Chapter-3

Result and Discussion

Part-1

Studies with Piroxicam
Results

3.1. Px causes cell specific and dose dependent cytotoxicity in MCF-7 cells.

To study the effect of Px on cell viability of breast cancer cell lines: MCF-7, MDA-MB-231, T47D and MDA-MB-468, MTT assay was performed. The cells were exposed to 10, 20, 30, 50 and 100 μM of Px for 72 h and processed for the assay. In MCF-7 cells, Px resulted in dose dependent decrease in cell viability ~ 30% at 30 μM and ~ 40 % at higher doses of 50 and 100 μM (Fig. 3.1. A). MDA-MB- 231 cells showed no detectable change in cell viability up to 50 μM Px concentration. At a much higher dose of 100 μM Px, about 30% decrease in viability was observed (Fig. 3.1.B). There was no significant change in viability occurred in T47D and MDA-MB-468 cells till 100 μM of Px exposure (Fig. 3.1. C, D). We chose MCF-7 and MDA-MB-231 cell lines for or subsequent studies to compare the effect of Px exposure on them.

The 30 μM dose was selected for subsequent experiments keeping in mind the peak plasma concentration (C_{max}) of Px which is 2.64 μg/ml which is ~ 24 μM (1). Px was exposed to the breast cancer cells for 6, 12, 24 and 48 h. Px is a long lasting drug and takes 48-72 h to get metabolised completely inside the body. So the maximum time point chosen for our study was 48 h.
Figure 3.1. **Px induces cell specific cytotoxicity in breast cancer cells.** Dose dependent decrease in cell viability of (A) MCF-7, (B) MDA-MB-231, (C) T47D and (D) MDA-MB-468 cells on exposure with Px (10, 20, 30, 50 and 100 μM) for 72 h as assessed by MTT assay (*, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001). Error bars represent mean ± SD of 3 independent experiments of duplicate/triplicate samples (C= control, Px= piroxicam).

3.2. **Px increases the intracellular reactive oxygen species (ROS levels) in MCF-7 cells.**

Fig. 3.2. A and B shows the time dependent effect of 30 μM Px on MCF-7 and MDA-MB-231 cells. MCF-7 cells showed a significant drop (*p*<0.01) in viability at 6 h of exposure which continued at later time points. In contrast no significant change in viability occurred in MDA-MB-231 cells. Px showed a time dependent increase in ROS production in MCF-
Cells showed prominent increase (~ 8 fold) in ROS levels after 3 h of Px exposure. There was a significant and steady surge in the ROS levels in MCF-7 cells at each time point compared to their corresponding control (Vehicle-DMSO treated). The rise in ROS levels in MCF-7 cells reached to ~ 60 folds after 24 h of Px exposure. The confocal imaging of cells also showed increase in ROS levels of cells on 3, 6, 24 and 48 h of Px exposure compared to the control (Fig. 3.2. E). In contrast Px failed to significantly alter the ROS levels in MDA-MB 231 cells till 48 h of exposure (Fig. 3.2. D). Thus cell specific effect of Px could be seen from the above results.
Figure 3.2. Px induces cell death and ROS increase in MCF-7 cells but not in MDA-MB-231cells. (A) & (B) Time dependent effect of Px (30 μM) on cell viability of MCF-7 and MDA-MB-231 cells for 6, 12, 24 and 48 h as assessed by MTT assay (*, p< 0.05; **, p< 0.01). (C) & (D) Time dependent increase in ROS levels of MCF-7 cells and MDA-MB-231 cells on exposure with 30 μM Px for 3, 6, 12, 24 and 48 h. Cells were processed for measuring fluorescent intensity using H2DCFDA dye (***, p< 0.001). Error bars represent mean ± SD of 3 independent experiments of duplicate/triplicate samples. (E) Time dependent increase in ROS levels of MCF-7 cells on exposure with Px for 3, 6, 24 and 48 h as observed by imaging using H2DCFDA dye. Scale bar represents 10μm (C= control, Px= piroxicam).

3.3. Px treatment causes upregulation of Akt phosphorylation in MCF-7 cells.

We studied the effect of Px on Akt as it is one of the key signalling players in many cancers. Effect of Px on Akt phosphorylation levels (Ser 473 and Thr 308) were analysed. The cells were treated with 30 μM Px for different time points 6, 12, 24 and 48 h and the levels of phospho- Akt (Ser 473 and Thr 308) were examined. It was surprising that the levels of phospho-Akt (Ser 473) were enhanced following exposure with Px in MCF-7 cells (Fig. 3.3.A Upper panel). The increase in the levels of pAkt on exposure to Px was observed from as early as 6 h which further increased to ~2 fold in 12 and 24 h exposure (Fig. 3.3.C). Even though the pAkt level decreased slightly on 48 h exposure, it was still higher than that of control. MCF-7 cells also showed steady increase in the levels of pAkt.
(Thr 308) (Fig. 3.3.A Lower panel). The result was unexpected as increased Akt phosphorylation is usually associated with cell survival. Hence Px was causing cytotoxicity and Akt hyperphosphorylation/activation in MCF-7 cells at the same time. Conversely, in MDA-MB-231 cells no increase in Akt phosphorylation (Ser 473 and Thr 308) was detected (Fig. 3.3.B). Instead, a decline in Akt (Ser 473 and Thr 308) phosphorylation was detected starting from 12 h and continuing till 48 h of exposure (Fig. 3.3.D).

**Figure 3.3. Px induces Akt phosphorylation in MCF-7 cells but not in MDA- MB- 231 cells.** (A) & (B) Upper panel: Western blot analysis for the expression of pAkt (Ser 473),
Akt (total) expression; Lower panel: Western blot analysis for pAkt (Thr 308), Akt (total) levels in MCF-7, MDA-MB-231 cells on exposure to Px (30 μM) for 6, 12, 24 and 48 h. (C) & (D) Densitometric analysis of pAkt (Ser 473). Quantified bands were normalized with their respective Akt levels and the fold change was calculated compared to control (*, p< 0.05; ***, p< 0.001). Error bars represent mean ± SD of 3 independent experiments (C= control, Px= piroxicam).

3.4. Px induces apoptosis in MCF-7 cells.

To identify the mode of cell death taking place in MCF-7 cells, the effect of Px on markers of apoptosis was analysed. The cells were treated with Px for different time periods (6, 12, 24 and 48 h) and DNA content of the cells were analysed with the help of propidium iodide (PI) staining. We observed that Px causes a time dependent increase in the sub G0/G1 population of cells compared to control (Fig. 3.4.A). There was a prominent enhancement (~ 3 fold) in the sub G0/G1 population of Px treated cells compared to the control at 12 h of exposure. The cells exposed to 48 h of Px showed ~ 6 fold surge in the sub G0/G1 population (Fig. 3.4.B). Cells were treated with 30 μM of Px for 6, 12, 24 and 48 h and the levels of caspase-3 (cleaved) was analysed using western blot. A time dependent increase in the levels of caspase-3 was observed indicating the induction of apoptosis in cells from 12 h (~ 1.6 fold) of exposure to Px (Fig. 3.4.C). Caspase-3 activity assay was also done with Px treated cells (6, 12, 24 and 48 h). The assay is based on spectrophotometric detection of the chromophore p-nitroaniline (p-NA) after cleavage from the labeled substrate DEVD-p-NA. The fold change in the p-NA light (O.D at 405 nm) was calculated w.r.t control (untreated cells). The assay showed apoptosis induction from 12 h showing ~ 1.7 fold increase in activity which increased to ~ 2.3 fold in 24 h (Fig. 3.4.D). ssDNA levels of the cell on exposure to Px was also observed. The cells were treated with 30 μM of Px for 24 h and the ssDNA levels was determined using the ELISA
based apoptosis detection kit. The increase in ssDNA levels in the cell is a prominent marker for apoptosis. The cells treated with Px showed ~ 2 fold increase in the ssDNA levels compared to control indicating the occurrence of DNA fragmentation on treatment with Px (Fig. 3.4.E).

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)
Figure 3.4. Px induces apoptosis in MCF-7 cells. (A) Representative cell cycle analysis of cells on exposure of cells to Px (30 μM) for 6, 12, 24 and 48 h using PI dye. (B) Significant increase in sub GO/G1 population of cells in Px treated cells compared to control (**, p< 0.01; ***, p< 0.001). (C) Upper panel: Western blot analysis of caspase-3 (cleaved) levels on exposure to Px (30 μM) for 6, 12, 24 and 48 h. Lower panel: Densitometric analysis of the caspase-3 (cleaved) levels. Quantified bands were normalized with their respective β-actin levels and the fold change was calculated compared to control (***, p< 0.001). (D) Determination of caspase-3 activity by quantitative colorimetric assay on Px exposure (***, p< 0.001). (E) Increase in ssDNA levels of cells on exposure to Px at 30 μM or DMSO for 24 h as determined by ELISA based assay (**, p< 0.01). Error bars represent mean ± SD of 3 independent experiments of duplicate/triplicate samples (C= control, Px= piroxicam).

3.5. Px treatment causes disruption of mitochondrial membrane potential.

The early critical event in apoptosis is the loss or disruption of mitochondrial membrane potential (Δψm) in the cells which eventually leads to initiation and activation of apoptosis cascades. We wanted to determine whether Px treatment had any effect on the mitochondrial membrane potential of MCF-7 cells using the JC-1 staining. Fig. 3.5.A shows that exposure of Px causes a time dependent increase in the proportion of green fluorescence positive cells in the lower right quadrant (indicating the loss of mitochondrial
potential) in MCF-7 cells. There was ~ 5 fold increase in the ratio of green and red fluorescent-positive cells on exposure of Px for 6 and 12 h and ~ 15 fold on 24 h exposure (Fig. 3.5.B) compared to control.

Figure 3.5. Px induces Mitochondrial Membrane damage in MCF-7 cells. (A) Increase in the mitochondrial membrane damage in cells on exposure to Px (30 μM) for 6, 12 and 24 h as assessed using JC-1 dye. (B) Significant increase in the ratio of JC-1 Green and JC-1 Red fluorescence (***, p< 0.001). Error bars represent mean ± SD of 3 independent experiments of duplicate samples (C= control, Px= piroxicam).
3.6. Px causes ROS induced Akt activation and apoptosis induction.

As Px causes both ROS generation and apoptosis induction in MCF-7 cells, we used the widely used ROS scavenger/antioxidant N-acetyl cysteine (NAC) to establish whether the apoptosis induction was ROS mediated or not. We used two concentrations of NAC: 5 and 10 mM to observe its effect on the ROS levels induced by Px for different time intervals. Fig. 3.6.A shows that both NAC at 5 and 10 mM concentration resulted in ~2 fold reduction in Px induced ROS levels. Increasing NAC concentration from 5 to 10 mM did not change the amount of reduction in ROS. Possibly, at 5 mM concentration NAC has reached its peak efficacy as an anti-oxidant. 10 mM dose of NAC was selected for subsequent experiments.

To study the effect of ROS inhibition we did viability tests with cells treated with Px alone or in combination with NAC at 5 and 10 mM doses. While Px alone showed ~ 30 % decrease in cell viability, the pre treatment with NAC at 5 mM and 10 mM reduced the decrease to ~ 5 and 15 % respectively (Fig. 3.6.B). NAC when added alone showed no significant effects. Thus inhibition of the ROS levels by NAC abrogated the Px mediated cytotoxicity.

To elaborate the signaling cascade responsible for apoptosis induction and Akt activation we inhibited the ROS induction by pretreating the cells with NAC- 10 mM, followed by Px exposure and examined the levels of pAkt (Ser 473), caspase-3 (cleaved) and Akt (total) by western blot (Fig. 3.6.C). ROS inhibition by NAC caused significant reduction (~ 3 fold) in the levels of pAkt (Fig. 3.6.D). Also the Caspase-3 (cleaved) expression was reduced ~ 30 fold compared to Px treated cells (Fig. 3.6.E). The level of Akt (total) however remained unchanged (Fig. 3.6.C).
Figure 3.6. Px mediated ROS release causes decrease in cell viability, increase in Akt phosphorylation and apoptosis in MCF-7 cells. (A) Effect of antioxidant NAC on ROS levels of cells on pre-treatment with NAC at 5 and 10 mM or DMSO for 2 h followed by Px exposure for 1, 2, 3, 4, 5, 6 and 22 h using H$_2$DCFDA dye (***, p < 0.001). (B) Effect of NAC on cell viability of Px exposed cells as assessed by MTT assay (***, p < 0.001). Error bars represent mean ± SD of 3 independent experiments of duplicate/ triplicate
3.7. Akt activation is essential for Apoptosis induction by Px.

Px causes ROS mediated Akt phosphorylation and apoptosis induction in MCF-7 cells. To validate the role of Akt activation in apoptosis induction, Akt was knocked down by Akt 1/2/3 siRNA (the siRNA targets all the three subtypes of Akt i.e., Akt1, Akt2 and Akt 3). Cells were transfected with three concentrations of siRNA: 7.5, 15 and 22.5 nM and the expression of total Akt was analysed by western blot (Fig. 3.7.A Upper panel). The total Akt levels of MCF-7 cell were reduced ~ 2 fold at 15 and 22.5 nM (Fig. 3.7.A Lower panel). The concentration of 22.5 nM of siRNA was then selected for subsequent experiments. The Akt siRNA transfected cells were then treated with Px and the levels of pAkt (Ser 473 and Thr 308), Akt (total), PARP (cleaved) and caspase-(cleaved) were analysed by western blot. The siRNA transfected cells which were further exposed to Px for 24 h showed almost negligible pAkt (Ser 473 and Thr 308), caspase-3 (cleaved), PARP (cleaved) levels compared to Px treated cells (Fig. 3.7.B). There was ~ 4 fold decrease in the PARP (cleaved) levels in Akt siRNA and Px treated cells compared to Px alone (Fig. 3.7.C). To further validate, we also used a specific chemical inhibitor of Akt, triciribine which inhibits phosphorylation/ activation of Akt1/ 2/ 3. The cells were pre-treated with triciribine followed by Px exposure and analysed for the levels of pAkt (Ser 473 and Thr 308), PARP (cleaved), caspase-3 (cleaved) and Akt (total) by Western Blot (Fig. 3.7.D).
There was ~ 2 fold decrease in the PARP (cleaved) levels in triciribine and Px treated cells compared to Px only (Fig. 3.7.E). Another hallmark of apoptosis is the lowering of mitochondrial membrane potential. We studied the effect triciribine on mitochondrial membrane potential of MCF-7 cells using JC-1 dye. The cells treated with Px for 24 h showed intense green fluorescence compared to control cells which showed red fluorescence when observed under the fluorescence microscope. The triciribine treated cells showed negligible green fluorescence like the control cells. The cells which were pre-treated with triciribine followed by Px exposure showed reduction in the green fluorescence compared to cells treated with Px alone (Fig. 3.7.F). We also tested the possibility whether inhibition of Akt could reverse the decrease in cell viability brought about by Px. Indeed, it was observed that the presence of the Akt inhibitor significantly reduced the Px mediated cell death (Fig. 3.7.G). Thus it was established that Akt activation was a prerequisite for lowering of mitochondrial membrane potential (Δψm) and apoptosis induction by Px in MCF-7 cells.

![Graph showing fold change in Akt after Akt siRNA treatment](image)
Figure 3.7. Akt activation causes apoptosis and mitochondrial membrane damage in MCF-7 cells. (A) Upper panel: Western blot analysis of Akt (total) expression using Akt siRNA at 7.5, 15 and 22.5 nM. Lower panel: Densitometric analysis of Akt (total) levels. Quantified bands were normalized with their respective Akt levels and the fold change was calculated compared to control (*, \( p < 0.05 \); **, \( p < 0.01 \)). (B) Upper Panel: Western blot analysis of pAkt (Ser 473), PARP (cleaved), Akt (total) and caspase-3 (cleaved) expression; Lower panel: Western blot analysis of pAkt (Thr 308), Akt (Total) levels on transfecting cells with Akt siRNA (22.5 nM) or control siRNA followed with Px treatment at 30 \( \mu \)M for 24 h (C) Densitometric analysis of the PARP (cleaved) levels. Quantified bands were normalized with their respective \( \beta \)-actin levels and the fold change.
was calculated compared to control (***, \( p< 0.001 \)). (D) Upper panel: Western blot analysis of pAkt (Ser 473), PARP (cleaved), Akt (total) expression; Lower panel: Western blot analysis of pAkt (Thr 308), caspase-3 (cleaved), Akt (total) levels on pretreatment with triciribine or DMSO for 2 hs followed by Px exposure at 30 \( \mu \text{M} \) for 24 h. (E) Densitometric analysis of the PARP (cleaved) levels. Quantified bands were normalized with their respective \( \beta \)-actin levels and the fold change was calculated compared to control (**, \( p<0.01 \)). Error bars represent mean ± SD of 3 independent experiments. (F) Effect of Akt inhibition on the mitochondrial membrane potential of cells as observed by imaging using JC-1 stain. Scale bar represents 50 \( \mu \text{m} \). (G) Effect of Akt inhibition by triciribine on cell viability as assessed by MTT assay (**, \( p<0.01 \), ***, \( p< 0.001 \)). Error bars represent mean ± SD of 2-3 independent experiments of duplicate/ triplicate samples (C= control, Px= piroxicam, T= Triciribine).

3.8. Px causes PI3K independent activation of Akt.

To see whether the Akt activation was PI3 K mediated or not, we used PI-3K inhibitor Wortmannin (Wrt). However, Wortmannin did not inhibit the Akt hyper phosphorylation induced by Px (Fig. 3.8.A). There was no change in ssDNA levels of cells pre-treated with Wortmannin compared to Px exposed cells (Fig. 3.8.B). This suggests that Px mediated Akt activation and apoptosis induction was not through the PI3K pathway.

![Graph showing Fold Change in ssDNA](image)
Figure 3.8. PI3K independent Akt activation and apoptosis induction in MCF-7 cells.
(A) Western blot analysis of pAkt (ser473), and Akt (total) levels on pre-treatment with wortmannin or DMSO for 2 h followed by Px exposure at 30 μM for 24 h (B) Determination of ssDNA levels as determined by ELISA based assay (**p, 0.01). Error bars represent mean ± SD of 3 independent experiments of duplicate/triplicate samples (C= control, Px= piroxicam, Wrt= Wortmannin).

3.9. Differential effect of H\textsubscript{2}O\textsubscript{2} on MCF-7 and MDA-MB-231.

To further explore the difference between the two breast cancer cell lines we used hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) to induce ROS in the cells. Although there was enhanced Akt phosphorylation in both the cell lines on H\textsubscript{2}O\textsubscript{2} treatment (3.9 A, B), only MCF-7 showed significant caspase-3 activity on exposure to H\textsubscript{2}O\textsubscript{2} (3.9. C). MDA-MB-231 on the other hand showed significantly reduced caspase-3 activity in H\textsubscript{2}O\textsubscript{2} exposed cells than control (3.9.D). This demonstrates that ROS mediated Akt activation results in apoptosis induction in MCF-7 cells while MDA-MB-231 cells become resistant to apoptosis.
Figure 3.9. H$_2$O$_2$ causes Akt phosphorylation (Ser 473) in both MCF-7 and MDA-MB-231 cells but apoptosis induction in MCF-7 cells only. (A) and (B) Western blot analysis of pAkt (Ser 473), and Akt (total) levels on exposure to hydrogen peroxide for 6 h in MCF-7 and MDA-MB-231 cells. (C) and (D) Determination of caspase-3 activity by quantitative colorimetric assay on exposure to hydrogen peroxide for 6 h (**p, 0.001) in MCF-7 and MDA-MB-231 cells (C= control, H= Hydrogen peroxide).
Discussion

Px is a traditional NSAID used as anti-inflammatory and analgesic agent (2). Px can exert its anticancer effect by down-regulating Akt activation (3). Conversely, in this study we show that Px causes Akt hyperphosphorylation/activation which consequently leads to apoptosis induction. This is a recently discovered pathway, considering the fact that Akt hyperphosphorylation is conventionally associated with cell survival (4). We found that Px induces apoptosis in MCF-7 breast cancer cells via a recently discovered pathway of ROS mediated Akt activation.

The conventional role of Akt is to promote cell survival and uncontrolled proliferation and it helps the cancer cells to evade cell death. Akt is activated in most of the cancers (5, 6). Hence the clinical trial drugs which are used to treat multiple cancers including breast cancer are targeted to inhibit Akt activation (7, 8). In our study, Px surprisingly caused a gradual increase in the phosphorylation of the survival molecule, Akt along with apoptosis induction (Fig. 3.3 and 3.4). The enhancement of Akt phosphorylation by an anticancer agent like Px was intriguing. So to investigate whether the increased phosphorylation of Akt had any role in apoptosis induction, the Akt was knocked down by siRNA. It was observed that silencing of Akt abrogated the apoptosis induction. Similar results were obtained when we used a specific chemical inhibitor of Akt (Fig. 3.7). In fact, a recent publication has elucidated a mechanism underlying the pro-apoptotic function of Akt which involves caspase 3 activation through enhanced interaction of second mitochondria derived activator of caspases (Smac) with X-chromosome linked inhibitor of apoptosis protein (XIAP) (9). This pro-apoptotic function of Akt might be involved in the Px mediated apoptosis induction. Px mediated Akt activation and apoptosis induction was not through the PI3K pathway PI-3K inhibitor (Fig. 3.8). This suggests the involvement of
mechanisms other than PI3K dependent processes. PI3K- independent mechanisms of Akt activation have already been reported (10, 11). However Akt activation was certainly a prerequisite for apoptosis to take place in MCF-7 cells.

Several NSAIDs have been shown to induce ROS mediated apoptosis in cancer cells (12-14). However, significant ROS generation by Px in cancer cells has not been reported (15). Here we showed that Px induces ROS increase in a time dependent manner in MCF-7, human breast cancer cell line (Fig. 3.2). ROS increase by Px resulted in decreased cell viability compared to control and also several signatures of apoptosis such as increase in the expression of caspase-3, PARP, increase in capase-3 activity, ssDNA content and lowering of mitochondrial membrane potential were observed. Px exposure not only resulted in ROS accumulation in MCF-7 cells but also induced a gradual increase in the phosphorylation of the survival molecule, Akt accompanied by significant levels of apoptosis. To establish whether apoptosis induction and Akt activation was ROS mediated, the ROS scavenger, NAC (N-acetyl cysteine) was used. Inhibition with NAC resulted in inhibition of apoptosis as well as Akt phosphorylation (Fig. 3.6). Cancer cells have elevated endogenous ROS levels and can withstand increased oxidative stress. Possibly, a “threshold level” of ROS was crossed by Px treatment to induce cell death (Introduction chapter). When this rise in ROS was partially reversed by NAC to a level below the threshold point, the damaging effect of Px was partially negated and cell viability was significantly restored (Fig. 3.6).

Contrary to the conventional role of Akt as a cell survival molecule, a recent report showed that Akt activation induces premature senescence and sensitizes cells to ROS-mediated apoptosis by increasing intracellular ROS. The intracellular ROS was increased through the increased oxygen consumption and by inhibiting the expression of ROS scavengers downstream of FoxO, particularly sestrin 3 (4). This uncovers an ‘Achilles’
heel of Akt, since in contrast to its ability to inhibit apoptosis induced by multiple 
apoptotic stimuli, Akt could not inhibit ROS-mediated apoptosis. This interesting finding 
paved way for selectively killing those tumor cell which show hyperactive Akt with help 
of ROS inducers (4, 16, 17). Thereafter several other reports showing the ROS mediated 
Akt activation and subsequent apoptosis induction came into light (18-20). However, till 
date, this pathway has not been demonstrated to be induced by any NSAID. The fact that 
this newly discovered pathway is being followed by Px to induce apoptosis in MCF-7 cells 
is the novelty of our work. This effect of Px was cell type specific as 30 μM Px failed to 
cause a similar boost to Akt phosphorylation in MDA-MB-231 breast cancer cell line. On 
the contrary, Px downregulated Akt phosphorylation and had no significant influence on 
ROS levels and cell viability of MDA-MB-231 cells (Fig. 3.2). This bears similarity with 
the findings of a recent study which demonstrated differential Akt activation by 
chemotherapeutic agent Doxorubicin in MCF-7 and MDA-MB-231 cells. In fact, 
Doxorubicin enhanced phosphorylation of Akt in MCF-7 cells but failed to do so in MDA-
MB-231 cells (21). It was observed that chronically elevated ROS within the cells could 
lead to increased levels of Akt phosphorylation which may lead to an apoptosis resistant 
phenotype (22). \( H_2O_2 \) is known to induce ROS mediated Akt phosphorylation (20) and its 
exposure had different effect on the two breast cancer cell lines (Fig. 3.9). We also 
observe that the basal ROS levels of MDA-MB-231 cells were ~3 fold higher than MCF-
7 cells (Fig. 3.2 A and B). The higher basal ROS might be responsible for a strengthened 
antioxidant defence in MDA-MB-231 cells thus making it difficult for Px to induce further 
ROS and thereby preventing cell death by the ROS/Akt activation pathway. This suggests 
that Px has a cell type specific activity. The differential ability of the two types of cells to 
generate ROS may well be a determinant of this specificity of Px.
The underlying mechanisms of breast cancer as a disease are not uniform and could be multi-faceted (23). Although both MCF-7 and MDA-MB-231 cell lines are breast cancer derived, significant differences exist between the two cell lines not only in the presence or absence of estrogen hormone receptor but also in metastatic potential, invasive behavior, status of tumor suppressor gene p53 and sensitivity to drugs (24, 25). In fact, MDA-MB-231 cells are known to be more aggressive compared to MCF-7 and may respond differently to drugs (26).

The oxicam group of NSAIDs like Meloxicam, Tenoxicam and Piroxicam has been reported to activate PI3K/ Akt kinase pathway earlier but the rationale was different from our study. These compounds are potent drugs which can halt progressive dopaminergic neurodegeneration in Parkinson’s disease by activating the Akt pathway (27, 28). However in our study, we find that Akt activation by Px has opposite effects which is manifested in apoptosis of MCF-7 cells.

Our studies clearly demonstrated that Akt activation by Px was not leading to cell survival but rather it was a prerequisite for apoptosis. Thus Akt activation might be making the MCF-7 breast cancer cells more vulnerable to ROS mediated apoptosis instead of protecting them from the lethal doses of ROS. Therefore, Px induces apoptosis by an uncommon pathway of Akt activation, the detailed mechanism of which remains to be demonstrated in the future. To the best of our knowledge this is the first report on Px causing apoptosis by ROS mediated Akt activation. Px however failed to show any ROS release, Akt activation or cell death in MDA-MB-231 cells. This highlights the cell specific effect of Px. Indeed, our findings establish the importance of Px as a possible candidate for use in therapy of a specific type of breast cancer and also reveal apoptosis induction by Px by a recently discovered pathway.
Figure 4.0. Graphical representation of the summary of the part-1 of thesis
References


