Results
4. Results

In order to understand the role of 15-LOX-1 and 15-LOX-2 pathways on the growth and multiplication of cancer cells, the effects of 13-(S)-HPODE and 13-(S)-HODE (15-LOX-1 metabolites) and 15-(S)-HPETE and 15-(S)-HETE (15-LOX-2 metabolites) on chronic myeloid leukemia cell line were analyzed. The 15-LOX-1 and 15-LOX-2 metabolites were synthesized by incubating the commercially available soybean 15-LOX with LA and AA respectively as per the methods described in the methodology and the generated products were separated on HPLC and analyzed by MS analysis. These products were then employed to test their effects on chronic myeloid leukemia cell line-K-562.

4.1. HPLC and LC-MS analysis

The 15-LOX-1 [13-(S)-HPODE and 13-(S)-HODE] and 15-LOX-2 [15-(S)-HPETE and 15-(S)-HETE] metabolites were extracted and separated on HPLC as per the procedure. When the collected peaks were rerun on HPLC, 13-(S)-HPODE was eluted as a single peak at 20$^{th}$ min (Fig. 8 A), 13-(S)-HODE was eluted at 18$^{th}$ min (Fig. 8 B), 15-(S)-HPETE has given a single peak at 16.21 min (Fig. 8 C) and 15-(S)-HETE has given a single peak at 11$^{th}$ min (Fig. 8 D) under similar conditions. This is in accordance with their polarities. 13-(S)-HPODE and 15-(S)-HPETE, the hydroperoxy metabolites of 15-LOX, being polar to hydroxy metabolites [13-(S)-HODE and 15-(S)-HETE] were eluted at a later time point compared to their respective hydroxy metabolites.
Fig. 8. HPLC chromatograms of 15-LOX-1 (13-(S)-HPODE and 13-(S)-HODE) and 15-LOX-2 (15-(S)-HPETE and 15-(S)-HETE) metabolites. 
A. 13-(S)-HPODE; B. 13-(S)-HODE; C. 15-(S)-HPETE; D. 15-(S)-HETE.
When these peaks were pooled and monitored on UV-VIS spectrophotometer, they gave characteristic conjugated diene spectra with a peak at 235 nm (approximated) (Fig. 9 A & B). When these peaks were subjected for mass-spectral analysis, both 15-(S)-HPETE and 15-(S)-HETE have shown characteristic mass spectra (Fig. 10 A & B). 15-(S)-HPETE has shown majorily a mass fragment with m/z 335.2 (M-H⁺) including other mass fragments with m/z values 336.2 ((M), 318.2, 317.2, 281.2, 255.2. (Fig. 10 A) and 15-(S)-HETE has shown mass fragments with m/z values 319.2 (M-H⁺) and 320.2 (M) (Fig. 10 B). Similar analyses were performed with 13-(S)-HPODE and 13-(S)-HODE and their identity was confirmed. The mass spectrum of 13-(S)-HPODE has shown a base peak with an m/z value of 311.1 (M-H⁺) along with other mass fragments 294.2 and 293.2 (Fig. 10 C). 13-(S)-HODE has shown a characteristic mass spectrum containing a mass peak with an m/z value of 296.2 (M), base peak with an m/z value of 295.2 (M-H⁺) and other mass fragments include 279.2 and 255.2 (Fig. 10 D).
Fig. 10 A & B. LC-MS analysis of 15-LOX-2 metabolites of arachidonic acid (15-(S)-HPETE and 15-(S)-HETE). HPLC purified 15-LOX metabolites of arachidonic acid were subjected to LC-MS analysis under negative ion mode employing electron spray ionization (ESI). A. LC-MS chromatogram of 15-(S)-HPETE shows characteristic mass peak with an m/z of 336.2 (M) and a base peak with an m/z value of 335.2 (M-H⁺). B. Mass spectrum of 15-(S)-HETE shows a characteristic mass peak with an m/z value of 320.2 (M-) and a base peak with an m/z value of 319.2 (M-H⁺).
Results

Fig. 10 C & D. LC-MS analysis of 15-LOX-1 metabolites of linoleic acid (13-(S)-HPODE and 13-(S)-HODE). HPLC purified 15-LOX metabolites of linoleic acid were subjected to LC-MS analysis under negative ion mode employing electron spray ionization (ESI). C. Mass spectrum of 13-(S)-HPODE showing a base peak of m/z value 311.1 (M-H⁺) and other mass fragments with m/z values, 294.2 and 293.2. D. Mass spectrum of 13-(S)-HODE showing mass peak with an m/z value, 296.2 (M) and a base peak with an m/z value, 295.2 (M-H⁺).
Since soybean LOX is known to generate mostly S-stereospecific metabolites, the LOX metabolites obtained in the present study were considered as of S-configuration.

4.2. Effects of 15-LOX metabolites on the growth of K-562 cell line

To test the 15-LOX metabolite-induced growth inhibition and apoptosis in K-562 cells, we first assessed the effect of 15-LOX metabolites on the survival and proliferation of these cells by MTT assay. Cells were cultured in RPMI 1640 + 10 % FBS and incubated in 1 % FBS containing medium with 1-20 µM 15-(S)-HPETE and 13-(S)-HPODE, 1-160 µM 15-(S)-HETE and 13-(S)-HODE for 3-24 h and after the incubation, the cytotoxicity and cell proliferation were evaluated by the MTT assay. Under these experimental conditions, 15-(S)-HPETE and 13-(S)-HPODE inhibited the growth of K-562 cells rapidly by 3 h with IC$_{50}$ values of 10 µM & 15 µM respectively (Fig. 11 A & C). However, 15-(S)-HETE showed maximum cytotoxicity at much higher concentrations than its corresponding hydroperoxide 15-(S)-HPETE, with an IC$_{50}$ value of 40 µM by 6 h (Fig. 11 B). 13-(S)-HODE, the hydroxymetabolite of LA, on the otherhand, showed effect only beyond 40 µM i.e at 80 µM and 160 µM concentrations. (Fig. 11 D). These results were also confirmed by trypan blue dye exclusion assay (data not shown). Further studies to elucidate the mechanism behind 15-LOX metabolite mediated cell death were carried out with 15-LOX-2 metabolites, 15-(S)-HPETE and 15-(S)-HETE with the above mentioned doses and time periods.
Fig. 11. Effects of 15-LOX-1 (13-(S)-HPODE and 13-(S)-HODE) and 15-LOX-2 (15-(S)-HPETE and 15-(S)-HETE) on the growth of human chronic myeloid leukemia-K-562 cell line. Cells (5 X 10⁴) were treated with various concentrations (as indicated in the figure) of A. 15-(S)-HPETE, B. 15-(S)-HETE, C. 13-(S)-HPODE and D. 13-(S)-HODE and the cell viability was measured by MTT assay at 3, 6, 12 and 24 h post treatment. The values represent the mean ± S.E from three independent experiments.
4.3. Phase contrast microscopy

Phase contrast microscopy pictures of K-562 cells, treated with 15-(S)-HPETE (10 µM for 3 h) and 15-(S)-HETE (40 µM for 6 h) were taken to observe the altered morphological features. Cells grown in the absence of 15-LOX-2 metabolites were round in shape with characteristic features of lymphoid cells (Fig. 12 A). However, treatments with 10 µM 15-(S)-HPETE (Fig. 12 B) and 40 µM 15-(S)-HETE (Fig. 12 C) showed altered cellular morphology with cytoplasmic shrinkage and membrane blebbing. Many cells displayed protuberances of the plasma membrane that would eventually separate into membrane-bound apoptotic bodies. Apoptotic body formation was also clearly evident in the treatments.

Fig. 12. Phase contrast microscopic analysis of 15-LOX-2 metabolite treated K-562 cells. K-562 cells were treated with 10 µM 15-(S)-HPETE for 3 h or with 40 µM 15-(S)-HETE for 6 h and photographed. Arrows indicate a typical apoptotic cell with apoptotic bodies (Magnification - 400X). A. K-562 cells treated with 0.1 % absolute ethanol (control); B. K-562 cells treated with 15-(S)-HPETE (10 µM) for 3 h; C. K-562 cells treated with 15-(S)-HETE (40 µM) for 6 h.
Results

4.4. Fluorescence microscopic studies

A distinguishing feature of apoptosis is the condensation and fragmentation of nuclear chromatin, which can be monitored by fluorescence microscope when stained with specific DNA binding fluorescent dyes like DAPI. K-562 cells were exposed to 15-\((S)\)-HPETE (10 \(\mu\text{M}\) for 3 h) and 15-\((S)\)-HETE (40 \(\mu\text{M}\) for 6 h), and then assessed for morphological signs of apoptosis by staining with DAPI. Nuclear condensation and fragmentation, hallmarks of apoptosis, were clearly observed in cells treated with 15-LOX-2 metabolites (Fig. 13 B & C). Chromatin of apoptotic cells was segregated and compacted into sharply delineated masses, very close to the nuclear envelope, where as control cells have shown intact nuclei (Fig. 13 A).

![Fig. 13. Fluorescence microscopic analysis of 15-LOX-2 metabolite treated K-562 cells.](image)

Fig. 13. Fluorescence microscopic analysis of 15-LOX-2 metabolite treated K-562 cells. Nuclear morphology of K-562 cells was observed under a fluorescence microscope (Olympus BH2RFC) after treatment with 10 \(\mu\text{M}\) 15-\((S)\)-HPETE for 3 h and with 40 \(\mu\text{M}\) 15-\((S)\)-HETE for 6 h and stained with DAPI. The arrows indicate the apoptotic nuclei with fragmentation and condensation (Magnification - 400X). **A.** Control (vehicle treated); **B.** K-562 cells treated with 15-\((S)\)-HPETE (10 \(\mu\text{M}\)) for 3 h; **C.** K-562 cells treated with 15-\((S)\)-HETE (40 \(\mu\text{M}\)) for 6 h.
4.5. Flow cytometric analysis of 15-LOX-2 metabolite-induced apoptosis

The induction of apoptosis in 15-LOX-2 metabolite treated cells was further verified and quantified by flow cytometric analysis of DNA content. Loss of DNA is a typical feature of apoptotic cells. In the present study, K-562 cells treated with 15-(S)-HPETE (5 µM, 10 µM for 3 h) and 15-(S)-HETE (20 µM and 40 µM for 6 h) were taken for FACS analysis. Fig. 14, illustrates the DNA content histograms obtained after PI staining of permeabilized cells that

![Flow Cytometry](image)

**Fig. 14.** Flow cytometric analysis of DNA content in K-562 cells treated with 15-(S)-HPETE and 15-(S)-HETE for induction of apoptosis. K-562 cells (1.3 x 10^6 cells) treated with 5 µM and 10 µM 15-(S)-HPETE for 3 h and with 20 µM and 40 µM 15-(S)-HETE for 6 h respectively were fixed in 1 ml of 70% ethanol with 0.5 % Tween-20 at 4°C for 30 min and suspended in PBS. The cells were then stained with propidium iodide (PI) solution for 1 h and analyzed for DNA content by flow cytometry. Data represent the results from one of three similar experiments. **A.** Control (vehicle treated); **B.** K-562 cells treated with 15-(S)-HPETE (5 µM); **C.** K-562 cells treated with 15-(S)-HPETE (10 µM); **D.** K-562 cells treated with 15-(S)-HETE (20 µM); **E.** K-562 cells treated with 15-(S)-HETE (40 µM).
were treated with 15-LOX-2 metabolites. Typical sub-diploid apoptotic peaks were observed in K-562 cells treated with 15-(S)-HPETE (5 µM, 10 µM for 3 h) and 15-(S)-HETE (20 µM and 40 µM for 6 h). The FACS analysis of control cells, on the other hand, showed prominent G1, followed by S and G2/M phases. Only around 4 % of these cells showed hypodiploid DNA (sub G0/G1 peak) (Fig. 14 A). This value of 4 % hypodiploid DNA in control cells increased to 18.25 % and 50.37 % in case of 5 and 10 µM 15-(S)-HPETE (Fig. 14. B & C) treated cells and to 32.16 % and 42.36 % in case of 20 µM and 40 µM 15-(S)-HETE (Fig. 14. D & E) treated cells respectively. These studies thus reveal increase of hypodiploid apoptotic cells in response to 15-LOX-2 metabolite treatment in a concentration-dependent manner and the decrease of the cells in other phases of cell cycle.

4.6. 15-(S)-HPETE and 15-(S)-HETE treatment evoke cytochrome c release

One of the major apoptotic pathways is activated by the release of apoptogenic protein, cytochrome $c$ from mitochondria into the cytosol. The release of cytochrome $c$, one of the most important respiratory-chain proteins, from the mitochondria into the cytosol is the hallmark of cells undergoing apoptosis (Liu et al., 1996; Martinou et al., 2000). To specify the molecular basis of apoptosis, the release of cytochrome $c$ into the cytosol was analysed in K-562 cells treated with 15-LOX-2 metabolites by Western blot analysis employing cytochrome $c$ antibodies. As shown in Fig. 15 A, the levels of cytochrome $c$ in the cytosol were elevated within 1 h after treatment with 10 µM 15-(S)-HPETE (lanes 2 & 3) and the levels were further increased at later time points (2 h & 3 h). In case of 15-(S)-HETE treatment,
Results

Fig. 15. Effect of 15-(S)-HPETE and 15-(S)-HETE on cytochrome c release. Equal quantities of protein (30 µg) from the K-562 cells treated with 10 µM 15-(S)-HPETE (A) and 40 µM 15-(S)-HETE (B) for the indicated times were analyzed by 15% SDS-PAGE and immunoblotted with anti-cytochrome c antibody. β-Actin was used as control for equal loading of protein.

the same time dependent increase in the level of cytochrome c in the cytosol (lanes 2, 3, 4) in comparison to the control (lane 1) was observed in the Western blot analysis, but only at higher concentration (40 µM) (Fig. 15 B).

4.7. Caspase-3 activation in response to 15-(S)-HPETE and 15-(S)-HETE treatment

Cytochrome c leakage into the cytosol results in the activation of caspases (Li et al., 1997), a family of cysteine aspartate proteases, which cleave the cellular proteins and eventually steer a cell to undergo apoptosis. As 15-LOX-2 metabolite treatment of K-562 cells resulted in the leakage of cytochrome c, we examined for the activation of caspase-3. Indeed caspase-3 was activated during 15-LOX-2 metabolite-induced apoptosis as shown by Western blot analysis and caspase-3 activity assay. Western blot analysis carried out with antibodies specific to cleaved caspase-3, has clearly shown time dependent activation of caspase-3 in cells treated with both 10 µM 15-(S)-HPETE (Fig. 16 A) and 40 µM 15-(S)-HETE (Fig. 16 B).
Results

Fig. 16. Caspase-3 activation during 15-LOX-2 metabolite-induced apoptosis. Cells were treated with either 15-(S)-HPETE (10 µM) (A) or 15-(S)-HETE (40 µM) (B) for indicated time periods. After the treatments cell extracts were resolved on 12 % SDS-PAGE and probed with caspase-3 antibodies that specifically detect cleaved caspase-3 fragments. β-Actin was probed to confirm equal loading.

In order to quantify the caspase-3 activity, a fluorometric assay was carried with caspase-3 substrate, Ac-DEVD-AFC. As shown in Fig. 17 A, 10 µM 15-(S)-HPETE has resulted in almost 5-fold (5 ± 0.34, n=3, P < 0.05)

Fig. 17. Fluorometric analysis of 15-LOX-2 metabolites-induced caspase-3 activation. K-562 cells after treatment with either 10 µM 15-(S)-HPETE (for 1, and 2 h) or 40 µM 15-(S)-HETE (for 2 and 4 h) were lysed and assayed for caspase-3 activity with a fluorescence caspase-3 substrate – Ac-DEVD-AFC. Ac-DEVD-CHO, a specific inhibitor for caspase-3 was used as an assay control. A. 15-(S)-HPETE (10 µM) treatments, 1. Control (vehicle treated); 2. 10 µM 15-(S)-HPETE – 1 h; 3. 10 µM 15-(S)-HPETE – 2 h; 4. 10 µM 15-(S)-HPETE – 2 h + Ac-DEVD-CHO (1 µg/ml). B. 15-(S)-HETE (40 µM) treatments, 1. Control (vehicle treated); 2. 40 µM 15-(S)-HETE – 2 h; 3. 40 µM 15-(S)-HETE – 4 h; 4. 40 µM 15-(S)-HETE – 4 h + Ac-DEVD-CHO (1 µg/ml).
Results

increase in caspase-3 activity by 2 h post treatment (Fig. 17 A-2) compared to control (Fig. 17 A-1). Cells treated with 40 µM 15-(S)-HETE have shown 3.5 to 4 fold (3.5 ± 0.4, n=3, P <0.05)) increase in caspase-3 activity by 4 h (Fig. 17 B-3) when compared to control (Fig. 17 B-1). Ac-DEVD-CHO (1 µg/ml), a caspase-3 specific inhibitor, when added to the assay mixtures, completely inhibited the caspase-3 activity in both the treatments (Fig. 17 A & B-4).

4.8. Z-VAD-FMK, a broad-spectrum caspase inhibitor, prevents 15-LOX-2 metabolite-induced apoptosis

15-LOX-2 metabolite-induced apoptosis was completely abrogated when pretreated with 25 µM Z-VAD-FMK (a broad-spectrum caspase inhibitor) for 2 h (Fig. 18 A). As depicted in Fig. 18 B, upon treatment with 10 µM 15-(S)-HPETE and 40 µM 15-(S)-HETE, apoptosis from 6.2 % ± 1.2 in control raised to 43.5 % ± 3.4 (n=3, P < 0.05) and 37 % ± 2.5 (n=3, P < 0.05) respectively. Where as pretreatment with 25 µM Z-VAD-FMK resulted in the complete protection of cells from 15-LOX-2 metabolite-induced apoptosis (Fig. 18 B). As a result there were only 7.5 % ± 1.3 and 5.9 % ± 1.2 cells undergoing apoptosis with exposure to 10 µM 15-(S)-HPETE and 40 µM 15-(S)-HETE respectively, in the presence of Z-VAD-FMK. The above results clearly show that caspase-3 activation is an essential event in 15-LOX-2 metabolite-induced apoptosis.
Fig. 18. Protective effect of Z-VAD-FMK, a broad spectrum caspase inhibitor, on 15-LOX-2 metabolite-induced apoptosis. K-562 cells were preincubated with a cell permeable caspase inhibitor, Z-VAD-FMK (25 µM) for 1 h and then treated with either 10 µM 15-(S)-HPETE for 3 h or with 40 µM 15-(S)-HETE for 6 h and scored for apoptosis induction on flow cytometer using propidium iodide staining. A. Flow cytometric histograms of control and treated samples as indicated. B. Quantitative comparison of the induction of apoptosis by 15-(S)-HPETE and 15-(S)-HETE treated samples and the prevention of apoptosis in Z-VAD-FMK pretreated cells.
4.9. PARP cleavage in response to 15-LOX-2 metabolite treatment

Activated caspase-3 cleaves many vital cellular proteins including nuclear poly (ADP-ribose) polymerase (PARP), which has been implicated in many cellular processes such as apoptosis and DNA repair (Casciola-Rosen et al., 1996). PARP is a 116 kDa protein and is cleaved by caspases to generate 89 and 23 kDa fragments during apoptosis. To determine whether PARP is cleaved in 15-LOX-2 metabolite induced cell death, we treated K-562 cells with 10 µM 15-(S)-HPETE and 40 µM 15-(S)-HETE as indicated and PARP cleavage was monitored with PARP antibodies, which specifically recognize the 23 kDa fragment of the cleaved PARP and

![Fig. 19. Detection of PARP cleavage by Western blot analysis. K-562 cells (3.5 x 10^6) were seeded in 60 mm dishes and treated with 10 µM 15-(S)-HPETE (A) and 40 µM 15-(S)-HETE (B) for indicated time periods. 50 µg of total protein extract was separated on 12% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. Intact PARP (116 kDa) and the cleavage product of PARP (23 kDa) were detected using a goat polyclonal anti-PARP antibody. β-Actin was used control for equal loading.](image-url)
Results

uncleaved 116 kDa PARP. Fig. 19 A & B, illustrate the gradual increase in the proportion of the 23 kDa cleavage product and simultaneous decrease in the proportion of 116 kDa uncleaved PARP, with increasing time periods after 15-LOX-2 metabolite treatment. In the control cells, however, very minute or no 23-kDa fragment of PARP was detected, except the uncleaved 116-kDa protein (lane 1).

4.10. 15-LOX-2 metabolites induced DNA fragmentation in K-562 cells

In addition to morphological evaluation, apoptosis induction by 15-LOX-2 metabolites was ascertained by using an assay developed to measure DNA fragmentation, a biochemical hallmark of apoptosis. During

![Fig. 20. Analysis of DNA fragmentation in K-562 cells treated with 15-LOX-2 metabolites. After treatment of the cells with various concentrations of 15-(S)-HPETE for 3 h and 15-(S)-HETE for 6 h, DNA was isolated and separated on 1.5% agarose gels. DNA was stained with ethidium bromide and visualized under UV light. Lane. 1: Vehicle treated control; lanes. 2-3: Cells treated with 15-(S)-HPETE; lanes. 4-5: Cells treated with 15-(S)-HETE. Later stages of apoptosis, internucleosomal cleavage of cellular DNA by endonucleases to 180 bp or oligomers of 180 bp fragments could be detected.](image)
by agarose gel electrophoresis of nuclear DNA. As illustrated in Fig. 20, agarose gel electrophoresis of DNA extracted from K-562 cells treated with 15-(S)-HPETE (5 µM, 10 µM for 3 h) and 15-(S)-HETE (20 µM and 40 µM for 6 h) show a progressive increase in the non-random fragmentation into a ladder of 180-200 bp (lanes 2-5). Such a pattern corresponds to internucleosomal cleavage, reflecting the endonuclease activity, characteristic of apoptosis. Control cells did not show any internucleosomal DNA fragmentation (lane 1).

4.11. Reactive oxygen species (ROS) mediate 15-LOX-2 metabolite-induced cell death

As ROS have been implicated in rapid induction of cell death in several studies, a possible correlation between ROS and 15-LOX-2 metabolite-induced apoptosis was examined through DCFH-DA analysis. As shown in Fig. 21 A, there was a massive outburst of ROS within minutes of post treatment as evidenced by the shift in DCF fluorescence towards right with increasing time periods after treatment with 10 µM 15-(S)-HPETE (2 and 3). This shift accounts for 6.29 folds over control in 30 min (2) and 8.95 folds over control in 60 min (3). 15-(S)-HETE (40 µM), however resulted in the ROS production (61% in 60 min, 82 % in 1.5 h compared to control) (3 & 4 in Fig. 21 B) that is lesser compared to 15-(S)-HPETE but a significant increase and accumulation of ROS was observed (Fig. 21 B). This differential ability of induction of ROS by the hydroperoxy (15-(S)-HPETE) and hydroxy (15-(S)-HETE) metabolites might play a key role in their difference in growth inhibitory effects and induction of apoptosis.
Results

Fig. 21. DCFH-DA analysis of 15-(S)-HPETE and 15-(S)-HETE induced ROS generation in K-562 cells. K-562 cells were incubated with 10 µM DCFH-DA for 15 min and then washed and incubated in PBS (containing 10 mM glucose) with either 10 µM 15-(S)-HPETE or 40 µM 15-(S)-HETE for indicated time periods. DCF fluorescence was detected by flow cytometry after stipulated treatments using 530 nm emission filter. A. 1. Vehicle treated control; 2. 10 µM 15-(S)-HPETE – 30 min; 3. 10 µM 15-(S)-HPETE – 60 min; B. 1. Vehicle treated control; 2. 40 µM 15-(S)-HETE – 30 min; 3. 40 µM 15-(S)-HETE – 60 min; 4. 40 µM 15-(S)-HETE – 90 min

Fig. 22. Effect of 15-LOX-2 metabolites on glutathione depletion, as represented by GSH/GSSG ratio. GSH and GSSG levels were measured after treating K-562 cells with 15-(S)-HPETE (10 µM) and 15-(S)-HETE (40 µM) for indicated time periods. The cell lysates were treated with 10% sulfosalicylic acid (w/v) and the protein free extracts were used to measure GSH and GSSG levels by DTNB recycling assay. Significance over control was established at *P < 0.05.
We further tested, whether the ROS production in case of 15-LOX-2 metabolite treatment is associated with changes in cellular glutathione content. These studies revealed a drastic depletion of cellular GSH (reduced form of glutathione) content without any significant change in the levels of oxidized glutathione (GSSG) and thus resulting in a decrease in GSH/GSSG ratio (Fig. 22). To determine the role of ROS generation and glutathione depletion associated with it in 15-LOX-2 metabolite-induced apoptosis, K-562 cells were pretreated with 50 µM NAC (a glutathione precursor and an antioxidant) for 3 h followed by exposure to 10 µM 15-((S))-HPETE or 40 µM 15-((S))-HETE and analyzed for ROS production and inhibition of apoptosis. NAC pretreatment resulted in reduction of cellular ROS levels by 52 % in case of 15-((S))-HPETE (Fig. 23 A) and by 63 % in case of 15-((S))-HETE (Fig. 23 B) and reduced apoptotic induction by 63.2% and 47% in case of 15-((S))-HPETE (Fig. 23 A) and 15-((S))-HETE (Fig. 23 B) respectively. Apoptosis induced by 10 µM 15-((S))-HPETE (Fig. 25 A, page. 70) and 40 µM 15-((S))-HETE (Fig. 25 B, page. 70) was reduced by 66.8% and 55% respectively, when the cells were pretreated with 200 µM GSH. These studies unequivocally demonstrate the role of ROS and depletion of reduced glutathione in 15-LOX-2 metabolite-induced apoptosis. As shown in Fig. 26 A & 26 B (page. 71), these antioxidants inhibit the activation of caspase-3 and thereby abrogating the subsequent induction of apoptosis. The above studies thus clearly suggest a critical role for ROS in the activation of caspase-3 through cytochrome c leakage by perturbing mitochondrial permeability transitions.
**Results**

Fig. 23. DCFH-DA analysis of ROS production in K-562 cells pretreated with N-Acetyl cysteine (NAC– glutathione precursor). K-562 cells were pretreated with 50 µM NAC for 3 hr followed by treatment with 15-LOX-2 metabolites. **A:** 1. Vehicle treated control; 2. 10 µM 15-((S))-HPETE – 60 min; 3. 10 µM 15-((S))-HPETE – 60 min + 50 µM NAC; **B:** 1. Vehicle treated control; 2. 40 µM 15-((S))-HETE – 90 min; 3. 40 µM 15-((S))-HETE – 90 min + 50 µM NAC. Data presented represent one of the three independent experiments.

4.12. 15-LOX-2 metabolites activate NADPH Oxidase to generate ROS and subsequently induce apoptosis

To find the possible involvement of NADPH oxidase activation in ROS production, we studied the effect of DPI pretreatment, a pharmacological inhibitor of NADPH oxidase on 15-LOX-2 metabolite-induced apoptosis. Pretreatment of K-562 cells with 10 µM DPI for 1 h followed by treatments with 10 µM 15-((S))-HPETE for 1 h or 40 µM 15-((S))-HETE for 1.5 h, resulted in the reduction intracellular ROS levels and shift in the DCF fluorescence towards control as shown in Fig. 24 A & B. DPI pretreatment inhibited of ROS production induced by 15-((S))-HPETE by 85 % and that of 15-((S))-HETE by 76 %. These results substantially demonstrate that NADPH oxidase is the site of ROS production by 15-((S))-HPETE and 15-((S))-HETE, though the
Fig. 24. Inhibition of ROS production in K-562 cells by DPI, an NADPH oxidase inhibitor. K-562 cells were pretreated with 10 μM DPI, an NADPH Oxidase inhibitor for 1 h and followed by the treatment with 15-LOX-2 metabolites and analysed for ROS production. **A**: 1. Vehicle treated control; 2. 10 µM 15-(S)-HPETE – 60 min; 3. 10 µM 15-(S)-HPETE + 10 µM DPI. and **B**: 1. Control, 2. 40 µM 15-(S)-HETE – 90 min 3. 40 µM 15-(S)-HETE – 90 min + 10 µM DPI. Data presented represent one of the three independent experiments.

degree of NADPH activation varied with hydroperoxy and hydroxy metabolites. This differential ability of these metabolites to activate NADPH oxidase might have resulted in varying levels of ROS production in K-562 cells. Consequently, we sought to examine whether the inhibition of intracellular ROS production by DPI could abrogate 15-LOX-2 metabolite-induced apoptosis. When the cells pretreated with 10 μM DPI were analyzed by propidium iodide staining, a dose dependent decrease in the hypodiploid DNA content was observed as illustrated in Fig. 25 A & B. As depicted in Fig. 26 A & B, the abrogation of apoptosis by DPI pretreatment was preceded by concomitant inhibition of caspase-3 activation. As DPI, an NADPH oxidase inhibitor, was also shown to inhibit inducible nitric oxide synthase (iNOS)
(Stuehr et al., 1991), to determine the role of iNOS, we examined the effects of L-NMMA, a more specific inhibitor of iNOS on 15-LOX-2 metabolite-induced apoptosis. The results presented in Fig. 25 A & B, 26 A & B (page. 71) clearly showed that L-NMMA could neither rescue cells from cell death

![Figure 25](image-url)

**Fig. 25.** Inhibitory effects of NADPH Oxidase inhibitor (DPI), ROS inhibitors (NAC, GSH, catalase), calcium channel blockers (lanthanum chloride (LaCl₂), ruthenium red, verapamil), iNOS inhibitor (L-NMMA), MEK inhibitor (PD-184352) on 15-LOX-2 metabolite-induced apoptosis as analysed upon propidium iodide staining by flow cytometer. K-562 cells were pretreated with various classes of inhibitors - 10 µM DPI (1 h) / 50 µM NAC (3 h) / 200 µM GSH (3 h) / 200µM L-NMMA/ 100 U/ml catalase (1 h) / 200 µM lanthanum chloride (3 h) / 100 µM verapamil(3 h) / 100 µM ruthenium red (3 h) / 2 µM PD- 184352 (1 h) and followed by 15-LOX-2 metabolite treatment. **A.** K-562 cells treated with various inhibitors and/or 10 µM 15-(S)-HPETE as shown in the figure. **B.** K-562 cells treated with various inhibitors and/or 40 µM 15-(S)-HETE. Data represent mean ± SE from three independent experiments and the significant difference was established at *P* <0.05 compared with 15-(S)-HPETE and 15-(S)-HETE alone treated samples.
Results

Fig. 26. Inhibitory effects of 10 μM DPI (1 h) / 50 μM NAC (3 h) / 200 μM GSH (3 h) / 200 μM L-NMMA / 1000 U/ml catalase (1 h) / 100 μM verapamil(3 h) / 2 μM PD-184352 (1 h) on 15-LOX-2 metabolites induced caspase-3 activity. K-562 cells were treated with various classes of inhibitors followed by 15-LOX-2 metabolites as shown in the figure. A. K-562 cells treated with various inhibitors and/or 15-(S)-HPETE (10 μM); B. K-562 cells treated with various inhibitors and/or 15-(S)-HETE (40 μM). Data represent one of the three independent experiments carried out.

nor inhibit caspase-3 activation, excluding the role of iNOS in 15-LOX-2 metabolite induced apoptosis. These data further substantiates that NADPH oxidase is the site for ROS production that led to the induction of apoptosis and DPI could prevent these effects.
4.13. Role of Calcium channel blockers in 15-LOX-2 metabolite-induced apoptosis

The association and mutual influence of Ca\(^{2+}\) and ROS generation has been well documented (Supiniski et al., 1999; Lee et al., 2000) and the release of calcium from endoplasmic stores has been suggested to play an important role in LOX metabolite-induced apoptosis (Maccarrone et al., 2000). To examine the role of Ca\(^{2+}\) in the rapid induction of apoptosis by 15-LOX-2 metabolites, K-562 cells were pretreated with 200 \(\mu\)M lanthanum chloride (LaCl\(_2\)), a store operated Ca\(^{2+}\) channel blocker, 100 \(\mu\)M verapamil, an L-type voltage dependent Ca\(^{2+}\) channel blocker, 100 \(\mu\)M ruthenium red, an endoplasmic reticulum calcium channel blocker for 3 h followed by 10 \(\mu\)M 15-\((S)\)-HPETE for 3 h or 40 \(\mu\)M 15-\((S)\)-HETE for 6 h. Following the completion of the above treatments, the cells were analysed for apoptotic induction by propidium iodide staining. Both LaCl\(_2\) and ruthenium red did not show any significant inhibition of 15-LOX-2 metabolite-induced apoptosis (Fig. 25 A & B, page. 70). Whereas pretreatment with 100 \(\mu\)M verapamil resulted in the reduction of apoptosis by 55 % from 10 \(\mu\)M 15-\((S)\)-HPETE treated samples (Fig. 25 A) and by 63 % in case of 40 \(\mu\)M 15-\((S)\)-HETE treatment (Fig. 25 B). This inhibition of apoptosis by verapamil was also observed to be associated with inhibition of caspase-3 activation as shown in Fig. 26 A & B, page. 71). However, pretreatment with any of these inhibitors including verapamil did not show any effect on ROS production (data not shown) suggesting that either ROS might precede Ca\(^{2+}\) influx or Ca\(^{2+}\) might
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independently act at mitochondrial level by bringing in permeability changes, leakage of cytochrome \( c \) and subsequent caspase-3 activation.


Rapid accumulation of intracellular ROS will result in impairment of cellular antioxidant defense system, turn detrimental to the cell survival and ultimately concedes the cell to undergo cell death. To determine the effect of 15-LOX-2 metabolite generated ROS on various antioxidant enzymes, cells were treated with either 10 \( \mu \)M 15-\((S)\)-HPETE or 40 \( \mu \)M 15-\((S)\)-HETE for indicated time periods and enzyme activities were measured in the cell extracts. 15-\((S)\)-HPETE (10 \( \mu \)M) has drastically decreased catalase activity where as 15-\((S)\)-HETE (40 \( \mu \)M) did not significantly inhibit catalase activity (Fig. 27). These results suggest a possible accumulation of \( \text{H}_2\text{O}_2 \) in K-562.

Fig. 27. Effect of 15-LOX-2 metabolites on catalase activity. K-562 cells were treated with either 10 \( \mu \)M 15-\((S)\)-HPETE or 40 \( \mu \)M 15-\((S)\)-HETE for indicated time periods and cells were lysed, catalase activity was assayed and the values are represented as units/ mg.protein. One unit of catalase was defined as the amount of enzyme, which decomposed one \( \mu \)mol of \( \text{H}_2\text{O}_2 \) per minute at 25°C and pH 7.0. The values represent the mean \( \pm \) S.E from three independent experiments and the significant difference was established at \( ^* \) \( P <0.05 \) compared with the control group (0.1 % EtOH).
cells treated with 15-(S)-HPETE as result of inactivation of catalase, thus leading to the rapid induction of apoptosis. To test this possibility, we pretreated the K-562 cells with 1000 U/ml of catalase for 1 h, followed by 15-LOX-2 metabolites and subjected these cells for cell death analysis by PI staining. The data presented in Fig. 25 A & B (page. 70) illustrates that catalase pretreatment almost completely abrogated (by 80 %) apoptosis induced by 15-(S)-HPETE (10 µM) and by 55 % incase of 15-(S)-HETE (40 µM) substantiating the hypothesis that accumulation of H$_2$O$_2$ might result in the induction of apoptosis. Corroborating this data, pre-incubation of the cells with catalase, inhibited caspase-3 activation by 71 % and by 42 % incase of 15-(S)-HPETE and 15-(S)-HETE respectively (Fig. 26 A & B, page. 71). Other antioxidant enzymes like superoxide dismutase and glutathione peroxidase were unaffected by 15-LOX-2 metabolites. Recent studies have shown that accumulation of ROS (H$_2$O$_2$) leads to the activation of extracellular signal regulated kinase (ERK) and its pertinence to induction of apoptosis through activation of transcription factors like AP-1 (Kitamura et al., 2002). The preliminary data presented in Fig. 25 A & B show that PD-184352, a MEK inhibitor, inhibits the apoptotic induction by 41% and 31% incase of 15-(S)-HPETE (10 µM) and 15-(S)-HETE (40 µM) treatments respectively, suggesting a possible role for ERK signaling in inducing apoptosis.

4.15. Cellular glutathione peroxidase levels and induction of apoptosis by 15-LOX-2 metabolites

The 15-(S)-HPETE, within in the cell gets eventually converted to 15-(S)-HETE by cellular GPx, but in view of their differential ability to induce
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ROS and apoptosis, it is conceived that cellular GPx levels play a critical role in 15-LOX-2 metabolite induced apoptosis. In order to test the role of GPx, the enzyme activity levels were measured in K-562 and other leukemic cell lines like U-937, HL-60 and Jurkat. These studies revealed very low levels of GPx activity in K-562, Jurkat and the highest levels in U-937 cells (Fig. 28).

![Graph of GPx levels in various cancer cell lines](image)

**Fig. 28.** Analysis of glutathione peroxidase levels in various cancer cell lines as indicated and the activity is represented as Units/mg.protein. One unit of GPx activity was defined as one nmole of NADPH oxidized per min. The values represent the mean ± S.E. from three independent experiments.

To ascertain the importance of these varying levels of GPx on growth inhibitory effects of 15-LOX-2 metabolites, we treated Jurkat and U-937 cells with different concentrations of 15-(S)-HPETE and 15-(S)-HETE and analyzed by MTT assay. Interestingly, as Fig. 29 illustrates [data with only 10 µM 15-(S)-HPETE and 40 µM 15-(S)-HETE (these are the IC₅₀ values observed with K-562 cell line) were presented], U-937 cell line with high levels of GPx was found to be highly resistant and Jurkat with low levels of GPx was more sensitive to 15-LOX-2 metabolite treatment. Jurkat with
similar levels of GPx as of K-562 cell line has shown growth inhibitory effects comparable to that of K-562 cell line upon 15-LOX-2 metabolite treatment. These results clearly suggest that cellular GPx levels play an important role in 15-LOX-2 metabolite-induced apoptosis.

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Fig. 29. Growth inhibitory effects of 15-LOX-2 metabolites on U937 & Jurkat cell lines. $5 \times 10^3$ cells were treated with $10 \, \mu$M 15-(S)-HPETE and 40 $\mu$M 15-(S)-HETE for 3, 6, 12, 24 h and analysed by MTT assay as described in methodology. Data presented represent the mean ± SE from three independent experiments and the significance over control was established at $(*) P <0.05$.

**4.16. Effect of 15-LOX-2 metabolites on transcription factors AP-1 and NF-kB**

Activator protein (AP-1) and nuclear factor-kB (NF-kB) are two important transcription factors that are sensitive to redox changes and get translocated to nucleus during oxidative stress and bring in transcriptional changes (Morel and Barouki, 1999). Several studies have shown a critical role played by these transcription factors in mediating induction of apoptosis (Baeuerlie and Baltimore, 1996; Ameyar et al., 2003). To examine the activation of these transcription factors during 15-LOX-2 metabolite-induced apoptosis, nuclear extracts were isolated from the cells treated with either 5 $\mu$M, 10 $\mu$M, 20 $\mu$M 15-(S)-HPETE or with 10 $\mu$M, 20 $\mu$M, 40 $\mu$M 15-(S)-
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HETE for 1 h and electrophoretic mobility shift (EMSA) analysis was carried out. As illustrated in Fig. 30 A, 15-(S)-HPETE (lanes 6 and 7) and 15-(S)-HETE (lanes 8, 9, 10) have shown significant activation of AP-1 in a dose

![EMSA analysis of Activator Protein-1 (AP-1) (A) and Nuclear Factor-κB (NF-κB) (B).](image)

K-562 cells were treated as indicated. After the treatments, nuclear extracts were isolated from treated cells and EMSA analysis was carried out as mentioned in the methodology.
dependent manner, compared to control (untreated, lane 4) and vehicle treated (lane 3) samples. However, activation of another redox sensitive transcription factor NF-kB was not observed in response to 15-LOX-2 metabolite treatments. As shown in Fig. 30 B 15-(S)-HPETE (lanes 8, 9 and 10) and 15-(S)-HETE did not show any significant change in the activation of NF-kB compared to the control (lane 4) and vehicle treated (lane 3) samples. These results clearly indicate that AP-1 but not NF-kB get activated during 15-LOX-2 metabolite-induced apoptosis.