptotic cell death by generation of ROS. ROS are now considered to function as second messengers and play vital role in cancer initiation and progression (Sen and Packer, 1996 and Droge, 2002). Both ROS inducers such as curcumin, gambogic acid (Khan et al., 2012; Peng and Gandhi, 2012) as well as ROS scavengers e.g. NAC (Halasi et al., 2013) have been shown to kill cancerous cells. Alterations in ROS, below or above a certain threshold induce apoptosis in several types of cancerous cells. ROS have been shown to induce apoptosis by activating caspase-3 (Higuchi et al., 1998), induce mitochondrial outer membrane permeabilization etc. (Maity et al., 2011). Therefore, ROS act as a double edged swords as they positively contribute towards the cancer initiation and progression and at the same time alterations of ROS below or above a specific threshold results in cancer cell death. ROS have been proposed to have important role in therapeutic control of bladder cancer (Wang and Choudhary, 2011). We also observed that GA leads to the ROS generation in bladder cancer cells.

Scavenging of GA induced ROS by NAC completely inhibited the cytotoxic effects of GA, suggesting that ROS are the key factors involved in GA induced cell death.

GA induced cell death was also blocked by a pan caspase inhibitor (z-VAD-fmk), indicating GA induces caspase dependent cell death in bladder cancer cells. Caspases involved in activation of both intrinsic and extrinsic apoptotic pathway were activated by GA. NAC inhibited the GA induced activation of both initiator and executioner caspases, suggesting that GA induces cell death by ROS mediated caspase activation. GA also resulted in truncation of Bid, Bax migration, mitochondrial membrane hyper-polarization, and cytochrome c release in bladder cancer cells. All these GA induced molecular events are regulated by ROS as NAC effectively blocks all these events. Interestingly, we observed that z-VAD-fmk is not able to prevent GA induced mitochondrial hyper-polarization and cytochrome c release. Alterations in MMP and cytochrome c are the upstream steps in apoptotic cascade and are followed by activation of caspases. Pan-caspase inhibitor; z-VAD-fmk is a direct inhibitor of caspases; therefore z-VAD-fmk was observed to have no effect on GA induced mitochondrial hyper-polarization and cytochrome c release. Also as reported by Madesh and Hajnoczky, ROS may directly induce cytochrome c release (Madesh and Hajnoczky, 2001) and our results also indicate the same. Caspase mediated truncation of Bid facilitates migration of Bax to mitochondria (Kroemer et al., 2007), therefore, both steps are effectively blocked by z-VAD-fmk.

In our study we found that ROS generated by GA affected multiple survival pathways in bladder cancer cells. GA induces ROS and caspase dependent cleavage of XIAP and also inhibited the expression of anti-apoptotic protein, Bcl-xL. Bladder cancer cells have high expression of Bcl-xL, which makes these cells resistant to drug induced apoptosis (Yoshimine et al., 2013). Molecular chaperones are involved in regulation of various signalling pathways, proper folding and degradation of proteins (Niforou et al., 2014). Molecular chaperones like Hsp90, Hsp70 and GRP-78 are reported to have anti-apoptotic properties (Li and Lee, 2006). GA leads to cleavage of Hsp90 and GRP-78 and this cleavage was induced by ROS mediated caspase activation. In contrast to Hsp90 and

GRP-78, Hsp70 is not cleaved by GA. In fact we observed that GA increases the expression of Hsp70 in bladder cancer cells. The GA induced expression of Hsp70 was mediated via ROS. The results from two caspase cleavage prediction softwares showed that Hsp70 has lesser number of caspase cleavage sites as compared to Hsp90. Therefore, Hsp70 is resistant towards caspase mediated cleavage/degradation. However, Sahara and Yamashima (Sahara and Yamashima, 2010) have shown have shown that Hsp70 is cleaved by calpains and not by caspases.

GA also leads to the inhibition of NF-κB at very early time points with maximum inhibition at 3 h. NAC restored the constitutive activation of NF-κB suggesting that GA mediated NF-κB inhibition is regulated by ROS. However, the two important regulatory proteins of NF-κB signalling axis (Hsp90 and IKK-α/β) are degraded only after activation of caspases (Broemer et al., 2004). Therefore, GA inhibits translocation of NF-κB to nucleus independent of caspase mediated degradation of IKK-α/β and Hsp90 cleavage. IKK-α/β phosphorylates IκB-α, which enhances its degradation via proteasomal pathway and makes NF-κB free to translocate to the nucleus. Palempalli et al 2009 have reported that GA inhibits NF-κB by inhibiting the kinase activity of IKK-β via its covalent modification. We also observed that GA inhibits NF-κB by inhibiting the phosphorylation of IκB-α. However, Phosphorylated form of IκB-α was reverted back by NAC, which would not have been possible if IKK-β was covalently modified by GA. Therefore, our results suggest that GA inhibits NF-κB by inhibiting phosphorylation of IκB-α but not via covalent modification of IKK-β as reported by Palempalli et al., 2009.

Cancer cells are known to employ various strategies for development of resistance against anticancer agents. Autophagy is one of the major cellular stress response up regulated in various cancer cells. In case of bladder cancer, the role of GA induced autophagy has not been studied in detail. Autophagy has been proposed to play a major role in cancer initiation, progression and drug resistance (Hanahan and Weinberg, 2011; Lunt et al., 2011; Sosa et al., 2014). Therefore, studying the role of autophagy in bladder cancer

progression and drug resistance may provide essential clues to develop the better therapeutic strategies against bladder cancer. GA induces ROS in bladder cancer cells and ROS are well known regulators of autophagy in various cancerous cells (Liu and Lenardo, 2007). GA induces autophagy up to 12 h, but at latter time points (18 and 24 h) autophagy was inhibited. GA induced cleavage of Atg4c and degradation of Beclin-1, however, the expression of Atg5 and Atg7 was not affected. Together the cleavage of Atg4c and degradation of Beclin-1 may explain why autophagy is inhibited after 12 of GA treatment. GA induced autophagy and JNK activation was inhibited by NAC suggesting that ROS are the common mediators of JNK activation and autophagy induction. Also autophagy induced by GA was inhibited by JNK inhibitor in bladder cancer cells implying that GA induces autophagy via the ROS mediated JNK activation. GA does not have any effect on the expression of another autophagy marker p62 even at time points when maximum autophagy was observed. NBR1 and p62 are adaptor proteins involved in the selective targeting of ubiquitinated proteins to autophagosomes and we observed that GA leads to the cleavage of both p62 and NBR1. Cleavage of Atg proteins was inhibited by NAC and z-VAD-fmk. Therefore, our results suggest that GA induced cleavage of Beclin-1, Atg4c, p62 and NBR1 was mediated by ROS dependent caspase activation.

Autophagy and apoptosis are intricately linked with each other and together determine the overall fate of the cell (Eisenberg-Lerner et al., 2009). Inhibition of autophagy has been reported to enhance drug induced apoptosis (Ojha et al., 2014), but other reports also suggest that inhibition of autophagy may partially or completely block apoptotic cell death (Loos et al., 2013). Inhibition of GA induced autophagy by Wm and CQ increases apoptosis at earlier time points with lower dose of GA. No such effect on cell death was observed upon inhibition of proteasomal pathway by MG132. Therefore, GA induced autophagy acts as a strong cell survival response in bladder cancer cells. Autophagy is a multifaceted process and has been reported to enhance or inhibit caspase activation depending on the stimulus (Rosenfeldt and Ryan, 2011). Our results clearly showed that inhibition of autophagy by pharmaceutical inhibitors (Wm and CQ) or by siRNA approach

increases activation of caspases at earlier time points. Therefore, GA induced autophagy delays caspase activation in bladder cancer cells while proteasomal pathway has no such effect.

GA mediated caspase activation results in cleavage of two important molecular chaperones Hsp90 and GRP-78 but no cleavage was observed in Hsp70, another chaperone protein. On the contrary, the expression of Hsp70 was increased by GA in bladder cancer cells. Various studies have shown that Hsp70 is involved in the regulation of both apoptosis and necrosis (Basu et al., 2000; Takayama et al., 2003). So, we wanted to study the functional significance of Hsp70 in GA induced apoptosis and autophagy. Inhibition of Hsp70 by Pif-μ resulted in shift of GA induced annexin positive population towards PI positive. However, combined treatment of GA and Pif-μ has no additive or synergetic effect on total cell death. When cells were analyzed under inverted microscope we observed that GA leads to distorted cell shape, de-adherence of cells, with prominent apoptotic bodies. However, with combined treatment of Pif-μ and GA, cells became round but remained adhered to the plate. Pif-μ alone treated cells were not detached from the plate but became round in shape. The plasma membrane of these cells was intact as observed by distinct boundaries. These morphological observations were further validated by transmission electron microscopy. Necrosis is characterized by rupture of plasma membrane, but no such effects (plasma membrane rupture) were observed in cells treated with GA alone, Pif-μ alone or in combined treatment of GA and Pif-μ. Together, these results indicate that although the inhibition of Hsp70 by Pif-μ shifts annexin positive population (induced by GA) towards PI positive (Induced by combined treatment of GA and Pif-μ) but these PI positive cells are not necrotic in nature.

Cell death induced by Pif-μ alone or by combined treatment of GA and Pif-μ was inhibited by NAC, indicating that ROS are important regulators of cell death. However, cell death induced by Pif-μ alone or by combined treatment of GA and Pif-μ was not inhibited by broad range caspase inhibitor. These results indicate that Pif-μ alone in presence of GA induces caspase independent cell death. ROS are important in all forms of

cell death whether it is caspase dependent (in case of GA) or caspase dependent (in case of Pif-μ alone and combined treatment). With the combined treatment of GA and Pif-μ cells are committed to death at earlier time point, which directly correlates with the higher level of ROS generated at early time points by combined treatment of Pif-μ and GA.

Hsp70 is known to play av important role in maintenance of lysosomal membrane integrity (Juhasz et al., 2013). Lysosomes are now regarded as important regulators of apoptosis, necrosis and caspase independent cell death. Lysosomal membrane permeabilization (LMP) is reported to initiate caspase independent cell death. Massive alterations in LMP are reported to induce necrotic cell death (Boya and Kroemer, 2008), while small changes activate apoptosis via cathepsins (Johansson et al., 2010). However, we do not observed any change in LMP in T24 bladder cancer cells after treatment with GA alone, Pif-μ alone or by combined treatment of GA and Pif-μ. These results indicate that changes in LMP are not involved in the cell death induced by Pif-μ alone or in combination with GA.

Inhibition of Hsp70 by Pif-μ inhibited the GA induced cleavage of Hsp90, GRP-78, and XIAP. Interestingly, GA induced PARP cleavage was only partially inhibited by Pif-μ. Pif-μ alone did not induce cleavage in Hsp90 and GRP-78 however; PARP was cleaved by Pif-μ. Pif-μ inhibited the GA induced activation of both caspase-3 and caspase-8 as confirmed by our colorimetric assays. Pif-μ alone was not able to activate the caspase-3 and caspase-8. These results further confirm that cell death induced by Pif-μ alone or in combination with GA is caspase independent. GA induced Hsp90 cleavage was completely blocked by Pif-μ but not Hsp70 siRNA. The reason for this difference may be that Hsp70 siRNA was not able to inhibit the expression of Hsp70 significantly in the presence of GA. This may be explained by the fact that GA increases the expression of Hsp70 so strongly that siRNA is not able to shut it completely.

Pif-μ induced PARP cleavage was inhibited by NAC and z-VAD-fmk, suggesting that PARP cleavage induced by Pif-μ is not mediate by caspase-3 and caspase-8, although

other caspases may be involved. GA induced PARP cleavage was inhibited by Pif-μ at earlier time points (6 and 12 h) but not at 24 h. PARP cleavage induced by combined treatment of GA and Pif-μ was also reverted back by NAC and z-VAD-fmk. However, we could not recognize the specific caspase involved in the cleavage of PARP because all the caspase inhibitors effectively inhibited the cleavage of PARP. This may be because there is strong crosstalk between different caspases during cell death and inhibition of one caspase may drastically regulate the activation or inhibition of other caspases. We also observed accumulation of higher molecular weight form of caspase-3 (hm-caspase-3) when GA mediated caspase-3 activation was inhibited by Pif-μ. A study by Leu et al., 2009 have shown that Pif-μ results in aggregation of proteins like p62. The nature (whether it is aggregated form of caspase-3 or in complex with some other protein) and the functional significance of hm-caspase-3 is not clear at present and further studies will be needed to identify the same. We also observed that GA leads to the expression of another form of apoptotic protease activating factor 1 (Apaf-1) which was inhibited by Pif-μ. Pif-μ alone did not show such effect on Apaf-1. There is not a single report in the literature which has compared the status of Apaf-1 in healthy cells and apoptotic cells. However, there are two reports which have showed that Apaf-1 can be phosphorylated, but the significance of this phosphorylation is not clear (Mishra and Delivoria-Papadopoulos, 2010; Bratton and Salvesen, 2010). Therefore, Apaf-1 during apoptosis may be regulated by posttranslational modifications which may be important during the final stages of apoptosis.

GA inhibits autophagy at latter time points and this autophagy was reverted back by Pif-μ. Pif-μ alone induces autophagy in a time dependent manner. GA activated caspases inhibit autophagy at latter time points but in the presence of Pif-μ, caspases are not activated therefore autophagy is reverted back. GA induced accumulation of ubiquitinated proteins was inhibited by Pif-μ, suggesting that functional activation of autophagy leads to the degradation of these accumulated ubiquitinated proteins. NAC completely inhibited autophagy induced by Pif-μ alone or in combination with GA. Inhibition of GA induced autophagy significantly enhances GA induced caspase-3 and caspase-8 activation,

however no such effect was observed in case of combined treatment of GA and Pif-μ. GA induced decrease in procaspase-3 expression and PARP cleavage was reverted back by Pif-μ even in the presence of CQ at 12 h. Cleavage of GA induced PARP was also not reverted back by Pif-μ at 24 h and presence of autophagy inhibitor has no effect on PARP cleavage. Also the accumulation of hm-caspase-3 was enhanced in the presence of CQ, suggesting that this hm-caspase-3 may be degraded via autophagy.

Taken together, our results suggest that GA initially induces a strong cell survival autophagic response but subsequently as the oxidative stress increases GA leads to the activation of caspases. The activated caspases inhibit autophagy by cleaving/degrading three important Atg proteins (Atg4c, Beclin-1 and p62) and also cleaves other anti-apoptotic proteins like Hsp90, GRP-78 and XIAP. Activation of caspases represents a border line between two separate effects of GA. Before the activation of caspases, GA induces autophagy via ROS mediated JNK activation and also inhibits NF-κB activation by blocking phosphorylation of IκB-α. Once the caspases are activated, all the vital cell survival pathways are inhibited to ensure the effective onset of apoptosis. Interestingly, GA increases expression of Hsp70 which is very critical for the maintenance of caspase activation. Inhibition of Hsp70 in GA treated cells induces caspase independent cell death and also results in shift of annexin positive population towards PI positive, however, these cells are not necrotic in nature. Therefore, increased Hsp70 expression is necessary for effective induction of apoptosis by GA and inhibition of Hsp70 impairs the effectiveness of GA. GA induces apoptosis in primary cultured urothelial carcinoma cells from human tissue samples and importantly did not show any effect on normal urothelial cells. In conclusion, GA is a unique drug, which effectively inhibits the drug resistant autophagy process and induces apoptotic cell death in bladder cancer cell lines as well as in ex vivo cultured patient derived cancer cells. Therefore, we propose that GA has a strong therapeutic potential against bladder cancer and can be explored in animal model system of bladder cancer before going for Phase-I clinical trials.