Chapter 3

Ormeloxifene, a non-steroidal selective estrogen receptor modulator (SERM) induces apoptosis in myeloid leukemia cells
Chapter 3

3.1 Introduction

Apoptosis

The term apoptosis (a-po-toe-sis) was first used by Kerr, Wyllie, and Currie in 1972 to describe a morphologically distinct form of cell death (153). Understanding of mechanisms involved in the process of apoptosis in mammalian cells transpired from the investigation of programmed cell death that occurs during the development of the nematode *Caenorhabditis elegans* (154). In this organism 1090 somatic cells are generated in the formation of the adult worm, of which 131 cells undergo apoptosis or “programmed cell death”. These 131 cells die at particular points during the developmental process which is essentially invariant between worms demonstrating the remarkable accuracy and control in this system. Apoptosis has since been recognized and accepted as a distinctive and important mode of “programmed” cell death, which involves genetically determined elimination of the cells.

The process of programmed cell death or apoptosis is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. Apoptosis is considered a vital component of various vital processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death. Inappropriate apoptosis (either too little or too much) is a factor in many human disease conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer. The ability to modulate the life or death of a cell is recognized for its immense therapeutic potential. Therefore, research continues to focus on the elucidation and analysis of the cell cycle machinery and signaling pathways that control the cell cycle arrest and apoptosis. To that end, the field of apoptosis research has been moving forward at an alarmingly rapid rate. Although many of the key apoptotic proteins have been identified, the molecular mechanisms of action or inaction of these proteins remains to be elucidated.

Morphology of Apoptosis

Light and electron microscopy have identified various morphological changes that occur during apoptosis (155). During the early process of apoptosis, cell shrinkage and pyknosis are visible by light microscopy (153). With cell shrinkage, the cells are smaller in size, the cytoplasm is dense
and the organelles are more tightly packed. Pyknosis, result of chromatin condensation is the most characteristic feature of apoptosis. Extensive plasma membrane blebbing occurs followed by karyorrhexis and separation of cell fragments into apoptotic bodies during a process called “budding” (156). Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment. The organelle integrity is still maintained being enclosed within an intact plasma membrane. These bodies are subsequently phagocytosed by macrophages, parenchymal cells, or neoplastic cells and degraded within phagolysosomes. Macrophages that engulf and digest apoptotic cells are called “tingible body macrophages” and are frequently found within the reactive germinal centers of lymphoid follicles or occasionally within the thymic cortex (157). The tingible bodies are the bits of nuclear debris from the apoptotic cells. There is essentially no inflammatory reaction associated with the process of apoptosis or with the removal of apoptotic cells because: (1) apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue; (2) they are quickly phagocytosed by surrounding cells thus likely preventing secondary necrosis; and, (3) the engulfing cells do not produce anti-inflammatory cytokines.

Biochemical Features

Apoptotic cells exhibit several biochemical modifications such as protein cleavage, protein cross-linking, DNA breakdown, and phagocytic recognition (158). Caspases are widely expressed in an inactive proenzyme form in most cells and once activated can often activate other procaspases, allowing initiation of a protease cascade. Some procaspases can also aggregate and autoactivate. This is proteolytic cascade in which one caspase can activate other caspases, amplifies the apoptotic signaling pathway and thus leads to rapid cell death. Caspases have proteolytic activity and are able to cleave proteins at aspartic acid residues, although different caspases have different specificities involving recognition of neighboring amino acids (150). Once caspases are activated, there seems to be an irreversible commitment towards cell death. Ten major caspases have been identified and broadly categorized so far. They are initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) (159). The other caspases that have been identified include caspase-11 which is reported to regulate apoptosis and cytokine maturation during septic shock; caspase-12
mediates endoplasmic-specific apoptosis and cytotoxicity by amyloid-β; caspase-13 and caspase-14 (160-163). Extensive protein cross-linking is another characteristic of apoptotic cells and is achieved through the expression and activation of tissue transglutaminase (164). DNA breakdown by Ca$^{2+}$- and Mg$^{2+}$-dependent endonucleases also occur resulting in DNA fragments of 180 to 200 base pairs (145). A characteristic “DNA ladder” is visualized by agarose gel electrophoresis with an ethidium bromide stain and ultraviolet illumination.

Another biochemical feature is the expression of cell surface markers that result in the early phagocytic recognition of apoptotic cells by adjacent cells, permitting quick phagocytosis with minimal compromise to the surrounding tissue. This is achieved by the movement of the normal inward-facing phosphatidylserine of the cell’s lipid bilayer to the outer layers of the plasma membrane (146). Although externalization of phosphatidylserine is a well-known recognition ligand for phagocytes on the surface of the apoptotic cell, recent studies have shown that other proteins are also be exposed on the cell surface during apoptotic cell clearance. These include Annexin I and calreticulin. Annexin V is a recombinant phosphatidylserine-binding protein that interacts strongly and specifically with phosphatidylserine residues and can be used for the detection of apoptosis (147, 165). Calreticulin is a protein that binds to an LDL receptor related protein on the engulfing cell and is suggested to cooperate with phosphatidylserine as a recognition signal (166).

**Mechanisms of Apoptosis**

The mechanisms of apoptosis are highly complex and sophisticated that involves an energy dependent cascade of molecular events. There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. These two pathways are linked and the molecules in one pathway can influence the other (167). There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme dependent killing of the cells. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A. The extrinsic, intrinsic, and granzyme B pathways converge on the same terminal, or execution pathway.

The apoptotic pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, crosslinking of proteins,
formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells.

**Figure 1:** Major components of the core apoptotic cascade. The two major apoptotic pathways—the cell surface death receptor pathway (extrinsic) and the mitochondria-initiated (intrinsic) pathway are depicted. The extrinsic pathway emanates from extracellular stimuli, which are transduced through membrane-associated receptors of the TNFR superfamily. Intracellular stimuli induce apoptosis primarily through the mitochondria and, to a lesser extent, through the ER (adapted from C. J. Zeiss, Vet Pathol 40:5, 2003).
Extrinsic Pathway

The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily (168). Members of the TNF receptor family share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the "death domain" (169). This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways. Ligands and corresponding death receptors include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (169-172). Upon ligand binding, cytoplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to Fas receptor results in the binding of the adapter protein FADD and the binding of TNF ligand to TNF receptor results in the binding of the adapter protein TRADD with recruitment of FADD and RIP (173-175). FADD then associates with procaspase-8 via dimerization of the death effector domain. At this point, a death-inducing signaling complex (DISC) is formed, resulting in the auto-catalytic activation of procaspase-8 (176). Once caspase-8 is activated, the execution phase of apoptosis is triggered. Death receptor mediated apoptosis can be inhibited by a protein called c-FLIP which binds to FADD and caspase-8, rendering them ineffective (177). Another point of potential apoptosis regulation involves a protein called Toso, which has been shown to block Fas-induced apoptosis in T cells via inhibition of caspase-8 processing (178).

Intrinsic Pathway

Apoptosis can be initiated by intrinsic signals that are produced following cellular stress. Cellular stress may occur from exposure to radiation or chemicals or by viral infection. It might also be a consequence of growth factor deprivation or oxidative stress caused by free radicals (179). In general, intrinsic signals initiate apoptosis through the involvement of the mitochondria. The relative ratios of the various bcl-2 proteins can often determine how much cellular stress is necessary to induce apoptosis. The intrinsic signaling pathways that initiate apoptosis involve a diverse array of non-receptor-mediated stimuli that produce intracellular signals which act directly on targets within the cell and are mitochondrial-initiated events (180). The stimuli that initiate the intrinsic pathway produce intracellular signals that may act either in a positive or
negative fashion. Negative signals involve the absence of certain growth factors, hormones and cytokines that can lead to failure of suppression of death programs, thereby triggering apoptosis. Other stimuli that act in a positive fashion include radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals. All of these stimuli cause changes in the inner mitochondrial membrane that result in an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of two main groups of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol (180). The first group consists of cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi (149, 181, 182). These proteins activate the caspase dependent mitochondrial pathway. Cytochrome c binds and activates Apaf-1 as well as procaspase-9 forming an “apoptosome” (183). The clustering of procaspase-9 in this manner leads to caspase-9 activation. Smac/DIABLO and HtrA2/Omi are reported to promote apoptosis by inhibiting IAPs (inhibitors of apoptosis proteins) activity (184).

The control and regulation of these apoptotic mitochondrial events occurs through members of the Bcl-2 family of proteins (185). The tumor suppressor protein p53 has a critical role in the regulation of Bcl-2 family of proteins (186). The Bcl-2 family of proteins governs mitochondrial membrane permeability and can either be pro-apoptotic or antiapoptotic. To date, a total of 25 genes have been identified in the Bcl-2 family. Some of the anti-apoptotic proteins include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG while Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk are proapoptotic. These proteins have special significance since they can determine if the cell commits to apoptosis or aborts the process. It is thought that the main mechanism of action of the Bcl-2 family of proteins is the regulation of cytochrome c release from the mitochondria via alteration of mitochondrial membrane permeability.

Perforin/granzyme Pathway

T-cell mediated cytotoxicity is a variant of type IV hypersensitivity where sensitized CD8+ cells kill antigen-bearing cells. These cytotoxic T lymphocytes (CTLs) are able to kill target cells via the extrinsic pathway; notably FasL/FasR interaction is the predominant method of CTL-induced apoptosis (187). However, they are also able to exert their cytotoxic effects on tumor cells and virus-infected cells via a novel pathway that involves secretion of the transmembrane pore-forming molecule perforin with a subsequent exophytic release of cytoplasmic granules through
the pore and into the target cell (188). The serine proteases granzyme A and granzyme B are the most important component within the granules (189).

Granzyme B cleaves proteins at aspartate residues and therefore activates procaspase-10 and can cleave factors like ICAD (Inhibitor of Caspase Activated DNAse) (190). Granzyme B can utilize the mitochondrial pathway for amplification of the death signal by specific cleavage of Bid and induction of cytochrome c release (191). However, granzyme B can also directly activate caspase-3. In this way, the upstream signaling pathways are bypassed and there is direct induction of the execution phase of apoptosis. Both the mitochondrial pathway and direct activation of caspase-3 are critical for granzyme B-induced killing (192).

Granzyme A is also important in cytotoxic T cell induced apoptosis and activates caspase independent pathways. Granzyme A activates DNA nicking via DNAse NM23-H1, a tumor suppressor gene product (193). This DNAse has an important role in immune surveillance to prevent cancer through the induction of tumor cell apoptosis. The nucleosome assembly protein SET normally inhibits the NM23-H1 gene. Granzyme A protease cleaves the SET complex thus releasing inhibition of NM23-H1 resulting in apoptotic DNA degradation. In addition to inhibiting NM23-H1, the SET complex has important functions in chromatin structure and DNA repair.

**Execution Pathway**

The extrinsic and intrinsic pathways both end at the point of the execution phase, considered the final pathway of apoptosis. Execution caspases activate cytoplasmic endonuclease which degrades nuclear material and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3, caspase-6, and caspase-7 function as effector or “executioner” caspases, cleaving various substrates including cytokeratins, PARP, the plasma membrane cytoskeletal protein alpha fodrin, the nuclear protein NuMA and others, that ultimately cause the morphological and biochemical changes seen in apoptotic cells (194).

Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). Caspase-3 specifically activates the endonuclease CAD (Caspase Activated DNase). In proliferating cells CAD is complexed with its inhibitor, ICAD. In apoptotic cells, activated caspase-3 cleaves
ICAD to release CAD (190) which then degrades chromosomal DNA within the nuclei and causes chromatin condensation. Caspase-3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies. Gelsolin, an actin binding protein, has been identified as one of the key substrates of activated caspase-3. Caspase-3 cleaves gelsolin and the cleaved fragments of gelsolin, in turn, cleave actin filaments in a calcium independent manner. It results in disruption of the cytoskeleton, intracellular transport, cell division, and signal transduction (195). Phagocytic uptake of apoptotic cells is the last component of apoptosis. Phospholipid asymmetry and externalization of phosphatidylserine on the surface of apoptotic cells and their fragments is the hallmark of this phase. Although the mechanism of phosphatidylserine translocation to the outer leaflet of the cell during apoptosis is not well understood, it has been associated with loss of aminophospholipid translocase activity and nonspecific flip-flop of phospholipids of various classes (146).

Perturbations in regulation of apoptosis have been implicated in a number of diseases. Cancer is a disease that is often characterized by too little apoptosis. Cancer cells typically possess a number of mutations that have allowed them to ignore normal cellular signals regulating their enhanced growth and become more proliferative than normal. Under normal circumstances, damaged cells will undergo apoptosis, but in the case of cancer cells mutations may have occurred that prevent cells from undergoing apoptosis. In these cases there is no check on the cellular proliferation and consequently the disease can progress to the formation of tumors. In many cases these tumors can be difficult to kill since many cancer treatments rely on damaging the cells with radiation or chemicals and mutations in the apoptotic pathway often produce cells that are resistant to this type of attack. Understanding how apoptosis is regulated in cancer is therefore of major interest in the development of treatments for this disease. In fact, suppression of apoptosis during carcinogenesis is thought to play a central role in the development and progression of some cancers (196). There are a variety of molecular mechanisms that tumor cells use to suppress apoptosis. However, effective cytoprotective therapies for these diseases remain a major unmet medical need. To a significant extent, the limited success of cytoprotective drug development can be traced to the simplified view that cell death is either intrinsically regulated by apoptosis or that it is unregulated, caused by stress (197).
Myeloid leukemia is a hematologic malignancy characterized by block in differentiation and abnormal proliferation of clonal myeloid cells. Despite extensive clinical research with numerous combinations of cytotoxic agents the overall prognosis of myeloid leukemia patients remains poor, thus the hunt for better effective agents is ongoing (198). Ormeloxifene (3,4-trans-2,2-dimethyl-3-phenyl-4-p-(beta-pyrrolidinoethoxy) phenyl-7-methoxychroman) also known as centchroman, is a potent non-steroidal selective estrogen receptor modulator (SERM) used as oral contraceptive for birth control. Ormeloxifene (ORM) suppresses the receptors in the reproductive organs like the ovaries, uterus and breasts while it stimulates the estrogen receptors of other organs like bones (129, 135, 136).

In the present study we have explored anticancer activity of Ormeloxifene (ORM) and few other synthetic and natural compounds in myeloid leukemia cells. We show that ORM at an IC$_{50}$ of 7.5µM induces apoptosis in HL60, U937 and K562 cells. However, this induction of apoptosis was more prominent in K562 cells, therefore, we further chose K562 as a model cell line to understand mechanism of apoptosis induction by ORM. We show that ORM induces apoptosis in these cells through activation of Extracellular Signal-Regulated Kinase (ERK) leading to cytochrome-c release and subsequent mitochondria mediated caspase-3 activation. Further, we show that prior treatment of K562 cells with ERK inhibitor PD98059 drastically inhibits ERK phosphorylation, cytochrome-c relase and reduces ORM induced apoptosis. Chronic myeloid leukemia is associated with Bcr-Abl fusion protein which is an oncoprotein necessary for malignant transformation. We show that ormeloxifene treatment in K562 cells inhibits both phosphorylation as well as expression of Bcr-Abl fusion protein which actually is a constitutively active tyrosine kinase and helps in the survival and proliferation of myeloid cells. Thus, in summary, our data shows that ormeloxifene induced apoptosis in K562 cells involves inhibition of Bcr-Abl phosphorylation, phosphorylation of ERK and mitochondria mediated caspase activation.
3.2 Results

In house compounds of CSIR-CDRI were screened for the cytotoxicity in the myeloid leukemia cell lines K562, HL60 and U937. MTT assay was performed for the calculation of IC50 in these cells after 48h of treatment with the compounds. Human embryonic kidney fibroblast HEK293 cells as a control were treated with compounds in a dose dependent manner. The compounds selected for screening were those already known for their efficacy in other diseases and disorders.

CDRI-99/373 is an antiosteoporosis synthetic compound developed by CSIR-CDRI. It is antiresorbing agent. It decreases the expression of calcitonin receptors, the functional marker of activated osteoclasts, and also disrupts F-actin ring in vitro.

Ormeloxifene (ORM) (3,4-trans-2,2-dimethyl-3-phenyl-4-p-(beta-pyrrolidinoethoxy) phenyl-7-methoxychroman) also known as centchroman, is a potent non-steroidal selective estrogen receptor modulator (SERM) used as oral contraceptive for birth control. It is marketed under trade name Saheli by Hindustan Latex Limited. It is also known to have potential anticancer activity in breast cancer (133, 199).

Guggulsterone is a standardized fraction of the plant Commiphora mukul. It possesses hypolipidemic efficacy and is a cholesterol lowering drug. It is manufactured and marketed by Cipla Limited under trade name Gugulip. It is also known to induce apoptosis and differentiation in leukemic cell lines HL60 and U937 (200).

K058 is a natural compound synthesized by CSIR-CDRI. It is currently studied for its role in the treatment of metabolic disorder like diabetes.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>K562</th>
<th>HL60</th>
<th>U937</th>
<th>HEK293</th>
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Table 1: IC50 values of in house compounds of CSIR-CDRI. Data represents the mean ± SEM of three independent experiments.
IC$_{50}$ values were calculated and compound having IC$_{50}$ > 30μM were not considered for further study due to their low cytotoxicity on the leukemic cell lines. Based on MTT assay, IC$_{50}$ of ORM was calculated to be ~7.0μM in leukemic cells compared to >30μM for HEK293, a non leukemic human kidney fibroblast cell line (Table 1). Thus, ormeloxifene was used for further studies in leukemic cell lines.

3.2.1 ORM induces growth inhibition in myeloid leukemia cells

In order to determine growth inhibitory concentration (IC$_{50}$) of ORM, we performed MTT assay in various cell lines. Activity of ormeloxifene in different myeloid leukemia cell lines K562, HL60 and U937 was assessed.

![Graph showing cell viability](image)

**Figure 2:** Cell viability as determined by MTT colorimetric assay, shows % viable cells after myeloid leukemia cells were treated with increasing doses of ORM. Assay was performed on 3 replicates for each treatment and repeated twice. 50% growth inhibition was observed at around 7.5μM. Data represents mean ± SEM.
K562, HL60 and U937 and Human embryonic kidney fibroblast HEK293 cells as control were treated with ormeloxifene for 48h in a dose dependent manner. Percentage viability of cells was measured with MTT assay. Based on MTT assay, IC$_{50}$ of ORM was calculated to be $\sim$7.0µM in leukemic cells compared to $>$30µM for HEK293, a non leukemic human kidney fibroblast cell line (Figure 2). Thus, showing selectivity index of more than 4 for leukemic cells compared to HEK293.

In previously published reports, ORM has been shown to induce apoptosis in breast cancer cells (199) suggesting it to have anticancer property. We therefore hypothesized that ORM might have anti cancer property in the myeloid leukemia cells as well and thus performed a series of experiments to assess anti cancer activities of ormeloxifene in these cells.

### 3.2.2 ORM induces apoptosis in myeloid leukemia cells

#### 3.2.2.1 ORM induces apoptosis in a dose dependent manner

After calculating the IC$_{50}$ of ormeloxifene in myeloid leukemia cells we further sought to assess if this cytotoxicity is via induction of apoptosis in these cells. To assess this, we performed Annexin V–PI assay which detects both early and late apoptosis. Notably, Annexin V is a recombinant phosphatidylserine-binding protein that interacts strongly and specifically with phosphatidylserine residues and can be used for the detection of apoptosis. K562, HL60 and U937 were treated with 1, 5 and 7.5µM of ORM for 48h and Post 48h treatment; cells were washed, stained with Annexin V-PI and analyzed in FACS Flow Cytometer. As shown in figure 3 the FACS flow analysis showed that ORM induces apoptosis in these leukemia cells in a dose dependent manner. The number of apoptotic cells (FITC-Annexin-V and PI double positive cells) in all the ORM treated cells drastically increased from 5-6% in control to 35-55% in cells treated with 5 and 7.5µM ORM. Thus, FACS flow analysis revealed that ORM induces apoptosis in myeloid cells in a dose dependent manner. Notably, maximum numbers of cells undergoing apoptosis were observed in K562 cells.
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Figure 3: Myeloid leukemia cells were treated with increasing doses of ORM for 48h. After induction, Annexin V-PI double positive staining was performed to detect the apoptotic cells by flow cytometer. (unind-uninduced)

The percentage of apoptotic cell population was calculated and a graph was plotted comparing the percentage of apoptotic cells in each myeloid leukemia cell line (Fig 4).
This clearly indicates that the highest percentage of cells that show apoptosis after 48h of induction with 7.5μM of ORM belong to K562 cell line which is in accordance with observed cytotoxicity in Fig 1. This prompted us to use K562 as the representative cell line for all our further experiments.

**Figure 4:** Percentage apoptotic cells by FACs flow cytometry were calculated and a graph was plotted for the comparative analysis of apoptosis in each myeloid leukemia cell line. Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P<0.001 compared to control.

### 3.2.2.2 DNA strand breaks are detected during ORM mediated apoptosis

Further, for validation of apoptosis in K562 cells, we performed TUNEL assay which detects the presence of a multitude of DNA strand breaks and is considered to be the gold standard for identification of apoptotic cells (201). K562 cells at the peak of their growth were treated with 5 and 7.5μM ORM, washed and stained with dUTP as per manufacturer’s protocol. Further, dUTP labeled cells were sorted by FACS flow cytometer which showed that almost 19% of cells have nicked DNA as compared to 2.5% in untreated cells (Figure 5).
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Figure 5: K562 cells were treated with 7.5μM ORM for indicated time points, TUNEL assay was performed using the APO-BrdU kit (Invitrogen), a terminal deoxynucleotidyl transferase (TdT) based end labeling assay for DNA strand breaks. TUNEL positive cells were analysed in Flow cytometry.

In addition, we also assessed the TUNNEL positive cells by fluorescence microscopy. Cells were cytopun, fixed and stained with dUTP for microscopic detection of labeled apoptotic cells. ORM induced apoptotic cell death as identified by Flow cytometer is consistent with the microscopic analysis confirming that ORM indeed induces apoptosis in leukemia cells.
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Figure 6: K562 cells were treated with 7.5µM ORM for indicated time points, TUNEL assay was performed using the APO-BrdU kit (Invitrogen), a terminal deoxynucleotidyl transferase based end labeling assay for DNA strand breaks. Cells were cytospun and Image analysis shows increased no. of apoptotic cells in 7.5µM ORM treated condition after both 24 and 48hrs.

3.2.2.3 ORM mediated apoptosis involves mitochondrial membrane potential loss

In a previous study, ormeloxifene has been reported to induce apoptosis through mitochondria and caspase-mediated pathway in breast cancer cells (133). Therefore, we asked if ORM induced apoptosis in these leukemia cells is also mitochondria mediated.

In order to investigate this, we measured the mitochondrial membrane potential. Note that membrane permeabilization leading to loss of membrane potential promotes apoptosis. For this, K562, HL60 and U937 cells were treated with ORM in a dose dependent manner for 0, 24 and 48hrs.
Figure 7: ORM induces mitochondrial membrane potential. After induction with ORM for indicated time points K562 cells were stained with JC-1 dye and membrane potential loss was analyzed in FACS Flow cytometer. Lower panel graphically shows the changes in mitochondrial potential. Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control.

Cells were washed and stained with cationic dye JC-1. JC-1 dye forms aggregates in healthy cells with a high FL-2 fluorescence indicating a normal mitochondrial membrane potential. Upon onset of apoptosis, mitochondrial membrane potential collapses and results in reduction of FL-2 fluorescence with a concurrent gain in FL-1 fluorescence as the dye shifts from an aggregate to monomeric state (202).
Figure 8: ORM induces mitochondrial membrane potential. After induction with ORM for indicated time points, HL60 cells were stained with JC-1 dye and membrane potential loss was analyzed in FACS Flow cytometer. Lower panel graphically shows the changes in mitochondrial potential. Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control.

The retention of the dye in the cell can be monitored through the increase in FL-1 fluorescence in FACS cytometer. As seen in figure 7, 8 and 9 for K562, HL60 and U973 cells respectively, the ratio (FL2/FL1) of cells depicted against ORM concentration in the representative graph after 24 and 48hrs shows dramatic change in the mitochondrial membrane potential.
Figure 9: ORM induces mitochondrial membrane potential. After induction with ORM for indicated time points U937 cells were stained with JC-1 dye and membrane potential loss was analyzed in FACS Flow cytometer. Lower panel graphically shows the changes in mitochondrial potential. Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control.

Thus, the mitochondrial membrane potential loss is visible in all the three myeloid cells lines K562, HL60 and U937 which suggests that ORM induced apoptosis in these cells is associated with the loss in integrity of the mitochondrial membrane.
3.2.2.4 ORM promotes mitochondria mediated caspase activation

ORM induced mitochondrial membrane potential loss in K562 cells prompted us to hypothesize that ORM induced apoptosis might involve mitochondria mediated apoptosis. Therefore to address the involvement of mitochondria mediated apoptosis we measured caspase 9 and 3 activity using caspase glo assay kit (Promega).

![Caspase 9 and Caspase 3 Activity](image)

**Figure 10:** ORM mediated apoptosis involves caspase 9 and 3 activation as shown by relative luminescence units (RLU) measured using caspase glo assay. Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P<0.001 compared to control.

In this assay, the signal generated is proportional to the amount of caspase activity present. ORM treatment for indicated time points substantially enhanced caspase 9 and caspase 3 activity indicating ORM induced apoptosis in K562 to be apparently via mitochondria mediated caspase activation (figure 10).

To dissect and validate further, we assessed the changes in the expression of molecular signature proteins related to the mitochondria mediated apoptosis.
Figure 11: K562 cells were treated with 7.5μM ORM for indicated days; RIPA lysates were prepared and equal amounts of proteins were resolved in 10% SDS PAGE and immunoblotted with indicated proteins after stripping and reprobing the same membrane, lower panel graphically depicts relative expression of indicated proteins upon ORM treatment at different time points (densitometric analysis).

Mitochondria mediated apoptosis involves increased activation and expression of bax proteins leading to multiple pores in mitochondrial membrane which is followed by release of cytochrome-c from mitochondria intermembrane space into the cytosol (203).
In agreement with this we observed significant increase in the expression of bax and cytochrome-c levels (figure 11). In addition, we also observed differential expression of anti-apoptotic protein Bcl-2. Furthermore, since either intrinsic or extrinsic, both modes of apoptosis lead to PARP cleavage by activation of caspase-3 for execution of efficient apoptosis, we assessed the activation of caspase 3 and subsequent PARP cleavage by ORM. Immunoblot against caspase 3 and cleaved PARP clearly showed increase in caspase 3 and cleaved PARP products suggesting ORM indeed promotes efficient apoptosis.

Together with the loss of mitochondrial membrane potential, increase in bax expression, release of cytochrome-c followed by activation of caspase and PARP cleavage, these data demonstrate that ORM induced apoptosis is through mitochondrial mediated caspase activation.

3.2.2.5 Apoptosis related morphological changes are detected upon ORM treatment

Morphological changes pertaining to apoptosis upon ORM induction in K562 cells were assessed by hoechst staining. This dye stains minor groove of DNA with AT region selectivity. This dye binds to all the nucleic acid but AT rich dsDNA strands enhance fluorescence greater than GC rich strands of the DNA.

After induction with 7.5µM of ORM for 24h and 48h, cells were cytospun and stained with Hoechst dye. Microscopic analysis showed apoptosis related morphological changes such as formation of apoptotic bodies, membrane blebbing and chromatin condensation in ORM treated cells which further confirmed that ORM induces apoptosis in myeloid leukemia cells K562 (figure 12). White arrows indicate the vacuole and apoptotic bodies formed upon ORM treatment in K562 cells.
Figure 12: K562 cells were cytospun after treatment with 7.5μM ORM for 24 and 48h. Slides were stained with Hoechst stain and visualized under fluorescent microscope.

3.2.2.6 ORM induced apoptosis involves activation of Extracellular Signal Regulated Kinase (ERK)

In order to investigate further the signaling involved in ORM mediated apoptosis, we assessed the phosphorylation status of ERK; because in many cases where an anticancer drug/molecule induces apoptosis in K562 cells, ERK activation has apparently been involved (204).

To examine this, K562 cells were treated with 7.5μM ORM for indicated time points. Post ORM treatment, whole cell extracts were prepared, separated on 10% SDS PAGE and immunoblotted with pERK antibody which showed dramatic increase in ERK phosphorylation as shown in figure 13. Further, the same membrane was stripped and reprobed with cytochrome-c which showed a direct correlation between multitude of ERK activation and induction of
apoptosis (as measured by cytochrome-c release). Histogram in the lower panel depicts densitometry analysis of the relative expression changes in the immunoblot.

![Graph showing relative expression changes in immunoblot](image)

**Figure 13:** Whole cell extracts of ORM treated K562 cells after indicated time points were separated on 10% SDS PAGE and probed with phospho ERK, cytochrome-c and loading control (β-actin). Lower panel graphically depicts relative expression (densitometry) in immunoblots.

Next, K562 cells were treated with ERK inhibitor PD98059 for 1h prior to ORM treatment. This resulted in inhibition of ORM mediated ERK phosphorylation and dramatic block in cytochrome-c release with concomitant ERK inhibition by PD98059 suggesting that ORM mediated apoptosis involves ERK activation.
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**Figure 14:** K562 cells pretreated for 1h with 10μM PD98059 were treated with ORM for indicated time points, whole cell extracts were prepared and separated on 10% SDS-PAGE, immunoblotted and probed with pERK, cytochrome-c and β-actin; PMA 10ng/ml for 2h was used as positive inducer of ERK phosphorylation. Lower panel graphically depicts relative expression (densitometry) in immunoblots.

Histogram in the lower panel depicts densitometry analysis of the relative expression changes in the immunoblot.

This finding was further manifested by reversal of ORM mediated apoptosis in FACS Flow cytometer analysis where these cells were treated with 10μM ERK inhibitor (PD98059) 1h prior to the ORM treatment. Flow cytometry analysis performed in K562 cells after treatment with
ORM in the absence or presence of ERK inhibitor PD98059 showed that ORM mediated apoptosis is substantially inhibited in the presence of PD98059 (figure 15).

**Figure 15:** Percentage of Annexin V-PI double positive cells were assessed by FACS flow cytometer in the presence of ORM alone and together treated with ERK inhibitor. Lower panel graphically depicts the percentage of double positive cells. Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control.

Furthermore, because PD98059 treatment substantially reduced the apoptotic effects of ORM, we further determined the expression of cleaved PARP by pre treating K562 cells with PD98059 before induction with ORM. PARP cleavage in ORM treated cells pretreated with PD98059 was dramatically inhibited as seen in figure 16.
ORM induces apoptosis

Figure 16: Immunoblot against cleaved PARP with ORM alone and after 1h pretreatment with ERK inhibitor. β actin is used as a loading control.

Taken together, these data affirm that ORM induced apoptosis is mediated by activation of ERK and subsequent release of cytochrome-c leading to caspase-3 activation and thus culminating in increased apoptosis. Importantly these effects could substantially be reversed by ERK inhibitor PD98059.

3.2.3 ORM inhibits cell proliferation and induces G0-G1 growth arrest

3.2.3.1 ORM inhibits cells cycle at G0-G1 phase

ORM efficiently induced cytotoxicity in K562 cells. In addition we showed that this cytotoxicity is via induction of apoptosis. However, since K562 is a highly proliferative due to presence of the activated oncogenic Bcr-Abl fusion protein, we sought to examine if ORM also inhibits proliferation by inducing growth arrest in these cells. To address this, K562 cells were treated with different doses of ORM for indicated time points and were assessed by PI staining for the distribution of cells in different phases of cell cycle using FACS Flow Cytometer. Marked increase in the no. of cells (62%) in G0-G1 phase of the cell cycle as compared to control (38%)
was observed suggesting ORM induced cytotoxicity to be coupled with growth arrest in this phase of cell cycle (figure 17).

![Graph showing cell cycle distribution](image)

**Figure 17**: Distribution of cells in different phases of cell cycle in K562 cells as analyzed by Flow cytometry after 24 and 48h of culture is depicted. Sub-G1 designates the fraction of cells that is undergoing apoptosis. The percentage of cells in each phases of cycle is also depicted in the graph (lower panel). Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P<0.001 compared to control.

Note that no. of cells after 48h in sub-G1 state is also enhanced which indicates marked increase in the apoptotic cells and further strengthens our apoptosis related finding in this study.
Figure 18: Distribution of cells in different phases of cell cycle in HL60 cells as analyzed by Flow cytometry after 24 and 48h of culture is depicted. Sub-G1 designates the fraction of cells that is undergoing apoptosis. The percentage of cells in each phases of cycle is also depicted in the graph (lower panel). Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P<0.001 compared to control.

Furthermore, HL60 and U937 cells were also treated with ORM for 24 and 48h for the cell cycle analysis. After induction with ORM, cells were stained with PI solution and analyzed in flow cytometer. As observed with K562, marked increase in the percentage of cells in the G0-G1 phase of the cell cycle as compared to the control was observed in these cells as well.
Figure 19: Distribution of cells in different phases of cell cycle in U937 cells as analyzed by Flow cytometry after 24 and 48h of culture is depicted. Sub-G1 designates the fraction of cells that is undergoing apoptosis. The percentage of cells in each phases of cycle is also depicted in the graph (lower panel). Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control.

Ormeloixifene treatment in HL60 and U937 cells inhibits cell cycle proliferation which validates our previous data that ORM induction leads to cytotoxicity and apoptosis in these cell lines.

Based on our finding, we can conclude that like K562, ORM treatment is equally effective in the acute myeloid leukemia cells HL60 and U937.
3.2.3.2 p21 up regulation leads to cell cycle inhibition

To elucidate the mode of ORM mediated growth arrest, changes in the molecular proteins associated with block of proliferation and G0-G1 arrest were assessed. As K562 is highly proliferative cell line, we analyzed the changes in the mRNA expression level of c-Myc, a well known marker of proliferation (205, 206) and p21, a cyclin dependent kinase inhibitor known to be upregulated during G0-G1 growth arrest (207, 208) with RT-PCR and quantitative PCR (real time PCR).

![Graphs showing mRNA expression of p21 and c-myc](image)

**Figure 20:** (a, b) Real Time PCR shows relative mRNA expression of p21 and c-myc upon ORM treatment after indicated time points. (c) RT-PCR analysis shows the downregulation of c-Myc and upregulation of p21 after ORM treatment in K562 cells.

The analysis revealed dramatic decrease in the mRNA level of c-Myc and increase in p21. In addition, concomitant decrease in cdk2 expression was observed with increase in p21 protein levels which again is associated with transition of cells from G1 to S phase.

We further corroborated our finding by assessing the changes in the protein expression of c-Myc and p21 upon 7.5μM ORM treatment for 24 and 48hrs compared to control cells.
Significant decrease in the protein levels of c-Myc upon ORM treatment was observed which corresponded well to the changes observed in the mRNA expression levels. p21 protein levels were also enhanced upon ORM treatment which approved the cell cycle inhibitory effects of ormeloxifene.

![Immunoblot with p21, c-Myc and cdk2 antibody showing reciprocal protein expression of p21 (upregulated) and c-Myc, cdk2 (down regulated). Lower panel graphically depicts relative expression levels (densitometry) in the immunoblots.](image)

Thus, taken together these data suggest that ORM inhibits K562 cell proliferation by inducing G0-G1 growth arrest via modulating the expression levels of molecules associated with it.

3.2.4 Ormeloxifene inhibits Bcr-Abl phosphorylation

The antiapoptotic activity of Bcr-Abl significantly contributes to the development of CML. Ber-Abl may function either by enhancing the proliferation potential of hematopoietic progenitors or
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by protecting these progenitors from apoptosis. Imatinib mesylate (STI571, Gleevec®) developed by Novartis is the first successful, rationally developed, receptor-targeted agent for chronic myelogenous leukemia (CML). Imatinib inhibits the constitutively active Bcr-Abl tyrosine kinase protein encoded by the fusion gene generated by the Philadelphia chromosome translocation, which actually is responsible for the pathogenesis of CML. Moreover, pre-clinical and clinical studies have shown that imatinib specifically inhibits the proliferation of cells expressing Bcr-Abl both in-vivo and in-vitro (209).

Based on this, we hypothesized that ability of ormeloxifene to induce apoptosis in K562 cells may also involve inhibition of Bcr-Abl. To investigate this, K562 cells were treated with 7.5µM ORM and were harvested after indicated time points (figure 22). Protein lysates were separated on 10% SDS PAGE and were probed with c-Ab! antibody. Like with Imatinib (IM) treated cells, marked decrease in Bcr-Abl protein expression after 48hrs was observed.

![Figure 22](image)

Figure 22: Whole cell extracts of ORM treated K562 cells after indicated time points were separated on 10% SDS PAGE and probed with c-Abl antibody, 293T alone served as negative control for Bcr-Abl (upper panel). IM- Imatinib mesylate, 10µM)

Furthermore, since constitutive tyrosine phosphorylation of Bcr-Abl is required for its leukemogenic function, we assessed if ORM can inhibit tyrosine phosphorylation of Bcr-Abl. To answer this, cells were again treated with 7.5µM of ORM and protein lysates were prepared after indicated time points (figure 23). Whole cell extracts were separated on 10% SDS PAGE and were probed with phosphotyrosine antibody.
Figure 23: (a) Whole cell extracts of ORM treated K562 cells after indicated time points were separated on 10% SDS PAGE and probed with phosphotyrosine antibody. (b) Same membrane was stripped and reprobed with c-Ab! antibody (Lower panel).

As shown in figure 23a dramatic decrease in phosphorylation of Bcr-Abl was observed. Reprobing of same membrane after stripping with Bcr-Abl again showed marked decrease in BCR-ABL protein expression (Figure 23b).

Taken together, these data suggest that ORM induced apoptosis in K562 cells is apparently associated with inhibition of both phosphorylation as well as protein expression of Bcr-Abl leading to inactivation of survival pathways.
3.2.5 Ormeloxifene mediated apoptosis is translatable in CML patients

Peripheral blood from two CML patients after their prior consent was kindly provided by Dr. Bhatt (CSMMU, Lucknow). Mononuclear cells were isolated from these cells using ficoll hypaque density gradient centrifugation as described in materials and methods.

Figure 24: Mononuclear cells isolated from two CML patients were cultured in RPMI and treated with 7.5μM ORM for three days. Hoechst staining shows visible apoptosis like features in treated cells as compared to control untreated cells.

Isolated mononuclear cells were cultured for 24h in RPMI-1640 supplemented with 10% FBS. Post 24h culture; cells were treated with 7.5μM ORM and cytospun on slides after 3 days. Cytospun cells were stained with Hoechst and visualized under microscope for morphological changes. Significant chromatin condensation and nuclear membrane blebbing were observed in ORM treated cells as compared to control cells.
3.3 Discussion

In the present study, for the first time we show that ORM, reportedly first non-steroidal SERM induces growth arrest and apoptosis in myeloid leukemia cells, K562 in particular. Like in breast cancer cells, 50% growth inhibitory concentration of ORM using MTT assay for myeloid leukemia cells was calculated at around 7.5μM. Note that, to induce similar extent of growth inhibition in HEK293 (considered as control) cells, more than 30μM of ORM was needed. The ability to evade apoptosis through a gain of antiapoptotic function or loss of proapoptotic signal is a hallmark of cancer. Therefore, studies involving therapeutic agents focus on selective growth inhibition and induction of apoptosis in cancer cells. Consistent with this, we also show that ORM induces apoptosis in myeloid leukemia cells U937, HL60 and K562. Interestingly, the induction of apoptosis was more prominent in K562 cells, therefore, we used it as a model cell line for our further studies. The rationale was that these cells harbor a fusion protein Bcr-Abl produced due to a reciprocal translocation between chromosomes 9 and 22 which imparts excessive proliferation and higher survival rate in these cells and hence anticancer effects of ORM would be more prominent.

Apoptosis is characterized by morphological and biochemical parameters such as chromatin condensation, membrane blebbing, membrane permeabilization, the formation of apoptotic bodies, chromatin fragmentation and phosphatidylserine exposure (203, 210). Consistent with this, flow cytometry analysis of TUNEL positive cells further confirmed the apoptosis inducing potential of ORM in K562 cells. TUNEL assay detects the presence of a multitude of DNA strand breaks and is considered to be the gold standard for identification of apoptotic cells (201). Studies demonstrating loss of mitochondrial membrane potential and caspase 9 and 3 activation further substantiated ORM mediated apoptosis in these cells.

Several studies indicate that apoptosis induced by a therapeutic agent in various cell types requires activation of the caspase pathways (209, 211). Therefore, we assumed that ORM induced apoptosis in myeloid leukemia cells, K562 in particular might also be mitochondria mediated. In line with this, we show that the loss of mitochondrial membrane potential in ORM induced cells is coupled with concomitant increase in Bax expression and release of cytochrome-c. A differential regulation of Bcl2 expression was also seen which may be attributed to K562 cell survival response against ORM. In addition, activation of Caspase 3; main executor of
apoptotic process and subsequent cleavage of PARP further confirmed that ORM induced apoptosis is indeed mitochondria mediated.

Our data nicely correlates the activation of ERK phosphorylation with release of cytochrome-c which initiates mitochondria mediated caspase activation. Note that ERK activation by various compounds is reported to be followed by release of cytochrome-c leading to apoptosis and this can be inhibited by blocking the ERK activity (204, 212, 213). Consistent with this, ORM treatment enhanced phosphorylation of ERK which is followed by increase in cytochrome c release. Furthermore, our data shows that inhibition of ERK phosphorylation by PD98059 reversed the ORM mediated apoptotic effects as shown in immunoblot showing inhibition of cytochrome-c release and reduced formation of cleaved PARP products. This is also obvious in FACS flow cytometer which shows dramatic reduction in annexinV-PI stained cells. Notably, apoptosis can be initiated by extracellular (non-mitochondrial) or intracellular (mitochondrial) signals and as such two major pathways can be distinguished, they are not isolated, and in fact have considerable overlap. Therefore, the possibility of apoptosis induction in these cells through non-mitochondrial mediated pathways may not be ruled out (214).

Significant advances made in cancer therapy during the last decade as our understanding of molecular biology and leukemogenesis evolved, suggests that leukemic cell proliferation and apoptosis should be targeted by antileukemic agents. In line with this ORM mediated inhibition of proliferation is also associated with arrest of cells in G0-G1 phase of cell cycle with reciprocal expression of p21 and c-Myc regulatory proteins known to be involved in progression of cells from G0-G1 to S phase. In addition, our finding that Ormeloxifene induces growth arrest and apoptosis in chronic myeloid leukemia cells is in accordance with inhibition of both phosphorylation and protein expression of oncogenic protein Bcr-Abl which is a hallmark signature protein of these cancer cells. Since Bcr-Abl provides proliferative and subsequent survival advantage to these cells, it makes sense that inhibition of this protein is a primary requirement. In agreement with this, we show that ORM does inhibit BCR-ABL protein which may further support the notion that ORM induces apoptosis and growth arrest in these myeloid leukemia cells.
Figure 25: Hypothetical model - Based on our findings we propose a hypothetical model of ORM mode of apoptosis induction in K562 cells.

Based on our findings, we propose a hypothetical model where treatment of K562 cells with ORM activates ERK leading to cytochrome-c release, caspase 9, 3 activation and subsequent PARP cleavage culminating in enhanced apoptosis of these cells. This ERK induced mitochondria mediated apoptosis can be substantially inhibited by use of ERK inhibitor. In addition, ORM also promotes G0-G1 growth arrest of these cells by reciprocally modulating the expression molecular signature proteins of G0-G1 to S phase cell cycle transition.

In conclusion, we propose a hypothetical model which shows that in addition to inducing G0-G1 growth arrest, ORM induces apoptosis in chronic myeloid leukemia cells K562 via phosphorylation of ERK leading to mitochondria mediated caspase activation. Although the
precise mechanistic details of ormeloxifene mediated effects may need intensive investigation, it offers significant hope for progress both on its own and in conjunction with classical cytotoxic chemotherapy for the treatment of leukemogenic malignancies.