CHAPTER - 4

APOPTOSIS OF COMBRETASTATIN A4 AND DERIVATIVES
4.1. INTRODUCTION

Apoptosis has its origin from a 'Greek' word meaning, "the falling of leaves from a tree or petals from a flower". As leaves falling from a tree die a characteristic death and are reabsorbed into the earth because they also are no longer necessary, apoptotic cells die via unique processes, and are reabsorbed because they are no longer necessary and must make room for new cells.

Apoptosis is scientifically defined as the cellular morphological process leading to deliberate controlled self-destruction (Lockshin and Williams, 1964). Apoptosis is a process of programmed cell death that plays a critical role in normal embryologic development, homeostasis, tumor surveillance, function of the immune system and organ regression in the senescence process (Van den Hoff et al., 2000). In order to maintain homeostasis of cell numbers in normal tissues, the ratio of cell proliferation to cell death must remain constant over time.

During apoptosis, several morphological changes occur that distinguish the apoptotic process from other cell death processes, such as necrosis. Necrosis induces major damage at the cell membrane that results in cell swelling and lysis. The intracellular contents released by necrosis provoke an inflammatory response. However, apoptosis is initiated by activation of endogenous proteases that result in the cleavage of chromatin into oligonucleosome length DNA fragments (Jiang and Wang, 2004). Chromatin is made to form into dense crescent shaped aggregates under the nuclear membrane. The plasma membrane integrity is initially preserved, but subsequent convolution of the plasma membrane results in the formation of clusters of membrane-bound cellular organelles referred to as "apoptotic bodies" (Jaattela, 2004).

Since cell swelling does not occur and the cytoplasmic contents are not released into the extracellular space, inflammatory reaction is not observed. Otherwise physiologic cell turnover, development and homeostasis would result in extensive inflammation throughout the body. Apoptotic bodies are phagocytosed by tissue macrophages and possibly viable neighboring cells (Jiang and Wang, 2004). While apoptotic bodies provide an ultrastructural evidence of apoptosis, DNA fragmentation is now accepted as the
biochemical marker of apoptosis. DNA fragmentation is demonstrated by the characteristic ladder pattern on DNA by agarose gel electrophoresis; the ladder represents DNA fragments of nucleosomal size of 180 base pairs or its multiples (Narula et al., 1997). Other significant changes in apoptotic cells include phosphatidylserine translocation in the plasma membrane, initiation of caspase cascade and activation of PARP (Thornberry and Lazebnik, 1998; Wieder et al., 2001).
4.2. REVIEW OF LITERATURE

Several different apoptotic pathways are reported. The compressive of apoptosis are described below.

4.2.1. Apoptotic pathways

A complex intracellular signaling network is initiated by a variety of apoptotic stimuli from inside or outside the cell, in addition to the morphological changes that occur during the apoptotic process. These stimuli include developmental death signals, stimulation of cell surface death receptors, deprivation of survival signals or DNA damage incurred from chemotherapy drugs or radiation. Previous studies indicate that two distinct pathways lead to apoptosis. The extrinsic pathway or death receptor-mediated apoptotic pathway, is evoked upon stimulation of death receptors in the tumor necrosis factor receptor family. The intrinsic pathway or mitochondrial apoptotic pathway, is evoked by stimuli that cause DNA damage or internal damage to the cell (Fig. 4.1).

4.2.1.1. Extrinsic or death receptor pathway

The extrinsic death receptor-mediated apoptosis pathway is stimulated by ligand binding to members of the tumor necrosis factor receptor superfamily including TNFR-1, Fas/CD95, and TRAIL receptors DR4 and DR5 (Ashkenazi, 2002). Trimerization of death receptors is required for its activation. Receptor activation is stimulated by binding of extracellular trimeric ligands to TNF receptors by bringing three death receptors in close proximity, whereupon they form an active, trimerized death signaling complex (Jiang and Wang, 2004; Jaattela, 2004). Death receptors contain a conserved DD which upon activation, recruits the death adaptor proteins such as TRADD (in the case of TRAIL) and FADD (in the case of Fas ligand) (Naismith and Sprang, 1998). Apoptotic signals are transmitted through death domains via death receptor pathway. The adaptor proteins themselves contain death domains that have a high affinity for active membrane bound death domains. The conglomeration of trimerized ligand, receptor and adaptor proteins is known as the DISC (Stanger et al., 1996). In addition to their N-terminal death domains, death adaptor proteins also contain C-terminal DEDs. The N-terminal region of the zymogen form of caspase-8 protease contains a DED that binds with high affinity to the
DISC (Sun et al., 1999). Recruitment of procaspase-8 molecules to the DISC brings them in close proximity to each other, where they have the capacity to autoactivate via a process termed “proximity-induced autoactivation”. The activated caspase-8 then cleaves and activates the executioner caspase, caspase-3. Activation of executioner caspases leads to downstream proteolysis of cellular substrate proteins (Jaattela, 2004).

4.2.1.2. Intrinsic or mitochondrial pathway

The intrinsic pathway is mediated by mitochondrial apoptosis. Signals in this pathway are initiated within the cells, such as from DNA damage induced by chemotherapeutic agents or radiation. These agents disturb the mitochondrial membrane to release mitochondrial apoptogenic proteins, which leads to mitochondrial dysfunction (Chen et al., 2000; Jiang and Wang, 2004;). Cytochrome c is one such apoptogenic factor, functions in normal cells as an electron carrier in the mitochondrial respiratory electron-transport chain (Myllykallio et al., 1999). After the induction of apoptotic stimuli, cytochrome c is released from mitochondria into the cytosol. Cytochrome c release is closely regulated by multiple genes to prevent its accidental stimulation. Bcl2 family members, which have both anti and pro-apoptotic genes are partly responsible for cytochrome c release from mitochondria. Subsequent to apoptotic stimulus, some pro-apoptotic factors are activated via oligomerization (e.g. Bax and Bak) or via cleavage (e.g. Bid). Anti-apoptotic factors (e.g. Bcl2 and BclXL) are inactivated via cleavage or via binding of regulatory proteins that suppresses their inhibitory function (Adams and Cory, 1998). Outer mitochondrial membrane contains Bcl2 and BclXL that inhibit cytochrome c release. In previous studies, it has been shown that release of cytochrome c from the mitochondria requires calcium efflux from the endoplasmic reticulum (Boehning et al., 2003; Mattson et al., 2003). Cytochrome c binds to Apaf-1, which stimulates its oligomerization to form cytochrom c Apaf-1 complex (Perkins et al., 2000). The cytochrome c Apaf-1 complex then binds the zymogen form of caspase-9, forming a complex known as the apoptosome. Formation of the apoptosome evokes catalytic activation of caspase-9, a process necessary for chemotherapy-induced apoptosis. Once activated, caspase-9 cleaves and activates executioner caspases, including caspase-3 and 7. Activation of the executioner caspases culminates in cell death (Perkins et al., 2000). It has been reported that p53 mediates apoptosis of cardiac myocytes stimulated with angiotensin II (Pierzchalski et al., 1997;
Leri et al., 1998). These studies strongly suggest that p53, Bax and mitochondria play key roles in apoptosis of cardiac myocytes.

**Extrinsic Pathway**

**Intrinsic Pathway**

![Diagram of Extrinsic and Intrinsic Pathways of Apoptosis](image)

**Figure 4.1: Intrinsic and Extrinsic pathways of apoptosis**

Intrinsic and Extrinsic pathways of apoptosis. Extrinsic pathway is triggered by death receptor engagement and intrinsic pathway occurs when various apoptotic stimuli trigger the release of cyt c from mitochondria. Apaf-1, apoptosis activating factor 1; Bak, bacille Calmette–Guérin; Bax, Bcl2 associated protein; Bid, pro-apoptotic Bcl2 family member; Bim, pro-apoptotic Bcl2 family member; DIABLO, direct IAP binding protein with low PI; FADD, Fas associated death domain protein; IAP, inhibitors of apoptosis; Smac, second mitochondria-derived activator of caspase; tBid, truncated beta interaction domain.
4.2.2. p53 and apoptosis

The tumor suppressing protein p53 plays pivotal roles in apoptosis. It transmits signal for cell death or apoptosis during cellular stress, DNA damage, hypoxia and nucleotide deprivation (Regula and Kirshenbaum, 2001). It is composed of a conserved N-terminal transactivation domain, a proline rich domain, DNA binding domain, a tetramerization domain and a basic C-terminal tail. The transcription activation of p53 appears to be an important mechanism to induce apoptosis, although apoptosis has occurred in its absence as well (Gupta et al., 2001). Although the upstream events leading to p53 activation such as phosphorylation is not clear (Deiry, 1998) downstream events involve activation of pro-apoptotic proteins like Bax and Fas (Miyashita and Reed, 1995). In the past few years, studies have substantiated the fact that transcription activation of p53 is caused by phosphorylation in the N and C terminal domains. In fact p53 has been shown to be phosphorylated on the serine residues by a number of kinases, including MAP kinases at Ser 15, 20, and 389 (Huang et al., 1999; Shieh et al., 1999; She et al., 2000), check point kinase-2 at Ser 20 and 33 (Hirao et al., 2000) and DNA-dependent protein kinase at Ser15 and 37 (Shieh et al., 1997; Smith and Jackson, 1999).

In contrast, MDM2, which is a negative regulator of p53, plays a unique relationship with p53 (Haupt et al., 1997). MDM2 interacts with p53 at the N-terminal transactivation domain to inactivate the transcription activity and to induce ubiquitin-mediated proteolytic p53 degradation. Conversely, p53 binds to the specific promoter region of MDM2 gene and stimulates its transcription (Momand et al., 2000). Nevertheless the interaction between p53 and MDM2 is blocked by phosphorylation of p53, there by protecting p53 degradation. This is brought about by genotoxic or oxidative stress, leading to the accumulation and elevation of intracellular p53 protein levels (Shieh et al., 1997).

Recent studies have shown the involvement of p53 in cardiac myocyte apoptosis. p53 mediated apoptosis was observed during cardiac failure, ischemia reperfusion and cardiac hypertrophy (Pierzchalski et al., 1997; Leri et al., 1998; Fortuno et al., 1999). One study in dog heart showed the expression of ventricular p53 and its target gene Bax significantly increased, causing an increase in myocytes apoptosis, followed by rapid ventricular pairing (Leri et al., 1998). In another study involving mice transfection of p53 into
ventricular myocytes with the adenovirus induced apoptosis through mitochondria-dependent pathway (Regula and Kirshenbaum, 2001). An over expression of Bcl$_2$ blocked p53 apoptosis (Kirshenbaum and de Moissacet, 1997). These studies substantiated the fact that p53 plays an important role in myocyte apoptosis.

4.2.3. Bcl$_2$ family proteins and apoptosis

Studies in the recent years have revealed the role of mitochondria and its connection with Bcl$_2$ family proteins in apoptosis in various cell lines (Green and Reed, 1998). The role of mitochondria in ischemic cardiac myocytes has been demonstrated in regard of two aspects. 1) Release of cytochrome c into cytosol resulting in the activation of caspases (Borutaite et al., 2001), mitochondrial inner membrane potential ($\Delta\psi$M) is decreased during apoptosis (Susin et al., 1998) and the loss of membrane potential ($\Delta\psi$M) results in the opening of a large multi-conductance channel, also known as mitochondrial PTP. Although the mechanism of PTP is still enigma, studies have hypothesized its regulation with the Bcl$_2$ family proteins (Marzo et al., 1998). The Bcl$_2$ family proteins are found to be located in the outer membrane of the mitochondrial or in the cytoplasm (Reed et al., 1998). They can be divided into two classes viz pro and anti-apoptotic proteins based on their functions. The anti apoptotic proteins include Bcl$_2$ and Bcl$_{xL}$, while the pro apoptotic proteins include Bax, Bad, Bid, Bak, Bcl-x, Bik, and Bim (Adam and Cory, 1998). The Bcl$_2$ family proteins are involved in the regulation of two important aspects of the mitochondrial pathophysiology namely 1) PTP opening; 2) release of the apoptotic proteins from the mitochondria into the cytosol. In one experiment when mitochondria PTP protein complex was partially purified by reconstituting in liposomes, it was seen that some of the functions were maintained including the suppression of pore openings by recombinant Bcl$_2$ but not mutant Bcl$_2$ proteins. From this, it was inferred that Bcl$_2$ proteins directly participate in the regulation of PTP (Zamzami et al., 2000).

The release of cytochrome c is guided by the channels of Bcl$_2$ family proteins, which form different sized pores or ion-channels. Usually Bcl$_2$ tend to form small channels, whereas Bax tend to form larger channels. It has also been proposed that the translocation of Bad and Bax from the cytosol to the mitochondria and their interaction with Bcl$_2$ forming heterodimers (e.g. Bax/Bcl$_2$) is a critical factor in regulating cytochrome c release.
(Oltvai et al., 1993). Recently, investigators have shown that H$_2$O$_2$ and O$_2^{*\cdot}$ induce distinctly different apoptotic pathways in cardiomyocytes (Von Harsdorf et al., 1999). H$_2$O$_2$ but not O$_2^{*\cdot}$ exposure to myocytes promoted translocation of the pro-apoptotic proteins (Bax, Bad) to the mitochondria, formation of heterodimers with Bcl$_2$ and the subsequent release of cytochrome c. There is increased evidence connecting p53 to intracellular oxidant formation (Wang et al., 2000; Huang et al., 2000). p53 activation may directly induce the activation of Bax gene, which contains p53 binding site. As both O$_2^{*\cdot}$ and H$_2$O$_2$ induce p53, it is critical to understand how oxidants modulate the translocation of Bax/Bad from the cytosol to the mitochondria. This information is lacking in Dox-induced myocyte and endothelial apoptosis. Regardless of the mechanism, the pro-apoptotic Bcl$_2$ family proteins induce the release of cytochrome c into the cytosol, initiating the activation of caspases in the presence of Apaf-1 protein.

4.2.3. Apoptosis and caspases

During apoptosis, both intrinsic and extrinsic pathways culminate at common point, which lead to activation photolytic enzymes that are known as caspases. These caspases are responsible for the degradation of proteins and concomitant destruction of the cell. In most of the apoptotic stimuli caspases execute the induction of apoptosis. In the recent years, the role of caspases in apoptosis has been significantly portrayed (Yue et al., 1998; Earnshaw et al., 1999; Hacker, 2000; Communal et al., 2002). Caspases are a group of proteolytic enzymes that can specifically cleave pro-enzymes into active enzymes (Thornberry and Lazebnik, 1998). The pro-enzyme forms of all caspases contain an N-terminal prodomain, followed by a large and a small subunit. During activation, prodomain is cleaved and degraded, while the large and small subunits heterodimerize to form a complex containing two large subunits and two small subunits (Cohen, 1997).

Functionally caspases are classified into two groups viz. initiator caspases and executioner caspases. The initiator caspases are caspases-2 and 9 (for the intrinsic pathway) and caspases-8 and 10 (for the extrinsic pathway). They are activated following binding of ligand to death receptor or following cytochrome c release and apoptosome complex formation (Chang and Yang, 2000). Initiator caspases cleave following activation and activate executioner caspases (caspases-3, 6, 7 and 12), so called because they elicit
cell death through cleavage of substrates that are critical to the normal function of the cell. Initiator caspases differ structurally from the executioner caspases in that they have a very long pro-domain that contains essential sequences for adaptor binding, while the pro-domains of executioner caspases are short and act solely to inhibit caspase activation (Cohen, 1997; Chang and Yang, 2000).

In apoptotic cascade, caspases cleave more than 60 substrate proteins that are necessary for normal cellular function. This extensive list of substrates includes proteins necessary for DNA metabolism (caspase-activated DNase), cytoskeletal scaffold proteins (lamins, gelsolin), cell cycle regulators (Cdk inhibitors), repair and housekeeping proteins PARP and signaling molecules (MEKK1, MAP3Ks) (Chen et al., 2000; Chang and Yang, 2000; Creagh and Martin, 2001; Garden et al., 2002). Studies have shown that p53 mediated apoptosis is coupled with the activation of several caspases in different cell lines. In p53-induced apoptosis, mainly caspase-3 and caspase-9 are activated. For instance, presenilins treatment in HEK cells activates caspase-3, which is p53 dependent (Alves et al., 2002), similar activation of caspase 3 and caspase 9 seen in colon cancer cells (Bartke et al., 2001). Interestingly, p53 induces apoptosis through the activation of caspases-3 and 9 in human endothelial cells exposed to Dox (Lorenzo et al., 2002). According to Narula et al. (1999), caspase 3 is activated to result in apoptosis during human cardiomyopathy.

4.2.4. Pro-apoptotic activity of combretastatin A-4/A-4P

CA-4-P destabilizes microtubules and causes endothelial cell death. The mechanisms by which CA-4-P induces the death of proliferating endothelial cells was demonstrated by Kanthou et al., (2004). CA4P at >/=7.5 nM damages mitotic spindles, arrests cells at metaphase and lead to the death of mitotic cells with characteristic G(2)/M DNA content. Mitotic arrest was associated with elevated levels of cyclin B1 protein and p34(cdc2) activity. Inhibition of p34(cdc2) activity by purvalanol-A caused mitotic-arrested cells to rapidly exit mitosis, suggesting that sustained p34(cdc2) activity was responsible for metaphase arrest. Pharmacological prevention of entry into mitosis protected cells from undergoing cell death, further establishing the link between mitosis and cell death induction by CA-4-P. CA-4-P-mediated cell death shared characteristics of apoptosis but was independent of caspase activation suggesting the involvement of a non-caspase...
pathway(s). The above statement was also proven by Vincent et al., (2005), stating that CA4P-induced endothelial cell death was not associated with activation of caspase-3 or caspase-9, and the ratio of the level of the anti-apoptotic protein Bcl-2 to that of the pro-apoptotic protein Bax was not altered. The induction of apoptosis in endothelial cells by CA-4-P is associated with prolonged mitotic arrest. Therefore, by activating cell death pathways.
4.3. MATERIALS AND METHODS

4.3.1. Materials

Recombinant human FGF-2 was procured from R&D Systems. DMEM (Dulbecco’s modified eagles medium) and fetal bovine serum (FBS) was procured from Gibco BRL (USA). DMSO was from Merck (India). PI, DAPI, guanosine 5’-triphosphate (GTP) and PIPES were obtained from Sigma, (St.Louis, USA). Antibiotic solution (containing penicillin and streptomycin) was obtained from Hyclone (USA). NP-40 was purchased from Calbiochem. Combretastatin A-4 (CA-4), compound 11 and compound 42 were got synthesized at the National Chemical Laboratory, Pune, India. Sterile disposable tissue-culture grade plastic-ware was from Nunc (Denmark) or Tarsons (India).

4.3.2. Cell culture

Human Umbilical Vein Endothelial cells (HUVEC), Endothelial Cell Basal Medium (EBM), EGM singleQuot kit containing bovine brain extract, endothelial growth factor (hEGF), hydrocortisone, Gentamicin Amphotericin B and fetal bovine serum and X-Vivo medium were procured from Lonza, Walkersville, MD. HUVECs were cultured in endothelial cell basal (ECB) medium containing bovine brain extract, 20 µg/ml endothelial growth factor (hEGF), hydrocortisone, gentamicin (0.25 mg/mL), amphotericin B (0.25 mg/ml) and 2 % fetal bovine serum at 37°C, 5% CO₂ and 100% humidity. HUVEC monolayer from passages 2 4 was used in these studies. ECV304 (endothelial) cell line was generously gifted by Dr.Takahashi (Tokyo University, Tokyo, Japan). ECV304 was cultured in DMEM, containing L-glutamine, 25 mM HEPES, supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 mg/ml) at 37 °C, 5% CO2, and 100% humidity.

4.3.3. Cell cycle analysis by flow cytometry

Cell cycle of human endothelial cancer cells (ECV304) treated with drugs was analyzed by flow cytometry (Kanthou et al., 2004). Cells were plated at a density of 1.5 x 10⁶ cells in 90 mm tissue culture dishes in DMEM medium supplemented with 10% FCS and incubated overnight. Subsequently, the medium was replaced with fresh medium and cells were treated with CA4 and cyclopentenone analogues 11 and 42 at the concentration
of 1 μM for 24 h. Cells were trypsinized, washed in phosphate buffered saline (PBS) and fixed with ice-cold 70% ethanol. Fixed samples were extensively washed in wash buffer (3.5 mM trisodium citrate, 0.1% NP-40, 0.5 mM tris-HCl, and 1.5 mM spermine tetrahydrochloride pH 7.6). The pellet was resuspended in 250 μl of solution A (30 μg/ml trypsin prepared in wash buffer), mixed by tapping and incubated at RT for 10-15 min. After 15 min, 200 μl of solution B (500 μg/ml trypsin inhibitor and 100 μg/ml RNase prepared in wash buffer) was added to the cell suspension and further incubated for 15 min. Subsequently, 200 μl of ice-cold solution C (420 μg/ml PI, 3.33 mM spermine tetrahydrochloride) was added to the suspension and incubated in the dark at 4-10 °C for 1 h. Stained cells were acquired on a FACS Calibur flow cytometer (Becton Dickinson, USA). The low-level gate was set at 10% of the value of the G1 peak and the percentages of cells within the G1 and G2/M phases of the cell cycle were determined by analysis with Modfit software (Becton Dickinson).

4.3.4. Analysis of Apoptosis by DNA Fragmentation

DNA fragmentation was taken as an index of apoptosis (Katschinski et al., 1999). Human endothelial cancer cells (ECV304) were plated at a density of 2 x 10^6 cells in 90 mm tissue culture dishes in DMEM medium supplemented with 10% FCS. The cells were incubated for 5-6 h to allow complete reattachment to the dishes. Subsequently, the medium was changed to fresh medium supplemented with 10% FCS and cells were treated with analogues 11 and 42 at concentrations ranging from 10 nM to 1 μM for 24 h. The compound CA4 (100 nM) was used as a positive control for the experiment. After the treatment, both the strongly adherent and the loosely adherent cells were harvested, pelleted at 2500 rpm, and lysed in buffer containing 1 mM Tris-HCl, 20 mM EDTA (pH 8.0), and 1% NP-40. The fragmented DNA was precipitated and separated from intact chromatin using 5 M NaCl and isopropanol. The mixture was centrifuged at 12000 rpm for 20 min to obtain the DNA pellet. The pellet was washed with 70% ethanol and analyzed by gel electrophoresis using 1.75% agarose gel.

4.3.5. Visualization of Apoptotic bodies by DAPI Staining

Apoptotic bodies were visualized by staining the nuclei with DAPI (Kanthou et al., 2004). ECV 304 cells were plated at a density of 1 x 10^6 cells per well in a six-well plate
in DMEM medium and incubated in a CO₂ incubator at 37 °C for 6 h. Cells were treated in the presence or absence of CA4 and cyclopentenone analogues 11 and 42 at concentrations ranging from 10 nM to 1 μM for 24 h. The compound CA4 and analogues were dissolved in 100% DMSO, and the final concentration of DMSO was kept less than 0.1%. The loosely adherent cells were harvested, washed with PBS and fixed in 70% ice-cold ethanol for 2 h. DAPI main stock of 5 mg/ml was prepared in water and stored at -20 °C. The fixed cells were stained with 500 μl of 10 μg/ml DAPI prepared in methanol (working stock) and incubated at 37 °C for 20 min. Subsequently, the cells were washed and resuspended in 50 μl of PBS. A 10 μl amount of suspension was taken on a slide and observed under the NIKON fluorescence microscope (emission wavelength of 344 nm and excitation wavelength of 450 nm) for visualization of multiple damaged, fragmented and unevenly displayed micronuclei.

4.3.6. Annexin V-FITC apoptosis detection assay

Human Umbilical Vein Endothelial cells (HUVEC) were collected at 70% confluence and plated in a 6-well plate (Nunc, Denmark) at a density of 2 x 10⁵/well suspended in X-Vivo medium containing 1% fetal bovine serum (FBS), allowed to adhere overnight and then incubated with 5ng/ml FGF-2. CA4, compound 11 and compound 42 were added at concentrations ranging from 10 to 0.1nM (CA4) and 5000 to 100nM (compound 11 and 42). The untreated wells were served as controls. The plates were incubated at 37 °C, 5% CO₂ incubator. Samples were drawn at 6 hrs, 12 hrs and 24 hrs and the cells were measured for apoptosis using Annexin V-FITC Assay Kit (Catalog Number ANNEX100F, AbD Serotec, UK) by following manufacturer’s instructions. Briefly, after 6 hrs, 12 hrs and 24 hrs incubation with the above test compounds the medium was removed from respective plates and cells were harvested by using trypsin (0.25% trypsin, 0.03% EDTA). Trypsinized cells were centrifuged at 1000 rpm for 10 min. The cells were washed with ice-cold culture medium, centrifuged at 1000 rpm for 10 min, supernatant was discarded and cells were gently resuspended in ice-cold binding buffer at a concentration of 2-5 x10⁵ cells/ml, followed by 5 μl Annexin V: FITC to 195 μl cell suspension and incubated in the dark for 10 min at room temperature. Cells were resuspended in 190 μl 1X binding buffer and 10 μl of propidium solution after washing. The resultant suspensions of
samples were analyzed by flow cytometer (FACS Caliber, BD, USA) within one hour for maximal signal.

4.3.7. Active caspase-3 immunoassay

Human Umbilical Vein Endothelial cells (HUVEC) were collected at 70% confluence and plated in a 6-well plate (Nunc, Denmark) at a density of $2 \times 10^5$/well, suspended in X-Vivo medium containing 1% fetal bovine serum (FBS), allowed to adhere overnight and then incubated with 5 ng/ml FGF-2. CA4, compound 11 and compound 42 were added at concentrations ranging from 50 to 1nM (CA4) and 5000 to 100nM (compound 11 and 42). The untreated wells served as control. The plate was incubated at 37 °C, 5% CO₂ incubator. Samples were drawn at 6 hrs, 12 hrs and 24 hrs and measured for levels of active caspase-3 using Quantikine Human active Caspase-3 Immunoassay (Catalog Number KM300, R&D Systems, Inc., USA) by following manufacturer’s instructions.

4.3.8. Statistical Analysis

In vitro data

The results were expressed as mean ± SEM.
4.4. RESULTS AND DISCUSSION

4.4.1. Effect of compounds on induction of apoptosis in endothelial cells

The effect of CA4 and its derivatives compound 11 and 42 on the growth of endothelial cells suggested a potential apoptotic or cytotoxic action. Hence, the effect of these compounds on apoptosis was examined.

CA4 and derivatives compound 11 and 42 caused morphological changes like cell shrinking, granularity and formation of apoptotic bodies in as early as 24 hrs. This effect is characteristic of cells undergoing apoptosis. The effect on endothelial (ECV304 and HUVEC) cells is illustrative of this effect. To elucidate the mechanism by which CA-4P exerts its anti-proliferative effect on HUVECs, we investigated the effect of CA-4, compound 11 and compound 42 on inducing endothelial cell apoptosis.

4.4.2. Determination of Cell cycle arrests in Endothelial Cells at G2/M and Inhibition of Mitosis flow cytometry

The ability of cyclopentenone analogues 11 and 42 to modulate endothelial cell cycle progression was evaluated using asynchronous proliferating endothelial cultures (ECV304) exposed to the drug, which were then stained with propidium iodide (PI) and analyzed by flow cytometry. Cells were treated with CA4, 11, and 42 at the concentration of 1 \( \mu \text{M} \) for 24 h. There was an accumulation of 41, 54, and 59% of cells in the G2/M phase of the cell cycle observed with CA4, 11, and 42 respectively as compared to that of the untreated control (Figure – 4.2) in which only 18% accumulation was observed. Hence, both the derivatives 11 and 42 at a concentration of 1 \( \mu \text{M} \) led to enhanced mitotic arrest in G2/M phase in endothelial cell cultures.
Figure – 4.2: Cell cycle analysis

Effect on the cell cycle as determined by flow cytometry. (4.2a) Untreated cells (control); (4.2b) Cells treated with analogue 11; (4.2c) Cells treated with analogue 42; (4.2d) Cells treated with CA4
4.4.3. DNA fragmentation analysis

Proliferating endothelial cells were treated with CA4, 11 and 42 at concentrations ranging from 10 nM to 1 μM for 24 h. After the treatment, many rounded, loosely adherent cells accumulated in the cultures. Evaluation of apoptosis by DNA fragmentation was carried out in both strongly and loosely adherent cells. No DNA fragmentation was observed in strongly adherent cells at any of all the concentrations tested. However, the treatment with compounds CA4, 11, and 42 led to evident DNA fragmentation in the loosely adherent cells at all the concentrations studied. (Fig. 4.3). Similar apoptotic effects of CA4P (100 nM) were previously reported (Kanthou et al., 2004) in human umbilical vein endothelial cells (HUVECs).

![Image of DNA fragmentation analysis](image)

**Figure – 4.3: Evaluation of apoptosis by DNA fragmentation**

Evaluation of apoptosis by DNA fragmentation in ECV304 cells. (4.3a and 4.3b) Depiction of the DNA fragmentation pattern observed with treatment of cells with 11 and (4.3c and 4.3d) depiction of the DNA fragmentation pattern observed with treatment of cells with 42 in strongly adherent and loosely adherent cells, respectively, at concentrations of 10, 100, and 1000 nM. CA4 was included as a reference standard at the concentration of 100 nM. Lane 1, 100 bp DNA marker; Lane 2, control cells; Lanes 3-5, cells treated with 10, 100, and 1000 nM of analogues; and Lane 6, 100 nM CA4. Lanes 3, 4, 5, and 6 of 3b and 3d depict discrete DNA fragments, which are characteristic morphological marker of apoptosis.
4.4.4. DAPI staining for visualization of apoptosis.

CA-4 and analogues 11 and 42 were found to cause apoptosis as seen by staining micronuclei with DAPI (4′-6-diamidino-2-phenylindole) in loosely adherent ECV304 cells, in a dose-dependent manner at concentrations ranging from 10 nM to 1 μM (Fig. 4.4). Multiple damaged, fragmented and unevenly displayed micronuclei were observed in a significant number of cells. Hence, the cyclopentenone analogues 11 and 42 may possess similar apoptotic effects in endothelial cells that were comparable to that observed for CA4.

Figure – 4.4: Visualization of micronuclei in ECV 304 cells by DAPI staining

Visualization of micronuclei in ECV 304 cells by DAPI staining. Untreated cells were taken as control (4a). Cells were treated with CA4 (4b, 4c, 4d), 11 (4e, 4f, 4g), and 42 (4h, 4i, 4j) at the concentrations of 10 nM, 100 nM, and 1 μM, respectively.
4.4.5. Effect on Annexin V binding.

Apoptosis is a cell death process characterized by morphological and biochemical features occurring at different stages. Cells undergoing apoptosis break up the phospholipid asymmetry of their plasma membrane and expose phosphatidyl serine (PS), which is translocated to the outer layer of the membrane. This occurs in the early phases of apoptotic cell death during which the cell membrane remains intact. Annexin V has proven to be a useful tool in detecting apoptotic cells since it preferentially binds to negatively charged phospholipids like PS in the presence of Ca\(^{2+}\) (Vermes et al., 1995).

Sub-confluent monolayers of HUVECs were incubated with FGF-2 in the presence and absence of CA-4 (0, 0.1 and 10 nM), compound 11 and compound 42 (0, 100, 1000 and 5000 nM) and the number of apoptotic cells as quantified by annexin V / propidium iodide (annexin V/PI) staining was evaluated by flow cytometry. Surprisingly, a 24-hour incubation with CA-4, compound 11 and compound 42 did not induce endothelial cell death through apoptosis (Table-4.1). Similar results were also obtained for short-time incubation with CA-4, compound 11 and compound 42 (6 and 12 hours), did not interfere with cell viability.
Table – 4.1. Effect on Annexin V binding

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<th>Compound</th>
<th>Conc. (nM)</th>
<th>Time (h)</th>
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FGF-2-stimulated endothelial cells were treated with or without CA4 (0.1, 1 and 10 nM), compound 11 and 42 (100, 1000, 5000 nM) for 6, 12, 24 hours and analyzed for the presence of apoptotic cells by annexin V (AnnV) and propidium iodide (PI) staining using flow cytometry. Results obtained from pooled triplicates samples of the experiment (n=1).
4.4.6. Effect on induction of active caspase-3 in cancer cells

The caspase family of cysteine proteases plays a key role in apoptosis. Caspase-3 (CPP32, Yama, apopain) is a key protease that is activated during the early stages of apoptosis, and like other members of the caspase family, is synthesized as an inactive proenzyme that is processed in cells undergoing apoptosis by self-proteolysis and/or cleavage by another protease. Active caspase-3 proteolytically cleaves and activates other caspases, as well as relevant targets in the cytoplasm, e.g., D4-GDI and Bcl-2, and in the nucleus, e.g. PARP (Thornberry et al., 1998).

Endothelial cells (HUVECs) are known to express active caspase-3 in apoptosis caused by external agents (Inanami et al., 1999). It was found that when HUVEC cells were treated with a known apoptosis inducing agent, camptothecin, it led to levels of active caspase-3 in cell lysate which was 4.7 times more than that found in untreated control cells as early as 6 hrs (Fig. 4.5) (Inanami et al., 1999).

CA4 and its derivatives compound 11 and 42 induced endothelial cell death were not associated with activation of caspase-3 (Fig. 4.5). The data suggests that endothelial cell death mediated by CA-4, compound 11 and compound 42 treatment is not driven by caspase –3 enzyme, rather by a different route.
Figure – 4.5: Effect on caspase-3 activity

The effect of CA4, compound 11, 42 and positive control camptothecin on caspase-3 activity in FGF-2 stimulated HUVEC (endothelial) cells. The cells were treated with the compounds for 6, 12 and 24 hrs except camptothecin treated only for 6 hrs. Data shown are mean ± SEM of three independent experiments.
4.5. CONCLUSION

In this chapter, combretastatin A-4 and the two short-listed derivatives from Chapter-1 viz., compound 11 and 42 were tested for endothelial apoptosis (ECV304) and growth factor stimulated primary culture endothelial cell lines (HUVEC). Both compound 11 and compound 42 led to cell cycle arrest in proliferating endothelial cells (ECV304). At the concentration of 1 µM, the analogues displayed better activity than the parent combretastatin A-4. The analogues also induced apoptosis in ECV304 cells as shown by DNA fragmentation and DAPI staining of micronuclei of proliferating ECV304 cells, thereby indicating that the analogues may have similar apoptotic activity as that of combretastatin A-4. However, FGF-2 stimulated HUVECs did not get induced endothelial cell death through apoptosis when incubated with CA-4, compound 11 and compound 42, and it was also confirmed that cell death was not driven by caspase – 3 enzyme. A detailed investigation of the apoptotic markers will further unravel its mechanism of action at both molecular and genetic level.
Cancer is a devastating disease. In terms of cancer treatment, there are serious limitations in chemotherapy, namely the lack of selectivity of active ingredients and the development of resistance by cancer cells to these chemicals (Giaccone G et al., 1996). Current chemotherapeutic agents destroy both cancerous and non-cancerous cells. Thus, there is an urgent need to find new chemical agents that can differentiate between normal and cancerous cells in order to selectively kill the cancerous cells and drug resistant tumor with reduced toxicity (Tian et al., 2006).

Combretastatin A4, the most active compound in the group combretastatins (the stilbene group of phenypropanoids), which are anti-mitotic agents, isolated from the bark of the South African willow tree *Combretum caffrum*. Combretastatin was identified 27 years ago by Professor George R. Pettit, the Director of the Cancer Research Institute based at Arizona State University in the USA (Pettit et al., 1982). Scientists at the CRI have not only completed a total synthesis for combretastatin, but also have isolated it on scales large enough to allow clinical testing, and they have also produced water-soluble phosphate derivatives, which help the drug's delivery to patients. The cytotoxicity of combretastatin and its derivatives CA4/CA4P against cancerous cell lines was reported previously (Pettit et al., 1989; McGown et al., 1990; Nabha et al., 2001; Liou et al., 2004; Tron et al., 2005). Currently, CA4P is being evaluated in various Phase II/III clinical trials in combination with potent chemotherapeutic agents against anaplastic thyroid cancer, ovarian cancer, non-small cell lung cancer, head and neck, or prostate cancer; and solid tumors (http://www.oxiRene.com/trial.asp). Previous investigation demonstrated that simple modifications of the parent structure of combretastatin A-4 could produce a number of potentially important derivatives, which may improve the selective toxicity profile or introduce general toxic effects. However, results from a more extensive investigation using a greater number of derivatives are required for structure activity relationship (SAR) study for the design and ultimate synthesis of a more effective CA4-derived anti-tumor agent(s).

To date, 252 references have been published since 1982 and 187 references have been published since 1998 related to combretastatin research. The 102 references related to
chemistry efforts can be classified into three different categories including one-atom, two-atom and three-atom bridgeheads as linker between two aryl rings of combretastatins. A number of studies have been reported on the structure and activity relationship of combretastatins. Overall,

- Trimethoxy benzene moiety is essential for its activity. (Chaplin et al., 1999).
- The two aryl groups should be separated through a double bond and the cis isomer is preferred over the trans as cis is much more active than trans (Chaplin et al., 1996; Dark et al., 1997).

5.1. Summary of the present study

Keeping in mind the facts about combretastatin A4 and its analogues, some of the derivatives of this compound were got synthesized with necessary modifications and functional group substitutions.

During the present study, about 56 (Fiftysix) combretastatin A4 (CA4) novel derivatives or analogues with modifications in ring B belonging to class of 2,3-Diaryl-4/5-hydroxycyclopent-2-en-1-one were screened for primary cytotoxicity (Table - 2.1 and 2.2). Out of these, 2 (two) derivatives from the class of 5-hydroxycyclopent-2-en-1-one were selected from the primary screening and tested in a panel of cell lines in the secondary screening (Table - 2.3).

Based on the cytotoxicity (primary screen) data certain structure activity relationships were observed. The combretastatin A4 mimic, i.e., the compound 33 and its analogues 35-37, displayed comparable or even better cancer cell growth. Compound 11 displayed a promising cytotoxicity profile in a number of cancer cell lines (Table No. 2.4). In addition to 11, the corresponding oxime 42 also exhibited strong cytotoxicity against a number of cancer and endothelial cell lines (Table No. 2.4). The overall biological activity studies of the compounds in this group indicated that the compound with the A-ring possessing 3,4,5-trimethoxy systems and the B-ring with a 4-methoxy substitution was a very potent cytotoxic compound.
The *in vitro* anti-cancer potential of compound 42 in comparison with compound 11 was assessed using *in-vitro* cytotoxicity assays (secondary screening) in a panel of 10 tumor types comprising 16 human cancer cell lines. The results revealed that compound 42 showed an IC$_{50}$ < 1 μM in 6 out of 16 human cancer cell lines viz. PTC, MDA MB 453, PA1, SKOV3, DU145 and MiaPaCa2 whereas compound 11 showed its cytotoxic potential only against MiaPaCa2 (Table - 2.3).

Compound 11 and 42 were tested for their anti-tubulin property. CA4 and its derivatives viz. compound 11 and 42 inhibited tubulin polymerization. Based on IC$_{50}$ value of compound 42 was comparable to CA4 and may act as a cytotoxic agent through inhibition of tubulin polymerization. The anti-tubulin potential of compound 42 was evidently superior to compound 11 (Table - 2.6). Based on the above *in vitro* cytotoxicity data, anti-tubulin property and the results obtained from previous studies on better aqueous solubility, metabolic stability, and pharmacokinetic profile of compound 42 as compared to CA4 (Gurjar *et al.*, 2007), compound 42 was selected for its evaluation of anti-tumor activity in ovarian (SKOV3), prostate (DU145) and colon (PTC) xenografts *in vivo*.

Both CA4 and compound 42 showed tumor regression in ovarian and prostate tumors (Table - 2.7; Table - 2.8; Table - 2.11; Table - 2.12 & Figure - 2.5; Figure - 2.9). Unlike the parent compounds CA4P and CA4 that were inactive in colon SW1222 (Pedley *et al.*, 2001), colon26 (Ohsumi *et al.*, 1998) and HT-29 (Beauregard *et al.*, 2002) tumor xenografts in nude mice, the promising anti-cancer activities of our analogues in colon cancer cell line prompted us to explore their potential *in vivo*. Based on the *in vitro* cytotoxicity of 42 in primary tumor colon cells (PTC) obtained from patients with advanced metastatic colon cancer, the *in vivo* anti-tumor activity of 42 in PTC xenografted in nude mice was tested. Statistical results show that compound 42 significantly inhibited the growth of colon xenografts (p < 0.05) on day 19 and 22 p.i. (Table - 2.15; Table - 2.16 & Figure - 2.13).
The role of angiogenesis is more relevant to metastatic disease since metastasis involves cancer cell migration from primary tumor via the blood vessel to the secondary site of metastasis. CA4 derivatives compound 11 and 42 were selected for anti-metastatic studies (in vitro) based on the potent in vitro anti-cancer activity (described in Chapter-2) and its inhibitory effect of compound 11 and 42 on VEGF-A/FGF-2 stimulated endothelial cell proliferation after 24 hrs of treatment was studied at a concentration of 1000 nM, it was observed that both the analogues showed a significant inhibition of the endothelial cell proliferation (Figure – 3.3b-c). But, at this concentration, compound 11 showed toxic effects on unstimulated cells, whereas compound 42 was non-toxic showing its better tolerance by the unstimulated endothelial cells. An inhibitory effect of compound 11 and 42 on endothelial cell migration was demonstrated using wound assay (Figure – 3.4 and Figure – 3.5). The results elucidated that at a non-toxic concentration of 50 nM, compound 42 showed significant activity in comparison with compound 11. Whereas, a complete inhibition of endothelial cell migration was observed at a highest concentration of 1000 nM for compound 42. The anti-migration effect of the present study with the analogues are comparable with the previous findings on CA4/CA4P reported earlier in the literature.

The effects of CA4P on tube formation by primary endothelial cells (HUVEC) were known (Vincent et al., 2005). Hence, CA4 derivatives were subjected to TLS assay. A three-dimensional tube formation assay revealed that compound 11 and 42 blocks formation of a capillary tube network (Figure – 3.8 to Figure – 3.11). In addition, compound 11 and 42 can destabilize a pre-established endothelial network, disrupting endothelial cell lining, resulting in cell rounding. At a concentration of 50 nM, in both pre and post-TLS, compound 42 showed significant inhibitory effect in almost all the studied parameters in comparison with compound 11 (Figure – 3.14 to Figure – 3.17). Based on inhibition of endothelial cell proliferation, migration and capillary tube formation properties, compound 42 was found to have superior activity than compound 11 (Table – 3.2). Hence compound 42 was evaluated for its in vivo anti-angiogenic activity in comparison with its parent compound. Both CA4 and compound 42 caused significant
(P<0.05) inhibition of angiogenesis at a dose of 25 mg/kg as compared to untreated control (Figure – 3.18 to Figure – 3.20).

Combretastatin A4 and the two short-listed derivatives viz., compound 11 and 42 were tested for endothelial apoptosis (ECV304) and growth factor stimulated primary culture endothelial cell lines (HUVEC). Both compound 11 and compound 42 led to cell cycle arrest in proliferating endothelial cells (ECV304). At the concentration of 1 μM, the analogues displayed better activity than the parent combretastatin A4 (Figure – 4.2). The analogues also induced cell death apoptosis in ECV304 cells as shown by DNA fragmentation and DAPI staining for micronuclei of proliferating ECV304 cells, thereby indicating that the analogues may have similar apoptotic activity as that of combretastatin A4 (Figure – 4.3 and Figure – 4.4). Surprisingly FGF-2 stimulated HUVECs did not get induced endothelial cell death through apoptosis when incubated with CA4/ compound 11/compound 42, and it was also confirmed that cell death was not driven by caspase–3 enzyme (Table – 4.1 and Figure – 4.5). A detailed investigation of the apoptotic markers will further unravel its mechanism of action at both molecular and genetic level.

5.2. Conclusions drawn out of this thesis work

- In this thesis, 56 (Fifty six) CA4 novel derivatives with modifications in ring B belonging to class of 2,3-Diaryl-4/5-hydroxycyclopent-2-en-1-one were screened for cytotoxicity (primary screening) on a panel of human tumor cell lines and endothelial cell line.

- Out of these, 2 (two) derivatives viz. compound 11 and compound 42 (from the class of 5-hydroxycyclopent-2-en-1-one) were selected from the primary screening and tested in an extended panel of 10 tumor types envisaging 16 human cancer cell lines.

- Compound 42 showed an IC₅₀ < 1 μM in 6 out of 16 human cancer cell lines viz. PTC (colon), MDA MB 453 (breast), PA1, SKOV3 (ovary), DU145 (prostate)
and MiaPaCa2 (pancreas) whereas compound 11 showed its cytotoxic potential only against MiaPaCa2.

- Further, the above derivatives were also tested for their ability to inhibit tubulin polymerization. The IC\textsubscript{50} values of compound 42 were comparable to CA4 and the value of CA4 was found to be similar to that of previous published reports.

- Of the two derivatives tested, compound 42 was found to be the more potent. Both CA4 and compound 42 showed tumor regression in prostate and ovarian tumors. CA4 derivative compound 42 showed significant anti-cancer activity against colon cancer.

- Both the derivatives, compound 11 and 42 were found to inhibit vital processes of angiogenesis i.e. stimulated endothelial cell proliferation, migration and capillary tube formation.

- Compound 42 with its more potent anti-angiogenic activity \textit{(in vitro)} showed significant inhibition of vasculature \textit{in vivo} at 25 mg/kg dose.

- Compound 11 and compound 42 led to cell cycle arrest in proliferating endothelial cells (ECV304). At the concentration of 1 \textmu M, the analogues displayed better activity than the parent combretastatin A-4.

- The analogues also induced apoptosis in ECV304 cells as shown by DNA fragmentation and DAPI staining of micronuclei of proliferating ECV304 cells, thereby indicating that the analogues may possess similar apoptotic activity as that of combretastatin A4.

- In FGF-2 stimulated HUVECs neither the CA4, nor its derivatives viz., compound 11 and compound 42, induced endothelial cell death through apoptosis by caspase
- 3 enzyme. A detailed investigation of the apoptotic markers will further unravel its mechanism of action at both molecular and genetic level.

The comprehensive studies carried out on combretastatin A4 derivatives have enabled the selection of one compound from a library of about 56 new and novel combretastatin A4 derivatives. Based on the preliminary ADME (Gurjar et al., 2007), toxicity profiles, detailed in vivo and mechanism of action studies, compound 42 was found to have the potential to be taken up for formulation development, detailed efficacy and toxicity studies.

5.3. Future scope of work

Microtubules are long, filamentous tube-shaped polymers of α and β tubulin that are essential in all eukaryotic cells. They are crucial in the development and maintenance of cell shape, in the transport of vesicles, mitochondria and other components throughout the cells, in cell signaling, and in cell division or mitosis. Their importance in mitosis and cell division make microtubules an obvious and important target for anticancer drugs. The dynamic process of assembly and disassembly of microtubules to tubulin is blocked by various agents that bind to distinct sites such as binding sites for colchicines, taxol, and Vinca alkaloids, finally leading to arrest in mitosis and cell death by apoptosis or necrosis. Several microtubule targeting drugs, - namely, Paclitaxel, Epothilones, and Dolastatin are in clinical use or in various stages of clinical development (Hadfield et al., 2003). Thus, the design of tubuline-targeted cytotoxic drugs may constitute a novel strategy for earlier development. (Costantini et al., 2000).

Various clinical trials are going on to check efficacy of parent compound CA4P alone and combination with various existing anticancer drugs. These findings indicate that CA4 acts, as a sensitizer in chemotherapy-based combination regimens may be a novel strategy to enhance the efficacy of anticancer therapy. Further studies may be carried out to test whether any of the potent derivatives identified as a result of this study could be used in combination regimens.
Our studies have demonstrated the potential of developing compound 42 as an anti-cancer agent with promising anti-angiogenic activity. As, preliminary studies have indicated the apoptotic potential of compound 42 against endothelial cells, a detailed investigation of the apoptotic markers will further unravel its mechanism of action at both molecular and genetic level. Compound 42 exhibits drug-like physicochemical properties (Gurjar et al., 2007). Appropriate formulation studies need to be carried out to deliver the molecule systemically and preferably in a targeted fashion. While pre-clinical safety studies have demonstrated an acceptable safety index, a more detailed toxicology study needs to be performed to evaluate the safety of the compound upon long-term systemic administration. Finally, for the development of compound 42 for clinical assessment, detailed pharmacology and toxicology, including genotoxicity and reproductive toxicology studies need to be performed in order to generate data on the potential short and long term toxicities and other pharmacological actions etc.

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