ANTI-TUMOR STUDIES OF A SELECTION OF SUDANESE INDIGENOUS HERBS VIA THE ANGIOGENESIS PATHWAY

By

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5-FU	5-Fluorouracil
APC	Adenomatous Polypsis Coli
ATCC	American Type Culture Collection
BA	Betulinic Acid
Bad	Bcl-xL/Bcl2-associated death promoter
Bax	Bcl2 associated X protein
Bcl2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma extra large
bFGF	basic Fibroblast Growth Factor
bp	base pair
BSA	Bovine Serum Albumin
˚C	Degree Celsius
CAM	Cellular adhesion molecules
CCD-18Co	Normal human fibroblast
CD	Cluster of differentiation
CO_2	Carbon dioxide
COX	Cyclooxygenase
CV	Coefficient of Variation
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPPH	Di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme linked immunosorbant assay
Fas	Tumor necrosis factor superfamily receptor 6
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF-2	Fibroblast Growth Factor-2
FTIR	Fourier transform infrared spectroscopy

GAE	Gallic Acid Equivalents
HCT 116	Human colorectal carcinoma cell line
HER-2	Human Epidermal growth factor Receptor 2
HIF-1α	Hypoxia Inducible factor -1- alpha
HIFs	Hypoxia Inducible Factors
HPLC	High Performance Liquid Chromatography
HUVECs	Human umbilical vein endothelial cells
IC_{50}	Median inhibitory concentration
ICAM-1	Intercellular adhesion molecule-1
ICH	International Conference on Harmonisation
IFN-α and γ	Interferons alpha and gamma
Ig	Immunoglobulin
KBr	Potassium Bromide
K-ras/PI3-K	K-ras/Phosphatidylinositol 3-kinases
K-ras/RAF	K-ras/Murine leukemia viral oncogene homolog 1
K-ras/RAL	K-ras/ K-ras related protein
K-ras	Kirsten-rat sarcoma virus
LC-MS	Liquid Chromatography Mass Spectrometry
LD50	Lethal Dose 50
LOD	Limit of Detection
LOQ	Limit of Quantification
MAPK/ERK	MAPK-Extra cellular signal regulated enzyme kinase
MAPK/JNK	Mitogen-Activated Protein Kinase/Jun N-terminal Kinase
MAPK	Mitogen-Activated Protein Kinase
INF	Interferon
iNOS	Inflammatory nitric oxide
IL	Interleukin
LC_{50}	Median lethal concentration
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCF-7	Human hormone sensitive and invasive breast cancer cell
MEM	Minimum Essential Medium
MMPs	Matrix Metalloproteinase
MeOH	Methyl alcohol

mTOR	Mammalian Target of Rapamycin
MTT	Thiazolyl blue tetrazolium bromide
NF-kB	Nuclear factor kappa B
NIH	National Institutes of Health
Notch	A signal transduction pathway
NMR	Nuclear magnetic resonance
NO	Nitric Oxide
NSAIDS	Non-steroidal anti-inflammatory drugs
OD	Optical density
PBS	Phosphate buffered saline
PDGFR	Platelets Derived Growth Factor Receptors
PE	Plating Efficiency
PGs	Prostaglandins
PPM	Part Per Million
PS	Penicillin/Streptomycin
SD	Standard Deviation
SF	Survival Fraction
SPF	Specific Pathogen Free
STAT	Signal transducer and activator of transcription
TGF	Tumor growth factor
TGF-α	Transforming Growth Factor-alpha
TGF-β	Transforming Growth Factor-beta
TGFβR2	Transforming Growth Factor-β II Receptor
TKI	Tyrosine Kinase Inhibitor
TLC	Thin layer chromatography
TLRs	Toll like receptors
TNF	Tumor necrosis factor
UV-Vis	Ultraviolet-Visible
VEGF	Vascular Endothelial Growth Factor
VEGFR 1 and 2	Vascular Endothelial Growth Factor Receptors 1 and 2
VCAM-1	Vascular cell adhesion molecule-1

KAJIAN ANTI-TUMOR HERBA-HERBA ASLI SUDAN YANG TERPILIH MELALUI LALUAN ANGIOGENESIS

ABSTRAK

Tujuan kajian ini adalah untuk mengkaji enam tumbuhan ubatan Sudan untuk aktiviti antikanser. Bahagian berbeza daripada tumbuhan-tumbuhan ini telah dipilih untuk menyediakan 32 ekstrak menggunakan kaedah pengekstrakan berturutan dengan 4 jenis pelarut yang berbeza kekutuban. Antara keenam-enam tumbuhan ubatan Sudan yang telah diuji itu, *N. glauca*, *T. apollinea*, *C. hartmannianum* and *T. nilotica* telah didapati sangat aktif secara biologi dengan kesan-kesan "antiangiogenic" dan "antineoplastic" yang bererti. Aktiviti-aktiviti biologi tersebut didapati telah disebabkan oleh aktiviti antioksida berikutan kehadiran bahan-bahan fenolik dan flavonoid yang tinggi. Keputusan-keputusan kajian ini menunjukkan bahawa ekstrak etanol *T. apollinea* mempunyai kesan "antiangiogenic" yang tertinggi berbanding spesis tumbuhan lain dan ia juga merencatkan pertumbuhan kanser kolon "*in vivo*"secara bererti. Ekstrak tersebut didapati menghentikan "angiogenesis" dengan merencatkan penghijrahan sel, pembentukan saluran endothelial dan yang terpenting sekali, merencatkan pengungkapan "VEGF".

Sebatian-sebatian yang penting yang menyumbang kepada aktiviti ini adalah tiga "prenylated flavonoids" iaitu "(-)-pseudosemiglabrin (SSG)", "(-)-semiglabrin (SG)" dan "isoglabratephrin (IGP)". SSG telah didapati wujud dengan banyak dan adalah yang paling aktif sebagai "antiangiogenic" berbanding SG dan IGP. SSG merencatkan enzim "COX" dengan keutamaan tinggi terhadap enzim "COX-2". Ia juga menyekat penghasilan "cytokines" IL-1 dan TNF-α tetapi ia tidak mempunyai kesan yang bererti terhadap pengungkapan nitric oksida (NO). IL-1 dan TNF-α

adalah perantara-perantara keradangan yang penting yang memainkan peranan yang amat genting dalam karsinogenesis. SSG telah menunjukkan aktiviti antitumor "*in vivo*" yang kuat terhadap model xenograft kanser kolon ektopik dan ortotopik dengan yang kedua menunjukkan perencatan pengungkapan MMP-9. Antara ketiga-tiga sebatian tersebut, hanya SSG yang menunjukkan aktiviti sitotoksik terutamanya terhadap titisan-titisan sel kanser kolon (HCT116), kanser payudara (MCF7), kanser prostat (PC3) dan leukemia (HL-60). Sebatian tersebut menyasarkan fungsi mitokondrion dengan mengganggu keutuhan matriks sel. Sebatian-sebatian yang telah diasingkan itu digunakan untuk memiawaikan ekstrak etanol *T. apollinea* menerusi kaedah HPLC. Kajian ini telah menunjukkan sebahagian besar aktiviti "antiangiogenic" dan antitumor ekstrak etanol *T. apollinea* disumbangkan oleh SSG. Kesimpulannya, keputusan ini menunjukkan bahawa ekstrak *T. apollinea* dan sebatian aktifnya iaitu SSG sebagai ejen-ejen antitumor yang berpotensi yang mengubah aktiviti mereka melalui lata "antiproliferative" dan "angiogenesis".

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ABSTRACT

The aim of the present study is to investigate six Sudanese medicinal plants for anticancer activity. Different parts of these plants were selected to prepare 32 extracts using sequential extraction method with 4 solvents of different polarity. Amongst the six Sudanese medicinal plants tested, *N. glauca*, *T. apollinea*, *C. hartmannianum* and *T. nilotica* were found to be most biologically active with significant antiangiogenic and antineoplastic effects. Their biological activities observed were found to be attributed to their high antioxidant activity due to the presence of high amount of phenolic and flavonoid substances.

The results of present study indicate that the ethanol extract of *T. apollinea* possess the highest antiangiogenic effect when compared to the other plant species and it significantly inhibited the growth of colon cancer in *vivo*. The extract was found to halt angiogenesis by inhibiting cell migration, endothelial tube formation and most importantly the inhibition of VEGF expression. The key compounds that contribute to this activity are three prenylated flavonoids namely (-)-pseudosemiglabrin (SSG), (-)-semiglabrin (SG) and Isoglabratephrin (IGP). SSG was found to be the most abundant and the most active as antiangiogenic when compared to SG and IPG. SSG inhibits the COX enzymes with strong preference towards the COX-2 enzymes. It also suppresses production of cytokines IL-1 and TNF-α but it not significant effect towards nitric oxide (NO) expression, IL-1 and TNF-α are key inflammatory mediators that plays crucial role in carcinogenesis. SSG showed potent *in vivo* antitumor activity towards ectopic and orthotopic xenograft model of colon cancer with the latter indicating the inhibition MMP 9 expression. Amongst the three compounds only SSG showed cytotoxic activity particularly towards colon cancer

(HCT116), breast cancer (MCF7), prostate cancer (PC3) and leukemia (HL-60) cell lines. The compound targets the mitochondrial function by disrupting the integrity of its cellular matrix. The isolated compounds were used to standardize the ethanol extract of *T. apollinea* by HPLC method. The study indicated that the antiangiogenic and antitumor activity of *T. apollinea* ethanol extract is contributed mainly by SSG. All in all, the data shows that extract of *T. apollinea* and its active compound SSG, as potential antitumor agents that modulate their activity via the antiproliferative and angiogenesis cascade.

CHAPTER 1

INTORDUCTION

1.1 Cancer

Cancer is a group of diseases which is characterized by aggressive cell growth and division that exceeds normal cells. They invade and destroy neighboring tissues, and often spread to other locations of the body (metastasis). These three properties of cancer differentiate neoplasic tissues from benign tumors which are self-regulated, limited growth potential with no ability to metastasize (Gatta et al., 2013). Almost all cancers occur due to abnormality (mutation) in the genetic material of the transformed cells. The abnormalities are attributed to the effects of carcinogens, such as radiation, chemicals tobacco smoke or infectious agents (Mediana, 2008). Abnormality in genetic material may occur randomly due to error in DNA replication, or inherited, thereby originated in all cells from birth, which lead to genetic damage that, if not repaired, results in irreversible mutation. The altered cells grow till formation a colony, afterwards the mutated cells grow under the promotion of carcinogens or other factors via changing the environment in way that support the growth of mutated cells over normal cells (Mediana, 2008). The next stage is transformation' of mutated cells to cancerous cells, it may takes 5-20 years may require for the transition of benign carcinogenic phase to the fully developed malignant stage where the cancer can be detected clinically. The last stage called progression, where further genetically changes take place leading to increase the proliferation and metastasis (Marshall, 1991).

1.1.2 Hallmark of Cancer

Emerging evidence proposed six complementary and distinctive hallmarks that enable the initiation, growth and metastasis of tumor, these characteristic features provide solid platform of understanding cancer biology (Douglas Hanahan & Weinberg, 2011).

1.1.2.1 Sustaining Proliferative Signaling

The main characteristic feature of cancer cells constitutes the ability of chronic proliferation, due to the loss of control over the production and release of growth-promoting signals that regulate the process of cell growth and cell division, thus cancer cells no longer depend on external signals, while these signals in normal cells are well controlled to enable homeostasis of cell. Moreover, tumor cells become antagonist to anti-growth signals or they cannot respond to normal signals that regulate or limit the cell division(Douglas Hanahan & Weinberg, 2011).

1.1.2.2 Evading growth suppressors

In addition to the characteristic feature of inducing and sustaining positively acting growth-stimulatory signals, cancer cells must also circumvent powerful programs that negatively regulate cell proliferation; many of these programs depend on the actions of tumor suppressor genes. Dozens of tumor suppressors that operate in various ways to limit cell growth and proliferation have been discovered through their characteristic inactivation in one or another form of animal or human cancer; many of these genes have been validated as bona fide tumor suppressors through gain- or loss-of-function experiments in mice. The two prototypical tumor suppressors encode the RB (retinoblastoma-associated) and TP53 proteins; they operate as central control nodes within two key complementary cellular regulatory

circuits that govern the decisions of cells to proliferate or, alternatively, activate senescence and apoptotic programs.

The RB protein integrates signals from diverse extracellular and intracellular sources and, in response, decides whether or not a cell should proceed through its growth-and-division cycle (Burkhart & Sage, 2008; Sherr & McCormick, 2002). Both examples must reflect the operations of redundantly acting mechanisms that serve to constrain inappropriate replication of cells lacking these key proliferation suppressors.

1.1.2.3 Evading apoptosis

The major trait of cancer cells is increase cell populations not only by cell proliferation but also by the rate cell attrition. Apoptosis (cell programmed death) responsible for attrition, so apoptosis represent vital mechanism to eliminate damaged cells and avoid mutations that can lead to tumor formation. Cancer cells acquired resistance to apoptosis via different strategies. Mainly due to the loss of a proapototic regulator through p53 suppressor gene mutation (Harris, 1996).

1.1.2.4 Enabling replicative immortality

Almost all types of cancers are believed to originate from a single cell, which acquired capability of limitless replicative potentials. In order to become visible mass, the cells must divide almost unlimited times. Normal cells are unable to grow and divide indefinitely, because they are limited in the number of times they can efficiently copy all the cell's DNA, because telomeres i.e the caps of repetitive sequence of DNA at the end of each strand of DNA which are truncated during cell division. As the cell divides more and more its telomeres shorten leading the cell to

inter non-replicative state- a process termed senescence (Bodnar et al., 1998) . This mechanism limits cellular replicative potential. Malignant cells have routes to avoid cellular senescence to divide indefinitely and form tumors. This involve finding ways of repairing or lengthening the telomeres to protect them from being shortened, therefore facilitating indefinite replication(O'Sullivan & Karlseder, 2010). Telomerase is an enzyme which accumulates telomere sequences to the ends of chromosomes to avoid termination of replication; some normal cells express telomerase, such as embryonic stem cell, in which cells replicate indefinitely, but this not apply to adult cells. Many malignancies constitute mutations that lead to activation of telomerase, which results in replicative immortality (Hayflick, 1997).

1.1.2.5 Sustained Angiogenesis

Similar to normal tissues, tumor mass requires oxygen and nutrients. For a tumor to grow bigger than $2mm^3$ in size it must establishment blood supply in process by which the host's vasculature is extended and neovasculature grows into tumor i.e called angiogenesis. There are no distinct changes by tumor themselves, this process transitory and carefully regulated by tumor mass and the host tissue. Tumors initiates pathological angiogenesis by unbalancing angiogenesis inducers and countervailing inhibitors (D. Hanahan & Folkman, 1996). Changing the balance could be due to change in gene transcription. Evidence shows elevated expression of VEGF and/or FGFs in tumor tissues compared to normal tissue, down regulation of endogenous inhibitors such as thrombospondin-1 orb-interferon may associated with over expression of growth factors (Singh et al., 1995).

1.1.2.6 Invasion and metastasis

Cancer cells acquired the capability to become motile, migrate from primary tumor and invade adjacent tissues, and thence spreading to other site of the body

(metastasis), where they may form new tumors (secondary tumors). Metastasis and secondary tumors caused 90% of cancer patients mortality (Sporn, 1996). Invasion and metastasis are allied and complicated process. Invasion promoted by changes in the expression of surface marker which allow the cells to adhere to the surrounding tissues. Cancer cells invade blood vessels and move through circulatory system to different site of the body. These processes not completely understood, but it's known that several proteins which secreted to break down the tissue and allow invasion of cancer cells into blood vessels and eventually form new tumor at the site of settlement (a metastasis). These proteins include cell–cell adhesion molecules (CAMs), in addition to members of the immunoglobulin and calcium-dependent cadherin families both of which mediate cell-to-cell interactions, also integrins, which link cells to extracellular matrix substrates (Albelda, 1993).

1.2 Angiogenesis

Angiogenesis is the formation of new blood vessels from existing vasculature. Its biological process occurs in both pathological and health life. It's well-regulated in physiological process such as embryogenic development, female reproductive tract in uterus and placenta during pregnancy, in addition to wound healing process, while in disease stage it is deregulated in many pathological conditions including tumor growth and metastasis, rheumatoid arthritis, psoriasis, diabetic and obesity (Adair & Montani, 2010; Peter Carmeliet, 2005). Tumor angiogenesis is crucial for cancer growth and metastasis, thus targeting angiogenesis is of great therapeutic value, recently this field studied extensively to utilize pro- and antiangiogenic agents. Angiogenesis play a fundamental role in tumor growth as the tumor unable to grow more than 1-2 mm^2 without sufficient oxygen and nutrients, the newly formed blood vessels not only used for supplying nutrient and oxygen but

also can be utilized by tumor to metastasis to other locations (Ou et al., 1998). Thus targeting tumor angiogenesis became a cutting edge in cancer therapy, due to interdependence between cancer and angiogenesis and less side effect of inhibiting angiogenesis only under special circumstances such as pregnancy and not fully grown individuals. A huge effort has been assigned to screen or to develop antiangiogenic compounds to treat cancer and other angiogenic related diseases.

1.2.1 The Vascular Endothelial Growth Factor (VEGF)

VEGF is a signal protein that stimulate various steps in angiogenesis cascade such as cell proliferation, migration and cell survival of endothelial cells (Figg & Folkman, 2008). When VEGF over expressed it contribute to disease, for instance when solid tumors grow in size, the demand for oxygen increases causing release of oxygen free radical which in turn stimulate vascular endothelial growth factor (VEGF) that trigger tumor angiogenesis (Mukhopadhyay et al., 1995). It's reported that VEGF level is upregulated in RNA and protein of many types of malignancy. The elevated level of VEGF in cancer patients is associated with low survival rate and poor prognosis (Paley et al., 1997). The biological effects of VEGF family are mediated by three tyrosine kinase receptors, VEGFR-1, VEGFR-2 and VEGFR-3 (Wirzenius, 2007). VEGF-A is growth factor with specificity to endothelial cells that had been regarded as a heparin binding angiogenic growth factor (Wirzenius, 2007), it belongs to a gene family that includes placenta growth factor (PGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E. The main difference between these proteins is the type of receptors they bind to and activate; PGF, VEGF-B can bind with VEGFR-1 and VEGF-C, VEGF-D binds with VEGF-3, and VEGF-A can bind with two receptors: VEGFR-1 and VEGFR-2 (Shibuya, 2001).

VEGF play vital role in tumor angiogenesis, in turn tumor growth and metastasis, rendering it as good target for cancer therapy via deactivation of VEGF by neutralizing antibodies or by introduction of dominant negative VEGF receptors (K. J. Kim et al., 1993).

1.2.2 Angiogenesis Process Cascade

In the angiogenesis cascade, a number of biological processes occur. Figure1.2 depicts the steps of angiogenesis process. Angiogenesis initiated vasodilation of existing blood vessels to increase permeability to angiogenesis signals. Then, the pericytes that covers the blood vessels, detach followed by degradation of vascular membrane and extracellular matrix (figure 1.3 a) which allow for the activated endothelial cells to migrate into the perivascular space towards chemotactic angiogenic stimuli (Napoleone Ferrara, 1999). The migrated endothelial cells proliferate, loosely following each other into the perivascular space and form migration columns. Then, the endothelial cells differentiate; cells‘ shape change in a way that facilitates the cell-cell adherence which then forms a lumen (tube-like structure). Perivascular cells are attracted, and a vascular basal lamina is produced around the newly formed vessels. The details are still vastly obscure for the last stages when vascular sprouts fuse with other sprouts to form loops ((Bergers & Benjamin, 2003; Peter Carmeliet, 2005).

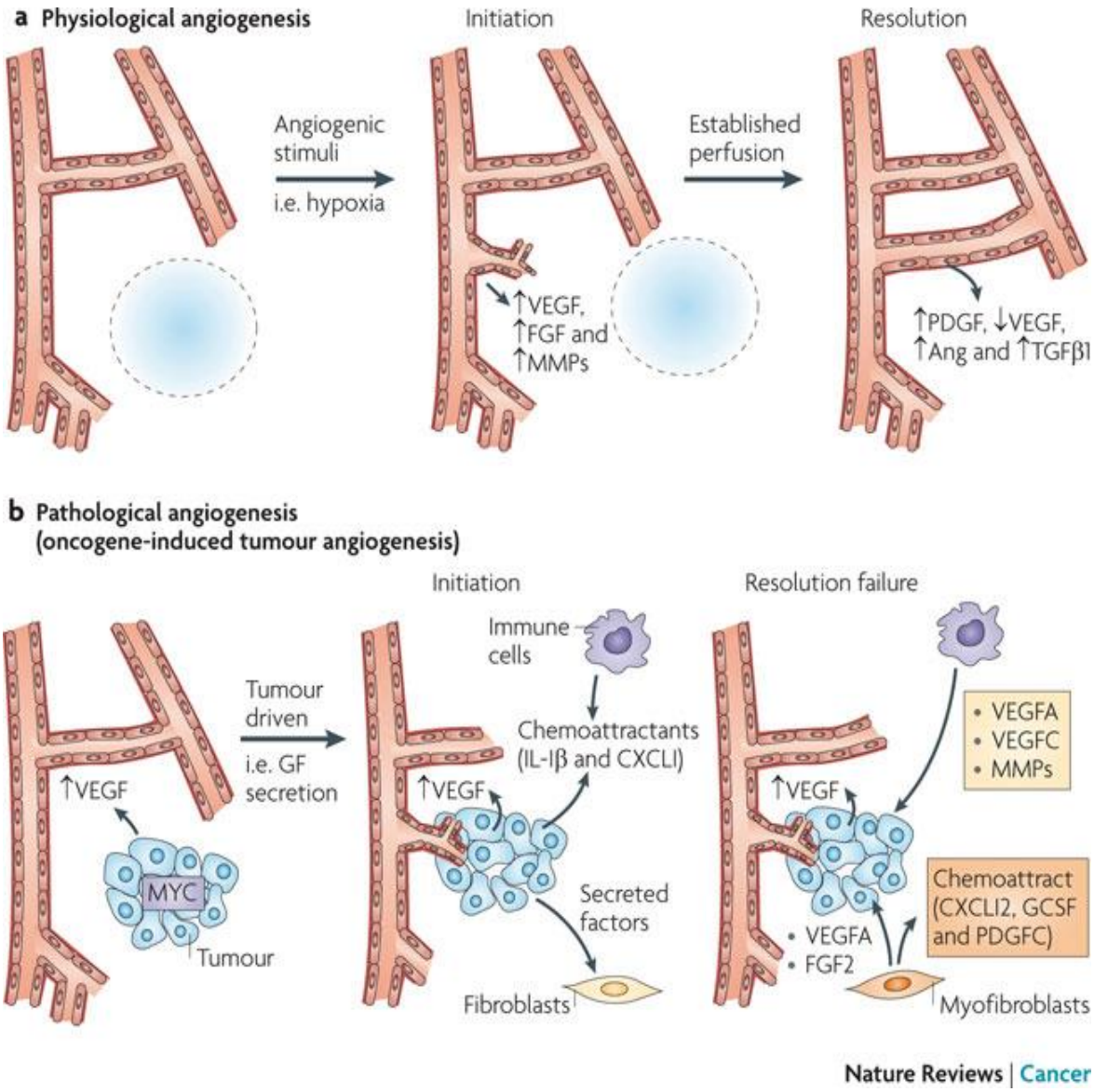

Figure 1.1: The angiogenesis process (physiological and pathological) cascade (Chung, Lee, & Ferrara, 2010)

1.2.3 Cancer is Angiogenesis Dependent

Studies proved that there is interdependency between tumor growth and neovascularisation. The blood supply and number/size of vessels must be proportional to tumor size (J. Folkman, 1990). The hypothesis has been proved by many studies. This proof that angiogenesis inhibitors can effectively utilized to combat cancer. Several preclinical studies proved that solid tumors need new blood vessels to grow. In one study, the tumors which implanted in corneas, the tumors

start to grow only after the new blood vessels reach the implant. It's noticed that introduction of angiogenic genes can transform dormant tumor into progressive and growing tumor. Moreover it's evident that some specific angiogenesis inhibitors can suppress tumor growth by modulating vascular compartment (Goel et al., 2011).

1.2.4 The Angiogenic Switch

Mutation in somatic cell leads to transformation of normal cells to cancerous cell, the latter divide rapidly to form tiny tumor called *in situ* carcinoma. The estimated time needed to develop complete tumogenesis from benign carcinogenic and to be detected clinically is about five to twenty years (Judah Folkman, 1995; Mediana, 2008). At dormant stage cancer cells start to proliferate at the same time the rate of cell death continues and maintain the mass of the tumor at stable state (Ribatti, 2009). At this situation, there is a balance between two opposite signals; angiogenesis signals like VEGF expression (N Ferrara, 2002) and antiangiogenesis signals (e.g., Endostatin, Angiostatin) (H. Kim et al., 2000). Therefore, the formation of new blood vessels starts when the net balance between these divergent signals is tipped in favor of angiogenesis initiation (Judah Folkman, 1995; Douglas Hanahan & Weinberg, 2011). Obviously, identification and impairing of factors and circumstances which enhance initiation of angiogenesis may keep the tumor in dormancy stage (Goel et al., 2011). Studies revealed that angiogenesis process can be initiated by different signal such as genetic mutation, metabolic stress (e.g hypoxia, hypoglycemia), mechanical stress (e.g., pressure generated by proliferating cells, and immune/inflammatory response (immune/inflammatory cells that have penetrate the tissue) (Douglas Hanahan & Weinberg, 2000). This environment facilitate synthesis and release of key angiogenic factor like VEGF (P. Carmeliet, 2005).

1.2.5 Hypoxia

Hypoxia is a condition in which the body or a region of the body is deprived of adequate oxygen supply required for cellular functions. Cells experience hypoxia when it is located too far from blood supply. As the tumor grow bigger the cells in centre of the tumor become hypoxic (Peter Carmeliet, 2005). Recent report demonstrated that hypoxia inducible transcription factors (HIFs) that activated in hypoxic cells play fundamental role in angiogenesis cascade (Bos et al., 2001). Ligation of the HIFs with the DNA activates major angiogenic factors such as PDGF, VEGF and nitric oxide synthase (Ahmed & Bicknell, 2009). HIF-1 and HIF-2 and the main principles of HIFs, HIF-1 expressed only in endothelial cells, the heart, small intestine, lungs and kidneys, whereas HIF-2 is expressed ubiquitously. HIF-1 is heterodimer containing two DNA binding protein receptors, HIF-1α and HIF-1β. HIF-1α expression is strongly organized by oxygen, whereas the HIF-1β is expressed constitutively (Stroka et al., 2001). In normal situation HIF-1α is swiftly degraded by enzymatic prolylhydroxylation. Nonetheless, in hypoxic stage the HIF-1α becomes stable and its half life increases significantly. Thus, HIF-1α dimerizes with HIF-1β. The heterodimer is then transferred to the nucleus and activates the promoter region of specific genes (Adair & Montani, 2010). The activation of HIF-α involved in the in the expression of (VEGF), which is a major growth factor in the angiogenesis. HIF represent a good target for angiogenic and cancer therapy. This hypnosis proved by study in mice implanted with cells infected with a polypeptide which abolish the binding of HIF-1α to its transcriptional co-activators, which showed significant reduction of tumor growth (Ryan et al., 2000) Some natural compounds such as Klugine, betulinic acid, taxol, isocephaeline and emetine

excelled their mechanism of action to inhibit hypoxia pathway (Assudani, Tyagi, & Vimalanathan, 2010).

1.2.6 Angiogenesis Targets

Tumor neovasculature is sophisticated multi-steps process. It could be inhibited by agents that interrupt the key steps of this process, such as (1) inhibition of growth factor signal production, inhibition of receptors production and /or inhibit the binding between signals and receptors, which modulate the activation of endothelial cells. (2) inhibition of endothelial cells proliferation, (3) inhibition of endothelial cells migration, (4) inhibition of endothelial cells differentiation to form a three dimensional tube-like structure and (5) induction of apoptosis in endothelial cells (Zhang & Bicknell, 2003).

1.3 Inflammation & Cancer

The functional relationship between cancer and inflammation is known since 1863, when Virchow hypothesized that cancer could be a precursor of chronic inflammation. This obvious when some kind of irritants associated with tissue injury and ensuing inflammation, many factors directly or indirectly enhance cell proliferation (Fran Balkwill & Alberto Mantovani, 2001), recruit inflammatory cells, increase production of reactive oxygen species leading to oxidative DNA damage, and reduce DNA repair. Subversion of cell death and/or repair programmes occurs in chronically inflamed tissues, thus resulting in DNA replication and proliferation of cells that have lost normal growth control. Normal inflammation is self-limiting, because the production of anti-inflammatory cytokines follows the pro-inflammatory cytokines closely. However, chronic inflammation seems to be due to persistence of the initiating factors or a failure of mechanisms required for resolving the

inflammatory response (Coussens & Werb, 2002). In prolonged tissue injury associated with wound healing, cells carry on proliferation while the tissue regenerates, the proliferating cells that sustain DNA damage and/or mutagenic assault continue to proliferate in microenvironments rich in inflammatory cells and growth factors that support their growth and up-regulate gene transcription factors such as NF-κB. In a sense, tumors act as wounds that couldn't to heal (Dvorak, 1986). The inflammation mediates host defense to pathogenic infection or irritants and regulates regeneration of afflicted tissue. It's evident from epidemiologic studies that there is obvious connection between inflammation and initiation of tumorigenesis. It estimated that 15% of cancer incidence worldwide attributed to microbial infection, of 1.2 million per year (Kuper, Adami, & Trichopoulos, 2000; Shacter & Weitzman, 2002). Continual infections within the host induce chronic inflammation. Leukocytes and other phagocytic cells degrade the DNA of proliferating cells, via their production of reactive oxygen and nitrogen species which combat infection. These species react to form peroxynitrite, a mutagenic agent (Maeda & Akaike, 1998). Thus, frequent tissue damage and regeneration of tissue, in environment rich of reactive nitrogen and oxygen species generated from inflammatory cells, interacts with DNA in proliferating epithelium resulting in permanent genomic alterations such as point mutations, deletions, or rearrangements. For instance, p53 mutations found frequently in individuals whom developed tumors due to choric inflammation such as inflammatory bowel disease (Yamanishi et al., 2002). Excess DNA damage by inflammatory cells is expression of macrophage migration inhibitory factor (MIF) from macrophages and T lymphocytes. MIF is a strong cytokine that up-regulates p53 function by suppressing its transcriptional activity (Hudson et al., 1999). Continual avoid of p53 regulatory

processes in the inflamed tissues can increase proliferation and prolong life span, also creating an environment with a poor response to DNA damage, leading to potential gene mutations.

Chemokines are multifunctional mediators mainly it direct the migration of leukocytes to inflamed site; however, chemokine biology extends to all cell types, including most human cancer cells (Ehrlich, 2000). This notion was supported by the role of chemokines during induction of tumor into an experimental animals deficient to T or natural killer (NK) cells, it exhibited typical inflammatory infiltrate; which indicate that the neoplastic cells produce chemotactic factors or induce their expression in neibouring cells of the host (Brummer, Vinoda, & Stevens, 2006) . Some cancer cells regulate their chemokines to recruit inflammatory cells as well as using these factors to enhance tumor growth and progression. For example in melanoma chemokines and their receptors (GROα/CXCL1, GROβ/CXCL2,GROγ/CXCL3 and IL-8/CXCL8) shown to have autocrine control over tumor cell proliferation (Richmond & Thomas, 1986). Blocking GROα or the CXCR2 receptor impair melanoma cell proliferation *in vitro* (Owen et al., 1997). *In vivo* studies demonstrated that overexpression of GROα, GROβ or GROγ in different tumor-derived cell lines enhances their colony-forming activity and tumorigenicity in nude mice (Owen et al., 1997).

Strong correlation between chronic inflammation and carcinogenesis most notably arising in patients with bowel inflammatory diseases, for example, chronic ulcerative colitis and Crohn's disease. Liver carcinoma is precursor of chronic hepatitis C infection. Schitosomiasis predisposes to bladder and colon cancer. An increase risk of stomach cancer is associated with chronic *Helicobacter pylori* infection (Ernst & Gold, 2000).

1.4 Inflammation & Angiogenesis

Inflammation is biological response of vascular tissues to injury, irritants or pathogens (Mariathasan et al., 2006). The inflammatory process induced in the afflicted tissue to facilitate the access of immune cells to the affected tissue in attempt to heal the affliction. The reaction is associated with multifactor network of chemical signals which include the release of pro-inflammatory cytokines namely TNF-α and IL-1α. Signaling pathways studies revealed strong association between angiogenesis and inflammation (Y. Kim et al., 2011). For example the activated macrophages during development of melanoma produce TNF-α, IL-1, arachidonate metabolites and extracellular proteases, melanocytes express IL-18 and VEGF-A, thereby induce angiogenesis (Torisu et al., 2000). Immune cells posses on the surface toll like receptors (TLRs), when it stimulated by bacteria antigen, it release pro-inflammatory cytokines from the activated dendritic cells that recruit the endothelial cells of the blood vessels to synthesize pro-inflammatory mediators such as prostaglandins and pro-inflammatory cytokines like IL-1 and TNF-α via up-regulation of gene transcription factors such as NF-$_k$B. Neoplastic cells mimic same mechanisms to trigger angiogenesis from the endothelial cells (Y. Kim et al., 2011).

Angiogenesis is highly regulated and strictly controlled. It is crucial for tissue development, growth and repair, although was found associated with chronic inflammatory diseases, such as rheumatoid arthritis, fibrosis and psoriasis, as well as with tumor growth and metastasis (Douglas Hanahan & Weinberg, 2000).

Cell adhesion molecules (CAM) are transmembrane receptors which facilitate adhesion to neighboring cells or the extracelluar matrix leading to multiple cellular processes such as cell migration, proliferation, morphogenesis, cell survival and gene expression. The over-expression of cell adhesion molecules plays a

fundamental role in recruitment of inflammatory cells from blood circulation to the inflamed tissue. Study in rat cornea revealed that endothelial adhesion molecules (E-seletin) and vascular cell adhesion molecule-1(VCAM-1) promote angiogenesis (Kräling et al., 1996). Blocking these CAMs by cyclic peptide or monoclonal antibodies not only inhibited inflammation, but also disrupt ongoing angiogenesis on chick chorioallantoic membrane (CAM) as well as ocular angiogenesis (Brooks et al., 1994). D-penicillamine is immunosupressor drug used to treat rheumatoid arthritis, showed profound antiangiogenic effect by inhibition endothelial cell migration, it believed that it suppress rheumatoid synovitis by quiescent the blood vessels from neovascularization for the emigration of chronic inflammatory cells (Matsubara & Ziff, 1987). Current drugs of rheumatoid arthritis such as methotrexate and gold compounds are proved to have anti-angiogenic effects (Colville-Nash & Seed, 1993). Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, indomethacin and ibuprofen are reported to inhibit inflammatory angiogenesis that is partly induced by PGE2, through direct effects on endothelial cells and modulation of COX-1 and COX-2 (Jones et al., 1999). Angiogenesis crucial for wound and ulcer healing, to supply oxygen and nutrients to the healing site, NSAIDs inhibited angiogenesis in gastric ulcer healing (Tarnawski et al., 1989). It evident from previous studies there is strong relation between inflammation and angiogenesis and antiiflammatory drugs have antiangiogenic efficacy and vice versa.

1.5 Antiangiogenic therapy

Antiangiogenic therapy is antitumor strategies that target the formation of know blood vessels that supply oxygen and nutrient to actively proliferating cancer cells, thus suppressing tumor growth and metastasis. Almost all anticancer agents

used in clinical settings for chemotherapy targeting rapidly dividing neoplastic cells, have serious adverse effects such as immunosuppression, intestinal disorder and hair loss (Kubota, 2012). On the other hand antiangiogenic therapy have very less side effects because vasculature is stable in adults under normal physiological condition except in the uterine endometrium during the menstrual cycle, beside antiangiogenic normally not cytotoxic, moreover some of them are antioxidant, which improve patient health.

1.5.1 Mechanisms of Anti-angiogenesis Therapy

Judah Folkman 1971 emphasized the strong relation of tumor vasculature and tumor growth, and if the tumor couldn't to establish its own blood vessels, it would wither and die, ever since there is growing number of antiangiogenic agents out of many studies. Antiangiogenic agents work by interfering with the key steps of angiogenesis. Vascular endothelial growth factor (VEGF) plays a central role in tumor angiogenesis (N. Ferrara, 2009). The expanding tumor tissue rapidly consume oxygen and tumor tissue specially the core of the tumor become hypoxic, which lead to activation of hypoxia-inducible factor (HIF) signaling in hypoxic-sensing cells stimulates VEGF expression, in turn it triggers microvessels growth (P. Carmeliet et al., 1998; Semenza et al., 1999). Anti-VEGF therapy is the most established antiangiogenic modalities. Several preclinical antitumor studies by administration of VEGF inhibitors have shown profound tumor suppression activity in different type of malignancies (N. Ferrara & Kerbel, 2005; K Jin Kim et al., 1993). Clinical studies on patient with metastasic colorectal cancer, administration of anti-VEGF monoclonal antibody (bevacizumab) showed significant efficacy in combination with 5-fluorouracil, subsequently U.S. Food and Drug Administration (FDA) approved bevacizumab in combination with 5-FU-based regimens for the treatment

of metastatic colorectal cancer (Peter Carmeliet & Jain, 2011; Venook, 2005). Some drugs induce apoptosis or inhibit the growth and migration of endothelial cells leading to shutdown of blood flow to the tumors, such as endostatin and Combretastatin A-4 (Tozer et al., 1999). Some drugs neutralize angiogenic molecules such as FGF and VEGF, where as some agents neutralize angiogenic factors that interfere with vascular basement membrane and extracellular matrix, hence affect cell invasion and migration such as MMPs inhibitor (Suramin) and some antibodies that interrupt adhesion molecules (anti-intergin alpha v beta 3).

1.5.2 Tyrosine kinase inhibitors

Tyrosine kinases are enzymes that catalyze the transfer of γ phosphate group from ATP to target proteins. They regulate different cellular processes and associated with the cytoplasmic domains of growth factor receptors as well as oncoproteins and many have the potential to cause transformation if mutated or hyperexpressed (Pawson, 2002; Workman, Brunton, & Robins, 1992). Therefore, Tyrosine kinases are an excellent target for cancer therapy, because they can modulate cancer-specific molecules and signaling pathways and may have limited nonspecific toxicities. Tyrosine kinase inhibitors (TKIs) are pharmaceutical small molecules orally active and well-tolerated. They have been found to have promising cytostatic and antitumor activity and now some of them approved for treatment of several malignancies such as breast, lung, kidney and pancreatic cancer as well as gastro-intestinal stromal tumors and chronic myeloid leukemia (Shawver, Slamon, & Ullrich, 2002). Examples of some tryrosine kinase inhibitors that inter clinical arena:

1.5.2.1 BCR-ABL Tyrosine Kinase Inhibitors

Translocation or abnormality (Philadelphia chromosome) is associated with 95% of patient with chronic myeloid leukemia (Epstein et al., 1999). The formation of the BCR-ABL oncogenes due to fusion of the BCR gene on chromosome 22 and the ABL tyrosine kinase gene on chromosome 9 leading to expression of protein-tyrosine kinases (BCR-ABL). The subsequent dysregulation of intracellular signaling enhances proliferation of myeloid cells and induce resistance to apoptosis of hematopoietic stem or progenitor cells (Druker et al., 2001). Imagtinib was found to inhibit or kill proliferating myeloid cell containing BCR-ABL protein which is unique to leukemic cells and expressed at high levels in patients (Drevs et al., 2000).

1.5.2.2 Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors

The epidermal growth factor receptor found implicated in most common human epithelial cancers and it has been associated with cancer cell proliferation, invasion, angiogenesis, metastasis and resistance to chemotherapy. Blocking (EGFR) emerged as promising therapeutic strategy against tumorigenesis. Studies revealed that (EGFR) can be inhibited by monoclonal antibodies or tyrosine kinase inhibitors (TKIs) (Ciardiello, 2000; Ranson, 2004). Several small molecules that block the ligand-induce activation of (EGFR) have been developed such as quinazolines, gefitinib and erlotinib, which are the most advanced in clinical development, are competitive inhibitors at the tyrosine kinase ATP binding site, also Irreversible inhibitors that bind to specific cysteines in the ATP-binding pocket of EGFR family receptors have been developed such as CI-1033 and EKB-569, in addition to exploring agents with homologous structures of EGFR receptor (Arora & Scholar, 2005; Janmaat & Giaccone, 2003).

1.5.2.3 Vascular Endothelial Growth Factor Tyrosine Kinase Inhibitors

Vascular endothelial growth factor (VEGF) is probably the most important growth factor that is involved in tumor angiogenesis, and it has a important role in the growth and progression of solid tumors (Hicklin & Ellis, 2005). Binding of the ligand VEGF-A to VEGFR-1 induces endothelial cell migration. VEGFR-2 believed to induce endothelial cell proliferation, permeability, and survival. Binding of VEGF to VEGFR-2 receptors leads to activation and autophosphorylation of intracellular tyrosine kinase domains, with initiation of intracellular signaling cascade (Bergsland, 2004; Parikh & Ellis, 2004). Tyrosine kinase inhibitors inter clinical arena are low molecular-weight that mimics ATP proteins and able to bind to the ATP-binding catalytic site of tyrosine kinase domain of VEGFRs, consequently block the intracellular signaling.

These (TKIs) agents include

- Semaxinib (SU5416) is small lipophilic molecule, with high protein-bound affinity and non-selective receptor tyrosine kinase inhibitor of VEGFs (Mendel et al., 2000). It showed profound antiangiogenic and antitumor efficacy in preclinical studies and it was the first VEGFR tyrosine kinase inhibitor to be investigated in clinical settings (Stopeck et al., 2002).
- Vatalanib (PTK787/ZK222584) which is a synthetic, orally bio-available agent, selective inhibitor of VEGFR-1 and VEGFR-2, although it most potent against VEGFR-2, also in higher concentrations it inhibits other tyrosine kinases such as PDGFR-β, c-MET and c-FMS (Lin et al., 2002). Preclinical studies have shown that vatalanib inhibited growth of implanted human tumor xenografts in nude mice and reduced microvasculature (Wood et al., 2000). Vatalanib entered the clinical arena as a single drug as well as

in combination with chemotherapy for treating colon cancer, advanced prostate and renal cell cancer, and relapsed/refractory glioblastoma multiforme, where VEGF overexpression has been demonstrated (Arora & Scholar, 2005; Bergsland, 2004).

- Sutent (SU11248): is multitargeted receptor tyrosine kinase inhibitor of VEGFR, PDGFR, c-KIT, and FLT-3 kinase activity as well as PDGFR-β, and c-Kit, with antiproliferative and antiangiogenic activity (O'Farrell et al., 2003). Phase III clinical trials with kidney cancer using sutent as a single agent and in combination chemotherapy are ongoing. It has demonstrated both efficacy and safety in these trials. It is also being studied in a phase III trial for imatinib-resistant GISTs. Sutent delayed the time of tumor progression on average from 1.5 to 6.3 months and also significantly reduced the death rate (Demetri et al., 2005).
- Sorafenib (BAY 43-9006): is a novel bi-aryl urea, it has double functions, its Raf kinase and VEGFRs inhibitor. Initially it was developed as Raf kinase inhibitor, subsequent studies shown that it can inhibit variety of kinase receptors, including VEGFR, EGFR, and PDGFR kinases (Strumberg et al., 2005; S. M. Wilhelm et al., 2004). Thus, Sorafenib inhibits cell proliferation as well as angiogenesis. Studies reveals that it has pronounce efficacy on: colon, pancreatic, lung, renal and ovarian neoplasm (S. M. Wilhelm et al., 2004). Intracellular activation of PDGFR receptors (α and β) results in cell transformation and generation of mitotic signal. These two receptor types are overexpressed in several solid tumors as well as in surrounding storma (Sedlacek, 2000).

1.5.2.4 Platelet-Derived Growth Factor Inhibitors

Signal transduction of Platelet-derived growth factor (PDGF) is through cell surface tyrosine kinase receptor (PDGFR) to stimulate various cellular functions, including growth, proliferation, and differentiation (Sedlacek, 2000). Leflunomide is small organic molecule that inhibits PDGF-mediated signaling events, including receptor tyrosine phosphorylation, DNA synthesis, cell cycle progression, and cell proliferation. basically immunosuppressive disease-modifying anti-rheumatic drug, it reduces signs and symptoms of the disease and attenuate structural damage (Dougados et al., 2005). Leflunomide proved to have antitumor in several preclinical studies. It effectively inhibited the growth of tumor xenografts that expressed PDGFR more than xenografts not expressing this receptor. A multi-institutional phase II study in hormone refractory prostate cancer patients with leflunomide revealed partial responses in 1 of 19 patients, a prostate- specific antigen decreased by more than 50% in 3 of 39 patients, as well as reduction in pain. The most frequently reported side effects with leflunomide were asthenia, nausea, anorexia, and anemia (Ko et al., 2001).

1.6 Programmed cell death (PCD)

Programmed cell death or apoptosis morphologically characterized by refraction of cell component, followed by DNA condensation and cytoplasmic shrinkage. Subsequently cell membrane shows blebs or spikes that are separate from the dying cell to form “apoptotic body”. The phospholipid asymmetry in the cell membrane of apoptotic cells no longer intact, moreover some changes appear in the mitochondrial outer membrane such as loss of its electrochemical gradient by formation of pores and leakage of cytochrome C into cytoplasm beside the appearance of phosphotidyl serine (PS) on the outer leaflet of the cell membrane. In

final step, the surrounding cell or macrophages phagocytose apoptotic bodies and the dying cell. Apoptosis is highly regulated process, arguably, involved the genetically programmed i.e., programmed cell death. In other words apoptosis is mediated by an intracellular program, which generally confers advantage during an organism's life-cycle (Formigli et al., 2000; Yi Lu, 2009).The events of apoptosis stand in contrast to necrosis, which describes uncontrolled process cell death due to exposure to harmful material or acute injury. The cytoplasm and mitochondria of the necrotic cell swell, and subsequently the cell bursts and many of its internal organelles spill over surrounding tissue, which more likely inflammatory responses are provoked (Raff, 1998). While there is no inflammatory reaction associated with apoptosis because there is no release of cellular content of apoptotic cell into the surrounding interstitial tissue, beside the apoptotic cell phagocytosed by adjacent cells thus likely preventing secondary necrosis, in addition the engulfing cells do not produce anti-inflammatory cytokines (Ortiz-Arduan & Neilson, 1994).

Table 1.1: depicts comparison between some of the major features of apoptosis and necrosis (Gold et al., 1994).

Feature	**Apoptosis**	**Necrosis**
Cell volume	Decreased	Increased
Plasma membrane integrity	Preserved	Lost
Plasma membrane structure	Characteristic blebbing	Lost
Cell-cell adhesion	Lost early	Typically preserved
Cell matrix adhesion	Lost early	Lost late
Exfoliation of cells	Early, as single cells	Late, as sheets of cells
Chromatin	Condensed	Preserved
Nuclear fragmentation	Characteristic	Absent
Cytosolic contents	Preserved	Released
Apoptotic bodies	Characteristic	Absent
Phagocytosis	Characteristic	Absent
Inflammatory response	Absent	Characteristic

Apoptosis and mitosis both play an important role in normal physiology fulfilling opposite but complementary needs to maintain homeostasis in the adult body. Therefore change in apoptosis rate (too little or too much cell death) may lead to pathology, hyper-activation of apoptosis could be a sign of some ailment such as neurodegenerative diseases (such as Parkinson's disease, Alzheimer's disease, Huntington's disease), ischemia-associated injury, Amyotrophic Lateral Sclerosis, and autoimmune disease like autoimmune syndrome (AIDS) as precursor of an infection with human immunodeficiency (HIV). In contrast apoptosis process is important in development of immune and nervous system when there is overproduction of cells, which cause death to the cells that no longer have reproductive antigen specificities or functional synaptic links (Nijhawan, Honarpour, & Wang, 2000; Opferman & Korsmeyer, 2003). Moreover, Apoptosis play fundamental role to eradicate pathogen-infected cells, in addition to its important role in wound healing in wiping the inflammatory cells and formation of granulation tissue into scar tissue (Elmore, 2007).

In cancer, apoptosis play a important role in suppressing carcinogenesis because the mechanisms of cell cycle are up-regulated either with an over proliferation of cells and/or decreased removal of cells (King & Cidlowski, 1998). Therefore suppression of apoptosis during carcinogenesis is thought to count for the development and progression of some cancers (Kerr, Winterford, & Harmon, 1994).

1.6.1 Apoptosis mechanisms

The mechanisms of apoptosis are very complex and sophisticated, involving an energy-dependent cascade of molecular events. Apoptotic signaling events

involve two main pathways: mitochondrial pathway (intrinsic) and death receptor pathway (extrinsic) (figure1.1)

1.6.1a Intrinsic Pathway

The intrinsic signaling pathways involve different non-receptor-mediated stimuli that produce intracellular signals that targets mitochondrial-initiated events within the cell. The stimuli that trigger the intrinsic pathway produce intracellular signals that may act in either direct or indirect fashion, the directly by radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals. The indirect fashion via the absence of specific growth factors and cytokines which are responsible of suppressing of cell death (like anti-apoptotic Bcl-2 family members including Bcl-2 and Bcl-XL), therefore masking these anti-apoptotic protein results in initiation apoptosis(Elmore, 2007). As a result of all these stimuli some changes occur in the inner mitochondrial membrane leading to mitochondrial permeability transition (MPT) pore, loss of mitochondrial integrity and release of cytochrome *c* , Smac/DIABLO and serine protease HtrA2/Omi(Du, Fang, Li, Li, & Wang; Garrido et al., 2006; van Loo et al., 2002). Cytochrome *c* activates procaspases-9, accumulation of procaspase-9 leads to activation of caspases-9.Smac/DIABLO and HtrA2/Omi are believed to promote apoptosis by inhibiting IAP (inhibitors of apoptosis proteins) activity. After triggering the apoptosis process, another group of pro-apoptotic proteins (AIF, endonuclease G and CAD will be released.

The second group of pro-apoptotic proteins, AIF, endonuclease G and CAD, are released from mitochondria. AIF cause DNA fragmentation and condensation of peripheral nuclear peripheral nuclear chromatin (Joza et al., 2001).