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TESI DOCTORAL

Novel mechanistic link between microtubule disruption and inhibition of tumor angiogenesis

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Badalona, Setembre de 2004

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CERTIFICA:

Que la Tesi Doctoral titulada: "**Novel mechanistic link between microtubule disruption and inhibition of tumor angiogenesis**", ha estat realitzada per en Daniel Escuin i Borràs sota la seva direcció, i és apta per a ser presentada davant del Tribunal i optar al grau de Doctor.

Dr. Rafael Rosell i Costa

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Abbreviations

2ME2	*2-Methoxyestradiol*
Ang	*Angiopoietins*
ARNT	*Aryl hydrocarbon Receptor Nuclear Translocator*
CHX	*Cyclohexamide*
ECM	*Extracellular Matrix*
EGF	*Epidermal Growth Factor*
ER	*Estrogen Receptor*
ET-1	*Endothelin-1*
FGF-2	*Fibroblast Growth Factor 2*
FGFa	*Fibroblast Growth Factors*
Glut-1	*Glucose Transporter-1*
HDAC	*Histones deacetylase*
HIF-1	*Hypoxia Inducible Factor*
HRE	*Hypoxia Response Element*
HUVEC	*Human Umbilical Vein Endothelial Cells*
ICAM-1	*Intercellular adhesion molecule-1*
IGF-1	*Insulin Growth Factor 2*
MAPK	*Mitogen-Activated Protein Kinase*
MAPs	*Microtubule Associated Proteins*
MMPs	*Matric Metalloproteases*
MTOC	*Microtubule-organizing center*
MVD	*Microvessel Density*
NE	*Nuclear Extracts*
PA	*Plasminogen*
PHDs	*prolyl-4-hydroxylases*
PI3K	*phosphatidylinositiol 3-kinase*
pVHL	*Von Hippel-Lindau protein*
RCCs	*Renal Cell Carcinomas*
TGF-α	*Transforming Growth Factor α*

Ties	*Tyrosine kinase with immunoglobulin- and EGF-like domains*
TOPO-I	*Topoisomerase I*
tPAs	*Tissue-type plasminogen activators*
Tx	*Taxol*
uPAs	*urokinase-type plasminogen activators*
VCAM-1	*Vascular cell adhesion molecule-1*
VCR	*Vincristine*
VEGF	*Vascular Endothelial Growth Factor*
VEGFR	*Vascular Endothelial Growth Factor Receptor*
WB	*Western Blotting*
WCE	*Whole Cell Extracts*
γ-TuRC	*γ-tubulin ring complex*

INTRODUCTION

Among the oldest of the world's writings mentioning both benign and malignant tumors are several Egyptian papyrus scrolls, dating from approximately 1600 BC. Hippocrates, an ancient Greek physician, and so called by some as the "father of modern medicine", first gave the name *karkinos* and *karkinoma* (the ancient Greek words for "crab") to a group of diseases that are now known as cancer.

The hallmarks of malignant neoplastic tissue are unregulated cell proliferation, invasiveness and metastasis to distant sites in the body. Cancer is a genetic disease. The majority of human tumors have a dramatically enhanced mutation rate, making cells genetically unstable. This genetic instability is caused by mutations in a very specific set of genes (oncogenes, tumor suppressor genes, etc…) whose protein products protect the genome from alteration. Some people inherit these mutations and have a raised incidence of cancer. However, most frequently, the destabilizing mutations arise *de novo* as a tumor develops, helping the cancer to accumulate mutations much more rapidly that its neighbors do. It is important to note that genetic instability does not itself give cells a selective advantage unless it has additional mutations that confer some competitive advantage. It appears that an optimum level of genetic instability exists for the development of cancer, making a cell mutable enough to evolve dangerously, but not so mutable that it dies.

In general, cancer arises by a process in which, an initial population of slightly abnormal cells evolve from bad to worse through successive cycles of mutation and natural selection. At each step, one cell acquires an additional mutation that gives it a selective advantage over its neighbors, making it able to thrive in its intratumoral environment that may be harsh, with low levels of oxygen, scarce nutrients, and the natural barriers to growth presented by the normal tissues. Upon reaching certain tumor size, tumors will develop hypoxic and necrotic regions because their vasculature can not supply oxygen and other vital nutrients to all the cells. Eventually, the tumor will be able to turn on the "angiogenic switch" which eventually will allow tumor cells to leave the primary tumor, invade the local host tissue, enter the circulation, arrest at the distant vascular bed, extravasate into the target organ interstitium and parenchyma and proliferate as a secondary colony.

It is known that hypoxia, a reduction in the normal levels of oxygen, in tumors tends to select for a more malignant phenotype, increases mutation rates, increases expression of genes associated with angiogenesis and tumor invasion, and is associated with a more metastatic phenotype of

human cancers. The primary mechanism of tumor response to reduced oxygen levels is via activation of hypoxia-inducible transcription factors (HIF). HIF proteins are critically important in tumor progression and angiogenesis as they regulate the transcription of >40 genes involved in angiogenesis, metabolic adaptation to hypoxia, survival and invasiveness. Among the angiogenic factors regulated by HIF-1 are members of the vascular endothelial growth factor (VEGF) family that play a predominant role in the angiogenic process.

In the case of cancer, angiogenesis –development of new blood vessels- is essential for the growth, progression and metastasis of a tumor. Tumor invasion and metastasis are the major causes of treatment failure and ultimately death in all human cancers. Therefore, agents that inhibit angiogenesis represent an attractive therapeutic modality. Based on successful preclinical data, several antiangiogenic factors alone or in combination with conventional therapies are now in clinical trials. Among those compounds is 2-Methoxyestradiol (2ME2), a naturally occurring derivative of estradiol currently in phase I/II clinical trials. 2ME2 is an orally active, well tolerated small molecule that possesses antitumor and antiangiogenic activity [1]. Although several mechanisms of action has been proposed, the process by which 2ME2 inhibits tumor growth and angiogenesis is still not well understood. 2ME2 has the ability to destabilize microtubules upon binding to colchicine binding-site of tubulin. Recently, it has also been recognized that microtubule-targeting drugs, especially those binding at the colchicine binding site, have the ability to shut down existing tumor vasculature by selectively inhibit the growth of endothelial cells in tumor vessels, producing massive tumor necrosis [2].

In this work we seek to understand the antitumor and antiangiogenic mechanism of action of 2ME2. Specifically, we want to characterize the molecular mechanism by which 2ME2 inhibits HIF-1 and angiogenesis and investigate whether there is cause-effect relationship between 2ME2's antiangiogenic and anti-tubulin properties

TUMOR ANGIOGENESIS

Angiogenesis, the development of new blood vessels from pre-existing vasculature, is essential during embryonic development. In the adult, angiogenesis occurs under physiological conditions in highly regulated processes such as wound healing and ovulation. With these exceptions, the vasculature is quiescent in adults and is tightly regulated by a balance of pro- and antiangiogenic factors. Under pathological conditions, angiogenesis supports the development of many diseases including cancer, rheumatoid arthritis, psoriasis, macular degeneration and diabetic retinopathy [3]. In the case of cancer, angiogenesis is essential for the growth, progression and metastasis of a tumor and the exquisite regulatory balance between pro- and antiangiogenic factors is disturbed in favor of angiogenic stimulators (Fig. 1).

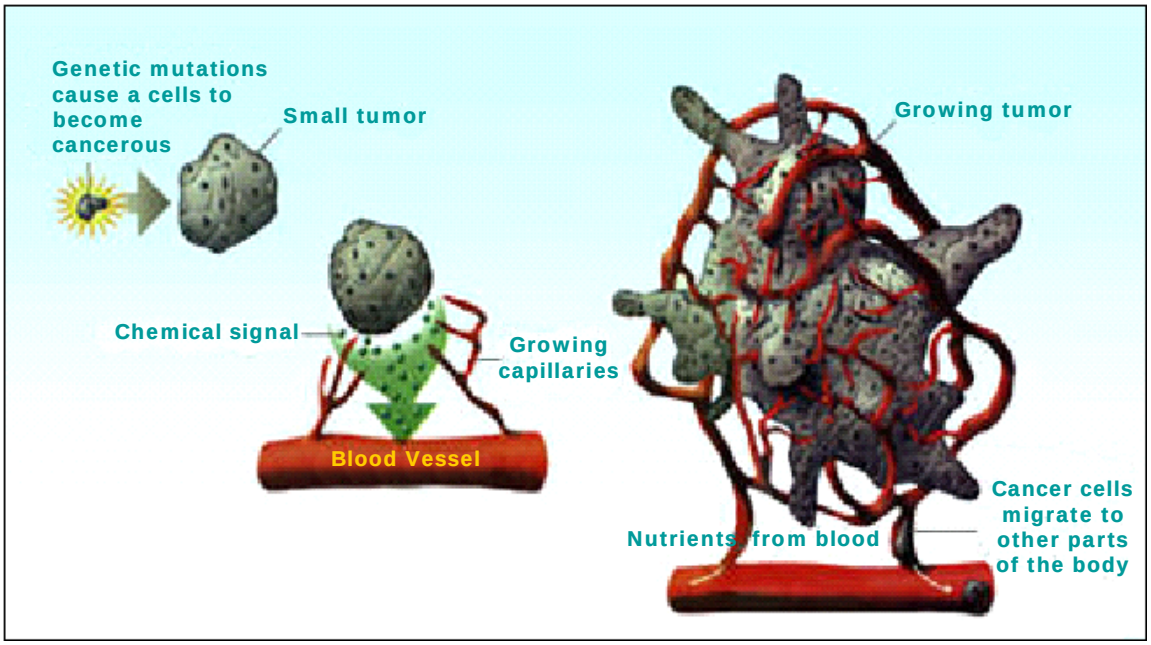

Figure 1. Tumor angiogenesis. Angiogenesis, the development of new blood vessels is required for the growth of a tumor beyond a certain size. Newly formed blood vessels feed cancer cells with nutrients and oxygen in order to sustain their growth. Newly formed blood vessels help cancer cells spread in the body to distant sites (metastasis).

The term “angiogenesis” was first used in 1787 by British surgeon Dr. John Hunter to describe blood vessels growing in the reindeer antler. The observation that angiogenesis occurs around tumors was made nearly 100 years ago [4]. But it is not until 1971, when Dr. Judah Folkman hypothesizes that tumor growth and metastasis are dependent upon angiogenesis and hence,

blocking angiogenesis could be a strategy to arrest tumor growth [5]. His theory, however, was initially regarded as heresy by leading physician and scientists. It is now widely accepted that the angiogenic switch is "off" when the effect of pro-angiogenic molecules is balanced by that of anti-angiogenic molecules and is "on" when the net balance is tipped in favor of angiogenesis. Various signals trigger this switch including metabolic stress (hypoxia, low pH, hypoglycemia), mechanical stress (pressure generated by proliferating cells) genetic mutations (activation of oncogenes or inactivation of tumor suppressor genes). How the interplay between environmental and genetic mechanism influences tumor angiogenesis and growth is a complex and largely unresolved matter. Pro- and antiangiogenic factors can emanate from cancer cells, endothelial cells, stromal cells, blood and the extracellular matrix. Their relative contribution is likely to change with tumor type and tumor site as well as with tumor growth, regression and relapse.

1.1. Regulation of angiogenesis

Angiogenesis is a complex process involving extensive interplay between cells, soluble factors, and ECM components and it can be divided into three steps: (1) breakdown of the extracellular matrix, (2) Proliferation of endothelial cells, and (3) migration of endothelial cells. The induction of this process is mediated by angiogenic factors released by both tumor and host cells, and depends on the net balance of positive and negative regulators.

1.1.1. Basement membrane breakdown: proteolytic enzymes

To initiate the formation of new capillaries, endothelial cells of existing blood vessels must degrade the underlying basement membrane and invade into the stroma of the neighboring tissue. These processes of endothelial cell invasion and migration require the cooperative activity of the plasminogen (PA) system and the matrix metalloproteinases (MMPs) [6].

The PA system is composed of an inactive proenzyme, plasminogen, which can be converted to plasmin by two serine proteases: urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). Plasmin displays a broad spectrum of activity and can degrade several glycoproteins (laminin, fibronectin), proteoglycans, and fibrin to activate pro-MMPs and to activate or release growth factors from the extracellular matrix.

MMPs represents a family of over 24 proteases that can process virtually any component of the ECM and are classified as collagenases, gelatinases, stromelysins, and matrilysins, based on their

ECM substrate specificity. They are Ca^{2+}- and Zn^{2+}-dependent proteases that are activated by the removal of an amino-terminal propeptide domain. Activation of MMPs is achieved either by autoproteolysis or processing by another MMP or a serine protease. MMPs activity is tightly controlled by endogenous inhibitors, such as β2 macroglobulin, and specific tissue MMP inhibitors (TIMPs) [7].

PAs and MMPs are secreted together with their inhibitors, ensuring a stringent control of local proteolytic activity, in order to preserve normal tissue structure. However, a large body of evidence suggests that this regulation is lost during tumor growth and metastasis; excessive MMP activity has been detected in colorectal, lung, breast, gastric, cervical, bladder, prostate cancer, and malignant glioblastoma. Moreover, in a number of these studies, a good correlation was found between the amount of MMPs and the aggressiveness/invasiveness of the tumor [7].

1.1.2. Endothelial cell migration and proliferation: angiogenic factors

Following proteolytic degradation of the extracellular matrix (ECM), endothelial cells start to migrate through the degraded matrix. They are followed by proliferating endothelial cells, which are stimulated by a variety of growth factors, some of which are released from the degraded ECM.

A variety of angiogenesis inducers have been described, which can be classified into three categories. The first category consists of the VEGF and angiopoietins families, which specifically act on endothelial cells. A second category of angiogenic inducers contains most direct-acting molecules, including several cytokines, chemokines, and angiogenic enzymes that activate a broad range of target cells in addition to endothelial cells. The prototype member of this group is FGF-2, one of the first angiogenic peptides to be characterized. The third group of angiogenic molecules includes the indirect-acting factors, whose effect on angiogenesis results from the release of direct-acting factors from macrophages, endothelial or tumor cells. The most extensively studied are TNF-α and TGF-β, which inhibit endothelial cell proliferation *in vitro*.

1.1.2.1. VEGF

VEGF is a potent and critical vascular regulator whose cellular concentrations are precisely regulated in a spatial, temporal and quantitative manner to avoid vascular disaster. Disruption of both VEGF alleles in mice results in almost complete absence of a vasculature. Disruption of

even a single VEGF allele in mice leads to embryonic lethality due to severe vascular abnormalities, providing to our best knowledge the only example of embryonic lethality due to a simple half-dosage effect [8, 9].

During tumor angiogenesis VEGF also plays a crucial role on the entire angiogenic process by stimulating ECM degradation, proliferation, migration and differentiation of the endothelial cells. It also induces the expression of uPA, PAI-1, uPAR, and MMP-1 in endothelial cells and regulates vascular permeability.

VEGF is one member of a family of four proteins that includes placenta-derived growth factors (PIGF), VEGF-B, VEGF-C and VEGF-D. VEGF gene encodes at least seven alternatively spliced forms consisting of 121, 145, 148, 165, 183, 189 and 206 amino acids respectively being $VEGF_{165}$the predominant form. The major isoforms are $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$. The smaller molecules of $VEGF_{121}$, $VEGF_{145}$ and $VEGF_{165}$ amino acids are secreted, while the larger molecules of $VEGF_{189}$ and $VEGF_{206}$ amino acids remain cell surface associated. Yet little is known about the quantity of VEGF-isoforms in different types of normal and malignant cells. The major variants $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ are expressed in nearly all investigated tissues, while the isoforms $VEGF_{145}$, $VEGF_{148}$, $VEGF_{183}$, and $VEGF_{206}$ were found only in a few cell types and seems to be of minor importance [10].

The active form of the various members of the VEGF is a 34-42kDa homodimer which have overlapping abilities to interact with cell-surface tyrosine kinase receptors. Two of them VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) have been identified on vascular endothelium, while a third one VEGFR-3 (Flt-3) is expressed on lymphatic vessels. In addition, there are a number of accessory receptors such as the neuropilins [11] which seem to be primarily involved in modulating binding to the main receptors, although roles in signaling have not been ruled out. Several studies have indicated that VEGFR-1 and VEGFR-2 have different signal transduction properties; VEGFR-2 seems to mediate the major VEGF-induced biological responses, whereas VEGFR-1 may have a negative role, either by acting as a decoy receptor or by suppressing signaling through VEGFR-2. VEGFR-3 on the other hand seems to have a critical role in the development of the lymphatic system.

Transcription of VEGF mRNA is regulated by tissue oxygen tension. Exposure to hypoxia induces VEGF expression rapidly and reversibly, through both increased HIF-1 mediated transcription and stabilization of the mRNA. Similar to VEGF, regulation of VEGF receptor

gene expression is regulated by hypoxia. In contrast, normoxia down-regulates VEGF production and even causes regression of some newly formed blood vessels. In addition to the hypoxia stimulus, VEGF levels are also induced by different growth factors and cytokines and therefore VEGF may function as a mediator for indirect-acting angiogenic factors.

1.1.2.2. Angiopoietins

Along with the VEGF family, another family of endothelial growth factors critical for vascular development is the angiopoietins. The angiopoietin (Ang) family consists of four members, Ang1 to 4, although very little is know about Ang3 and Ang 4. All angiopoietins bind to Tie2, a receptor tyrosine kinase. These ligands appear to have opposing actions in endothelial cells (ECs), as Ang1 and Ang4 function as agonists and activate Tie2, while Ang2 could either activate or antagonize Tie2 depending on the cell examined.

Studies in knockout mice have shown that embryos lacking Ang1 or Tie2 develop a rather normal primary vasculature but this vasculature fails to undergo normal further remodeling and stabilization of the blood vessels [12]. Transgenic overexpression of Ang2 in the embryonic endothelium resulted in embryonic death due to defects resembling those of Ang1 or Tie2 knockouts, demonstrating that Ang2 could act as a Tie2 antagonist *in vivo* at least in a context-dependent manner. Ang2 it has been proposed to be involved in initiating angiogenic remodelling by blocking the stabilizing influence of Ang1, allowing the endothelial cells to revert to a more plastic and destabilized state reminiscent of developing vessels [13].

1.1.2.3. Fibroblast growth factors (FGFs)

The fibroblast growth factors (FGF) family consists of at least 19 members and FGF-2 was one of the first angiogenic factors to be characterized. FGF-2 show angiogenic activity *in vitro* and *in vivo* and induce endothelial cell proliferation, chemotaxis, and uPA production and modulates integrin expression, gap junction intercellular communication, and VEGF, VEGFR-2, and uPAR up-regulation *in vitro* [14, 15]. FGF-2 is produced by many tumor cell lines *in vitro* and is thought to play a role in the growth and neovascularization of solid tumors [16].

1.1.3. Cell–cell and cell–matrix interactions: adhesion molecules

The processes of cell invasion, migration, and proliferation not only depend on angiogenic enzymes, growth factors, and their receptors, but are also mediated by cell adhesion molecules. To initiate the angiogenic process, endothelial cells have to dissociate from neighboring cells before they can invade the underlying tissue. During invasion and migration, the interaction of the endothelial cells with the ECM is mediated by integrins. Also, the final phases of the angiogenic process, including the construction of capillary loops and the determination of the polarity of the endothelial cells, which is required for lumen formation, involve cell-cell contact and cell-ECM interactions.

Cell adhesion molecules can be classified into four families depending on their biochemical and structural characteristics. These families include the selectins (P-selectin, E-selectin) [17], the immunoglobulin supergene family (ICAM-1, VCAM-1)[18], the cadherins [19], and the integrins. In all cases, members of each family have been implicated in neovascularization [20].

Integrins are a group of cell-matrix adhesion receptors that transmit information bi-directionally between the outside and the inside of the cell assisting vascular cells to build new vessels in coordination with their surroundings. Endothelial cells thus express several distinct integrins, allowing attachment to a wide variety of ECM proteins. The $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins positively regulate the angiogenesis switch, since their pharmacological antagonists suppress pathological angiogenesis.

Integrin $\alpha_v\beta_3$ was found to be particularly important during angiogenesis. $\alpha_v\beta_3$ is a receptor for a number of proteins including fibronectin, vitronectin, laminin, vWF, fibrinogen, and denatured collagen. $\alpha_v\beta_3$ is nearly undetectable on quiescent endothelium, but is highly up-regulated during cytokine- or tumor-induced angiogenesis. In activated endothelium, $\alpha_v\beta_3$ suppresses the activity of both p53 and the p53-inducible cell-cycle inhibitor $p21^{WAF1/CIP1}$, while increasing the Bcl2/Bax ratio, resulting in an anti-apoptotic effect [21]. Another receptor that has been implicated recently in angiogenesis is integrin $\alpha_v\beta_5$. Antibodies directed against $\alpha_v\beta_5$ were found to specifically block FGF-2- or TNF-α-induced angiogenesis, whereas antagonists of $\alpha_v\beta_5$ blocked VEGF-induced angiogenesis.

1.2. Formation of tumor vessels.

The increasing metabolic demands of growing tumor cells leads to the formation of severely hypoxic microenvironments and secretion of angiogenesis-stimulating factors. Although most

human cancers may persist *in situ* for large periods of time in a pre-vascular phase, growth beyond a certain size (1-3mm^3) requires new blood vessel formation. Tumor vessels develop by sprouting or intussusception (i.e. collateral growth bridges between vessel networks) from pre-existing vessels. Circulating endothelial precursors, shed from the vessel wall or mobilized from the bone marrow, can also contribute to that process. During physiological angiogenesis, VEGF maintains its position as the most critical driver of vascular formation, as it is required to initiate the formation of immature vessels by vasculogenesis or angiogenic sprouting. However, despite its requisite role in vascular formation, VEGF must work in concert with other factors, especially those of the angiopoietin family. For instance, Ang1 seems to be involved for further remodeling and maturation of this initially immature vasculature and stabilizing vessel walls. Disruption of this stabilizing signal coincides with reinitiation of vascular remodeling in the adult — as occurs in the adult female reproductive system or in tumors. Such de-stabilization seems to involve the autocrine induction — by the endothelium to be remodeled — of a natural antagonist of Ang1, termed Ang2. VEGFs and angiopoietins apparently recapitulate their developmental roles during vascular remodeling in the adult, and administration of individual factors to the adult allows them to reprise these roles but not to trigger the entire process. Thus, VEGF administration can initiate vessel formation in adult animals, but by itself promotes formation of only leaky, immature and unstable vessels. In contrast, Ang1 administration seemingly further stabilizes and protects the adult vasculature, making it resistant to the damage and leak induced by VEGF or inflammatory challenges.

In contrast to normal vessels, tumor vasculature is highly disorganized and structurally and functionally abnormal; vessels are tortuous and dilated, with uneven diameter, excessive branching and shunts. This may be due to an imbalance of angiogenic regulators, especially VEGF and angiopoietins. The temporal and spatial expression of these regulators is not as well coordinated in tumors as in physiological angiogenesis and induction of VEGF, in the absence of the entire angiogenic program, leads to formation of immature and leaky vessels. Moreover, tumor vessels lack protective mechanisms that normal vessels acquire during growth. For example, they may lack functional perivascular cells (pericytes), which are needed to protect vessels against changes in oxygen or hormonal balance, provide them necessary vasoactive control to accommodate metabolic needs, and induce vascular quiescence. Finally, the vessel

wall is not always formed by a homogenous layer of endothelial cells. Instead, it may be lined with only cancer cells or a mosaic of cancer and endothelial cells. As a consequence, tumor blood flow is chaotic and variable and leads to acute hypoxic and acidic regions in tumors in addition of chronic hypoxia (as it will be describe bellow).

HYPOXIA

Hypoxia is a hallmark of solid human cancers, as the microenvironment of rapidly growing solid tumors is associated with increased energy demands and diminished vascular supply, thus, resulting in focal areas of prominent hypoxia (reduced oxygen tension). The existence of hypoxic regions in human tumors was first postulated by Thomlinson and Gray 50 years ago, based on their observations on the presence of necrosis at a certain distance away from blood vessels [22]. Subsequently, the use of tissue oxygen electrodes showed that the median oxygen pressure in tumors was in the range of 10-30 mmHg, whereas in normal tissue it ranged from 24-66 mmHg [23]. Another important characteristic of hypoxic tumors is the shift from an oxidative metabolism to anaerobic glycolysis in order to allow tumor cells to cope with hypoxic environments. This increase in anaerobic glycolysis is achieved by the overexpressin of sugar transporters and enzymes important in carbohydrate metabolism. As a consequence, solid tumors excrete much larger quantities of lactic acid (a product of the glycolytic pathway) than their normal tissue counterparts. This phenomenon is known as Warburg effect and was named after being described by Otto Warburg [24].

It has been demonstrated that hypoxia in tumors tends to select for a more malignant phenotype associated with increased mutation rates, and increased expression of genes promoting angiogenesis and tumor invasion, leading to a more metastatic phenotype of human cancers [25]. In addition, hypoxic tumors are resistant to both chemotherapy and radiation. The effectiveness of chemotherapy is hampered as hypoxia selects for cells with a very aggressive and often metastatic phenotype, in addition to the fact that hypoxic areas lack functional vessels, inhibiting the delivery of chemotherapy drugs. Moreover, the multidrug resistance (*MDR1*) gene product P-glycoprotein is induced by ambient hypoxia [26], contributing to the overall drug-resistance phenotype of hypoxic tumors. Radiotherapy on the other hand, is severely compromised because oxygen is necessary to produce the reactive oxygen species that mediate the secondary toxic effects produced by ionizing radiation. Consequently, hypoxia is a major reason for the failure of cancer therapy and hypoxic tumors are therefore associated with poor patient outcome.

The main mechanism through which tumor cells respond to reduced oxygen levels is via activation of hypoxia-inducible transcription factors (HIF), which are critically important in

tumor progression and angiogenesis as its transcriptional activity promotes induction of genes involved in angiogenesis, glycolysis, growth-factor signaling, immortalization, genetic instability, tissue invasion and metastasis, apoptosis and pH regulation.

2.1. Hypoxia Inducible Factors (HIFs)

In mammalian cells there are at least three isoforms of the α-subunit of HIF (HIF-1α HIF-2α and HIF-3α). The first identified isoform, HIF-1α, was originally discovered as a high-affinity DNA binding protein localized to the 3′ hypoxia-responsive element (HRE) on the erythropoietin gene [27]. Two additional HIF-α subunits have subsequently been cloned and named HIF-2α (independently identified as EPAS-1 [28], HLF [29], HRF [30] or MOP2 [31] by four laboratories) and HIF-3α [32, 33]. HIF-1α and HIF-2α are closely related and are each able to interact with hypoxia responsive elements to induce transcriptional activity in response to hypoxia. In contrast, HIF-3α appears to be involved in negative regulation of the hypoxic response, through an alternative spliced transcript termed inhibitory PAS domain protein [34].

HIF-1α and -2α have high sequence identity and their functional domains are similarly organized and are regulated in a similar way (as described below). However, the modes of expression of HIF-1α and -2α differ substantially in various tissues of adult mice and during different developmental processes [29, 35]. HIF-1α is believed to be a universal master regulator for hypoxia-inducible gene expression, as it is expressed in a wide range of cell types [36]. In contrast, HIF-2α is most prominently expressed in the endothelial cells of various tissues, such as the brain, heart, kidney, and liver. HIF-2α mRNA is also observed in alveolar epithelial cells in the lung [29, 30]. These data suggest that HIF-1α and HIF-2α might have their own specific physiological functions *in vivo*. In addition, complete deficiency of both subunits results in developmental arrest and embryonic lethality. Histopathological analyses of homozygotic mutant embryos showed that HIF-2α deficiency causes either severe vascular defects in both the yolk sac and embryo proper [37] or pronounced bradycardia in the embryo due to defective catecholamine production [38]. In contrast, HIF-1α-deficient mice manifest neural tube defects and cardiovascular malformations [39, 40]. Overall, these results suggest that the two HIF-α isoforms play separate but essential roles during embryonic development.

2.2. Oxygen-dependent regulation of HIF-1

Hypoxia Inducible Factor-1 (HIF-1) is a transcriptional factor that plays a key role in adaptation to hypoxia and therefore in tumor progression and angiogenesis. HIF is a heterodimer composed of HIF-1α and HIF-lβ (also know as ARNT -aryl hydrocarbon receptor nuclear translocator) subunits both of which belong to basic helix–loop–helix (bHLH)/PAS domain transcription. HIF-lβ is constitutively expressed, whereas HIF-lα is maintained at low steady-state levels under normoxia through controlled degradative processes in the presence of oxygen (Fig. 2).

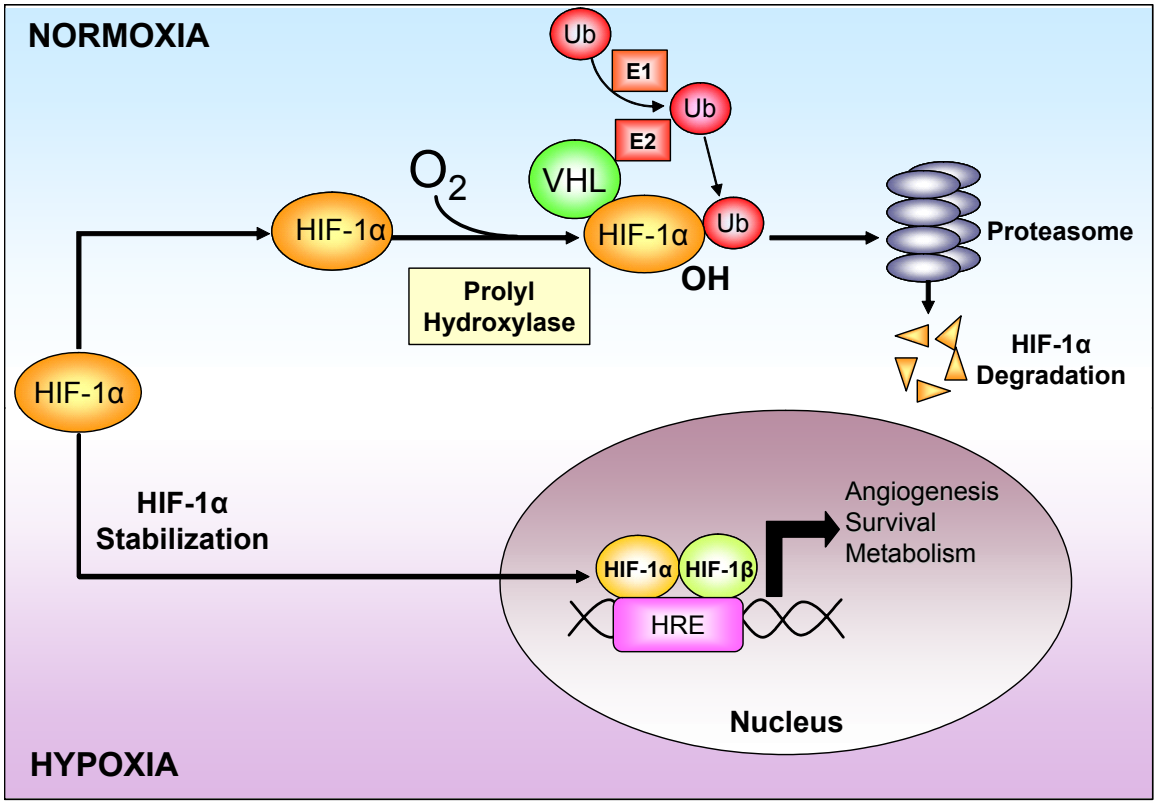

Figure 2. HIF-1 pathway. Under normoxic conditions HIF-1α is post-transcriptionally modified by specific prolyl-4-hydroxylases allowing its interaction with a complex containing the von Hippel-Lindau protein. pVHL ubiquitinates HIF-1α and therefore targeting it for proteasomal degradation. In the absence of oxygen, HIF-1α is stabilized allowing HIF-1α to heterodimerize with its partner HIF-1β and bind to the hypoxia response element, therefore activating the transcription of several genes implicated in the response to hypoxia.

This control is exerted by a family of specific enzymes known as HIF-lα prolyl-4-hydroxylases (PHD) [41-43] which are absolutely dependent on iron and oxygen as co substrate [44, 45], providing the molecular basis for the oxygen-sensing function of these enzymes. Hydroxylation of HIF-1α at residues 402 and 564 at the oxygen-dependent degradation (ODD) domain allows the interaction of HIF-1α with the β-domain of the Von Hippel Lindau protein (pVHL). pVHL acts as an E3 ubiquitin ligase giving the specificity to proteasomal degradation by targeting HIF-lα for ubiquitination. The interaction between HIF-1α and VHL is further accelerated by acetylation of lysine residue 532 through the N-acetyltransferase ARD1 [46]. In a second hydroxylation control HIF-1α/VHL complex also recruits Factor Inhibiting HIF-1α (FIH), which has been recently shown to hydroxylate Asn 803 residue. This hydroxylation abrogates the interaction of HIF-1α with the transcriptional coactivator p300/CBP inhibiting in this way HIF-1 transcriptional activity [47]. Interaction of HIF-lα/VHL with FIH also permits the recruitment of histones deacetylases (HDAC), which are known to function as transcriptional co-repressors [48] and that may also contribute to the loss of HIF-l a transcriptional activity under non-hypoxic conditions (Fig. 3).

Under hypoxic conditions PHDs are inhibited resulting in an exponential increase in the HIF-1α protein as the O_2 concentration decreases. Under these conditions HIF-lα heterodimerizes with its partner HIF-lβ and binds to the specific enhancer DNA sequences called hypoxia response elements (HRE) [A(G)CGTG] activating the transcription of more than 40 genes involved in angiogenesis, metabolic adaptation and survival of cells under hypoxia [49, 50].

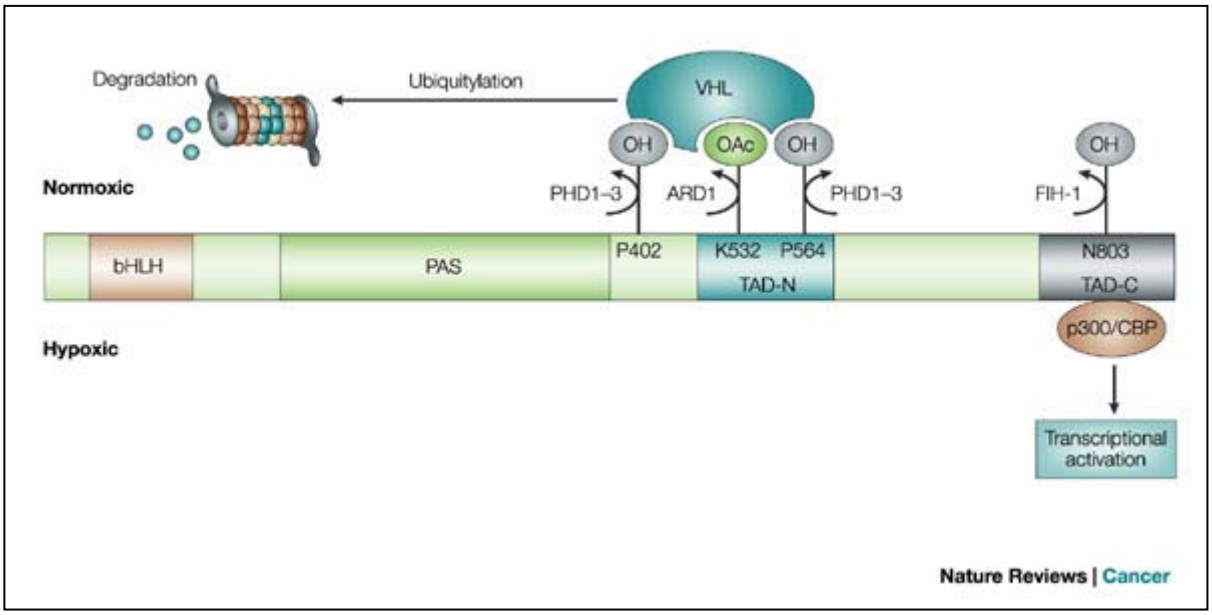

Figure 3. Oxygen-dependent regulation of HIF-1α. HIF-1α protein stability and transcriptional activity is regulated by oxygen-dependent post-translational modifications. Hydroxylation of Pro402 or Pro564 in HIF-1α is required for binding of VHL, which recruits an E3 ubiquitin–protein ligase that targets HIF-1α for degradation. VHL also recruits histone deacetylases (HDAC) that repress transactivation domain function. FIH-1, which binds to both VHL and HIF-1α, is also required for transcriptional repression. Hydroxylation of Asn803 in HIF-1α blocks its interaction with p300, a key co-activator that is required for transcriptional activation [49].

2.3. Oxygen-independent regulation of HIF-1

HIF-1α activity is also controlled in an O_2-independent fashion under the regulation of signaling pathways, both via the phosphatidylinositiol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways [51]. In contrast to hypoxia regulation, which affects all cells by stabilizing HIF-1α protein leading to an increase in HIF-1 transactivation function, this regulation is cell-type specific and mainly increases the rate of HIF-1α synthesis as well as the stimulation of the HIF-1α transactivation domain function [52, 53]. Among the growth factors and cytokines that activate these signal transductions pathways are insulin growth factor 2 (IGF2), transforming growth factor α (TGFα), epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), heregulin, insulin, insulin-like growth factor 1 and 2. These growth factors induce HIF-1α protein through its interaction with Receptor Tyrosine Kinase that will activate

PI3K-AKT-FRAP pathway or MAP kinase pathway, resulting in either an increase in HIF-1α protein synthesis or stimulation of the HIF-1α transcription activity, respectively (Fig. 4).

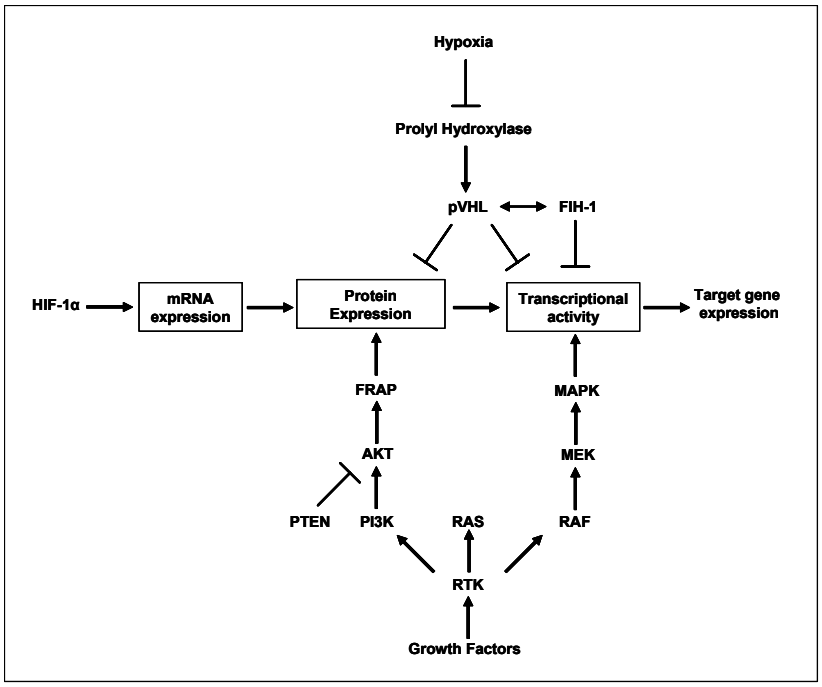

Figure 4. Oxygen-independent regulation of HIF-1α Oxygen- and growth factor-regulated signal transduction pathways determine HIF-1α protein expression and transcriptional activity. HIF-1α is induced by hypoxia (top) in all cell types. In contrast, activation of the PI3K or MAPK pathway (bottom) has cell- and stimulus-specific effects on HIF-1α[51]

Induction of HIF-1α under normoxia conditions has been also linked with *Helicobacter pylori*-induced gastric carcinogenesis. Reactive oxygen species (ROS) from *Helicobacter pylori* in gastric epithelial cells has been shown to induce constant HIF-1α expression under normoxia and subsequently activate HIF-1-mediated transcription [54]. However, the precise role that ROS species play on HIF-1α stability is still controversial, since ROS species have been reported to both inhibit HIF-1 (NAPDH oxidase hypothesis) [55] and activate it (mitochondria pathway) [56]. In either case, the effectors whose activity is regulated by ROS and transduce the signal directly or indirectly to HIF-l remains unknown.

2.4. HIF-1 and cancer

HIF-l is a critical, genome-wide transcription regulator identified for oxygen homeostasis responsive to hypoxic stress. HIF-1 controls the expression of >80 genes involved in angiogenesis, metabolic adaptation to hypoxia, tumor survival and invasion [49]. Several lines of evidence show clearly that overexpression of HIF-1α and HIF-1-dependent genes contribute to the lethal phenotype of many solid tumors. These lines of evidence include:

2.4.1. HIF-1-regulated genes.

More than 80 putative direct HIF-1 target genes have been identified based on functional studies of the HREs in the promoter region or by loss of hypoxia-induced genes in either VHL-null cells or cells transfected with specific siRNA that targets HIF-1α mRNA. Four groups of direct HIF-1 target genes that are particularly relevant to cancer encode angiogenic factors, including VEGF, glucose transporters and all the glycolytic enzymes enzymes, survival factors and invasion factors (Fig. 5)

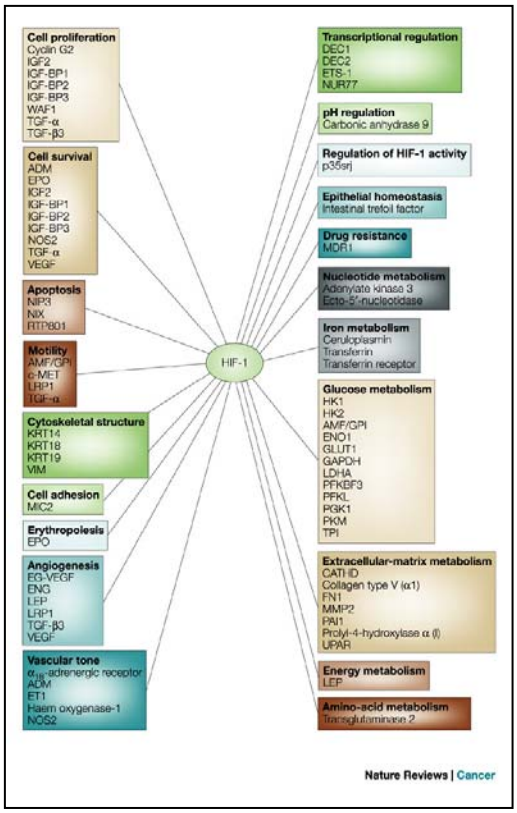

Figure 5. Genes that are transcriptional activated by HIF-1. HIF-1 is a transcription factor that controls the expression of a battery of more than 80 target genes whose protein products control oxygen delivery (via angiogenesis), metabolic adaptation to hypoxia (via glycolysis) and cell survival (via induction of growth factors)[49].

The products of the genes that HIF-1 regulates act at several steps in each of these processes. Expression of several HIF-1 target genes is induced by hypoxia in most cell types. However, for the majority of HIF-1 target genes, expression is induced by hypoxia in a cell-type-specific manner. HIF-1 activity is induced by hypoxia in almost all cell types and therefore HIF-1 alone cannot account for this cell-type-specific gene expression. Rather, it is the functional interaction of HIF-1 with other transcription factors that determines the subgroup of HIF-1 target genes that is activated in any particular hypoxic cell. HIF-1 can be viewed as a messenger that is sent to the nucleus to activate transcriptional responses to hypoxia. The details of this response are determined by the past (developmental) and present (physiological) programming of each cell. As a result, the total number of HIF-1 target genes cannot be ascertained by analysis of one or a few cell types. Perhaps 1–5% of all human genes are expressed in response to hypoxia in one or more cell types in a HIF-1-dependent manner. Heterogeneity in target-gene expression is observed even among cell lines that have been derived from cancers of the same histopathological type [49].

2.4.2. HIF-1α is overexpressed in human tumors

In humans, up-regulated HIF-1α protein expression is found in >70% of human cancers with the highest levels observed in metastases as compared to adjacent normal tissues [57, 58]. Immunohistochemistry of human tumor samples has shown that there is a significant association between HIF-1α overexpression and patient survival in oligondendrogliomas [59], breast [60-62], endometrial carcinomas [63], oropharingeal [64] and esophageal [65, 66]. In a study of nasopharyngeal cancer [67] poor survival was associated with expression of HIF-1α, while high expression levels of HIF-1α carbonic anhydrase IX (CAIX) and VEGF, both of which are HIF-1α target genes, correlated with a worse disease-free survival. Furthermore, high HIF-1α expression levels in colorectal carcinomas were correlated with decreased survival [68]. However, some discrepancies have also been reported between HIF-1α overexpression and mortality. For example, in patients with ovarian carcinoma, expression of HIF-1α was associated with increased apoptotic rate in the majority of the cases. However, strong expression of HIF-1α and overexpression of nonfunctional p53 showed an increased MVD and a shorter overall survival with a very low rate of apoptosis in those tumors [69]. In a head and neck tumor study, the authors proposed that the improved overall survival was due to an increased apoptosis rate in

those tumors overexpressing HIF-1α [70], while in a similar study HIF-1α expression was correlated with a poor prognosis and no correlation was found between p53 mutations and loss of bcl-2 [71]. A positive correlation between HIF-1α expression and several pro-apoptotic markers such as capase-3, Fas and Fas ligand was detected in patients with non-small cell lung [72]; while in a different study HIF-1α was associated with up-regulation of various angiogenic factors and with poor prognosis [73]. Finally, in three different studies overexpression of HIF-1α was associated with poor outcome in patients with cervical carcinoma [74-76] but no correlation was found between the level of HIF-1α and patient outcome using disease- free survival as the end point in those tumors [77]. Collectively, although different studies utilize different assays and end-points to assess the prognostic value of HIF-1α expression in cancer, there is nevertheless a strong trend correlating high HIF-1α expression with poor prognosis as well as chemoresistance.

2.4.3. Genetic alterations contributing to HIF-1α overexpression in cancer.

Overexpression of HIF-1α in human cancer can also be a consequence of genetic alterations in tumors resulting in loss-of- function in tumor suppressor genes or gain- of-function in oncogenes. Many oncogenes affect HIF-1α by enhancing both the phosphatidylinositiol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways [51]. Among them, some regulate HIF-1α at the protein stability or degradation levels, while others affect signaling pathways upstream of HIF-1α activation. Loss of the tumor suppressor function of PTEN has been shown to augment HIF-1 mediated gene expression [78] while restoration of PTEN function inhibits the expression of HIF-1α [79]. Loss of wt p53 (by gene deletion or mutation) enhances HIF-1α accumulation in hypoxia, and augments HIF-1 dependent expression of VEGF in tumor cells [80]. However, the most remarkable effects on HIF-1 regulation, are seen in clear renal cell carcinomas (RCCs) and cerebellar haemangioblastomas (HBs) that have lost the function of the von Hippel-Lindau (VHL) tumor suppressor gene. Approximately 70-80% of clear renal cell carcinomas (RCCs) have mutations in the pVHL. In the case of HBs, pVHL mutations are almost always found in those tumors associated with VHL-disease and the VHL gene is also assumed to be involved also in the development of sporadic HBs [81]. pVHL is recognized as the substrate of a ubiquitin E3 ligase that targets HIF-1α subunits for proteasomal

degradation under normoxic conditions. Loss of VHL function results in high levels of HIF-1α subunit that it is not oxygen regulated anymore. As a result, VEGF, the glucose transporter GLUT-1, carbonic anhydrase IX and other HIF-1 target genes are constitutively expressed, resulting in hypervascularity and aggressive tumor phenotypes in those patients. All experimental data indicate that HIF-1 is involved in the pathophysiology of these cancers, and the upregulation of HIF-1 activity seems to occur even in the earliest detected neoplastic lesions (for review see [82]).

2.5. Therapeutic strategies to target HIF

The striking upregulation of HIF-1α in so many different tumors and by so many different mechanisms highlights the critical role HIF-1 plays in tumor biology and makes HIF-1α a prime target for anticancer therapy. Several strategies have been employed by several groups most of which focus on the identification of small molecules that specifically inhibit HIF and HIF-transcriptome [83]. To date there are a few of those strategies for which there is significant knowledge on the mechanistic aspects of HIF inhibition and includes inhibition of the topoisomerase I [84], Hsp90 inhibitors [85-88], YC-1 [89-91], Nonsteroidal anti-inflammatory drugs (NSAIDs) [92, 93] and gene therapy [94-97].

MICROTUBULES

Microtubules are major dynamic structural components of the cytoskeleton involved in a variety of cell functions important for the development and maintenance of cell shape, cell reproduction and division, cell signaling, intracellular transport and cell movement. The biological functions of microtubules are regulated for the most part by their polymerization dynamics. Microtubules are built by the self association of individual α/β-tubulin dimmers. Tubulin is one of the most abundant cellular proteins accounting for 2-5% of total cell protein in most epithelial cells, while tubulin accounts for approximately 20% of total cell protein in the brain. The central role of tubulin in the cell division cycle, together with the fact that aberrant cell division is a hallmark of malignant cell growth has made tubulin and microtubules prime targets for cancer chemotherapy. In fact, microtubule-targeting drugs are the most effective class of anticancer agents. Among the most successful microtubule-targeted drugs, taxanes are arguably the most effective anticancer agents introduced in the clinic since cisplatin, due to their remarkable activity in a broad range of malignancies. The list of compounds that bind to tubulin is large and continues to expand. The overwhelming majority of them are natural products and their chemical structures are remarkably diverse. For no other cellular target including DNA, has a more diverse list of ligands been discovered. Microtubule-targeting drugs act by either destabilizing or hyperstabilizing microtubules, resulting in mitotic arrest at the metaphase/anaphase transition and subsequent apoptotic cell death.

3.1. Microtubules: structure and dynamics.

Microtubules are highly dynamic cytoskeletal fibers whose dynamic properties are based on the inherent structure and polarity of the microtubule itself. Microtubules are built by the self-association of αβ-tubulin dimers that associate in a head-to-tail fashion to form protofilaments. Protofilaments, usually in a number of 13, interact with each other laterally by contacts between monomers of the same type to form a hollow helical cylindrical microtubule with an outer diameter of 25nm. The assembly of the αβ-tubulin heterodimers creates a polarity on the microtubule that greatly influences the polymerization rates of the two ends of the microtubule. The faster growing end is referred to as the plus end (where the β-tubulin monomer is exposed)

and the slower growing end is referred to as the minus end (where the α-monomer is exposed) (Fig. 6).

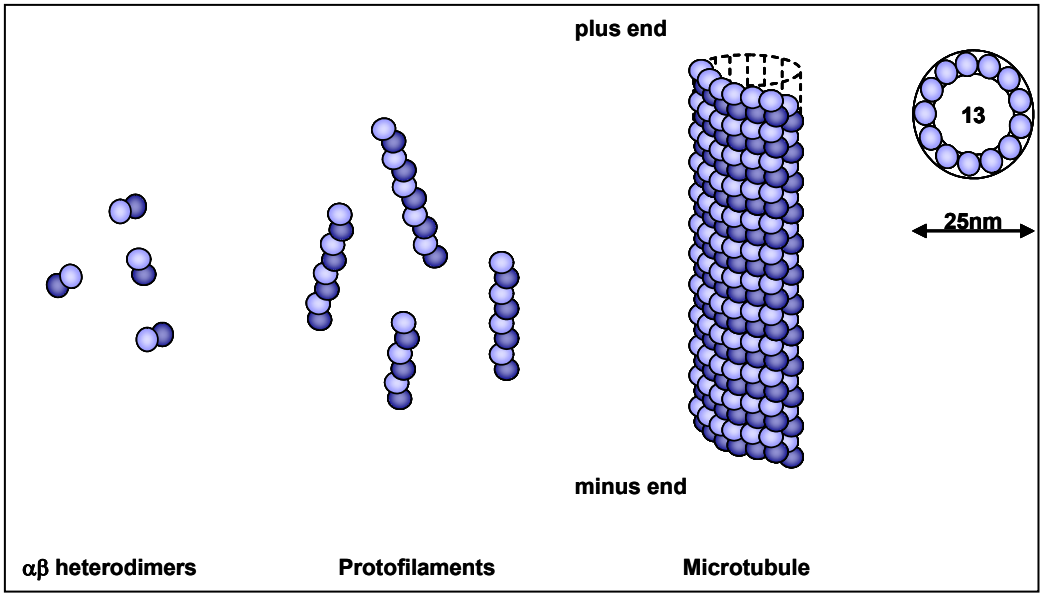

Figure 6. The structure of microtubule. Microtubules are hollow cylindrical structures, built from two kinds of similar 50-kd tubulin subunits, α- and β-tubulin that associate in a head-to-tail fashion to form a protofilament. Lateral association of protofilaments (usually 13) produce a microtubule with an outer diameter of 25 nm that clearly distinguishes from other cytoskeletal filaments. Microtubules are polar structures with a dynamic plus end and a minus end that can be stabilized by embedding it in a microtubule-organizing center (MTOC).

The αβ-tubulin heterodimer is the basic structural basic unit of the microtubule. Each subunit has a binding site for one molecule of GTP. Once the αβ-tubulin dimer is formed, the nucleotide in the α-subunit (GTP) is buried at the intradimer interface, and it can not be hydrolyze (non-exchangeable). In contrast, the nucleotide on the β-tubulin is partially exposed on the surface of the dimer and can be either a GDP or a GTP, thus, it is exchangeable (E-site). The energy released after the hydrolysis of the GTP at the β-tubulin site is used to polymerize the αβ-dimers and assembled them into microtubules. After hydrolysis, the nucleotide at the β-tubulin (E-site) is buried at the interdimer interface and becomes nonexchangeable.

The biological functions of microtubules in all cells are determined and regulated in large part by their polymerization dynamics. Microtubules are in a state of dynamic instability, in which

individual microtubules are either growing or shrinking and stochastically switch between the two states. The switch from growth to shrinkage is called a catastrophe, and the switch from the shrinkage to growth is called a rescue. Dynamic instability is due to the structural differences between a growing and a shrinking microtubule end. An accepted model is that if nucleotide hydrolysis proceeds more rapidly than subunit addition, the GTP cap is lost and the microtubule begins to shrink. But GTP-subunits can still be added to the shrinking end and if enough is added to form a new cap, then the microtubule growth resumes. In an intact microtubule, protofilaments made from GDP are forced into a linear conformation by the many lateral bonds within the microtubule wall, given a stable GTP-cap. Loss of the GTP-cap, however, allows the GDP-containing protofilaments to relax into their more curved conformation that leads to a progressive disruption of the microtubule.

In addition to the dynamic instability, microtubules have another kind of dynamic behavior, called 'treadmilling', which is net growth at one microtubule end and balanced net shortening at the opposite end. It involves the intrinsic flow of tubulin subunits from the plus end of the microtubule to the minus end and is created by differences in the critical subunit concentrations at the opposite microtubule ends. This behavior occurs in cells as well as *in vitro*, and might be particularly important in mitosis. Treadmilling and dynamic instability are compatible behaviors, and a specific microtubule population can show primarily treadmilling behavior, dynamic-instability behavior or some mixture of both. The mechanisms that control the degree to which a microtubule population shows one or the other behavior are poorly understood, but probably involve the tubulin isotype composition of the microtubule population, the degree of post-translational modification of tubulin and, especially, the actions of regulatory proteins.

Assembly of microtubules *in vivo* occurs at the microtubule-organizing center (MTOC). In most animals cells there is a single, well-defined MTOC called the centrosome, located in the cytoplasm near the nucleus. The centrosome is a complex structure organized by a pair of perpendicular centrioles surrounded by pericentriolar material containing an isoform of tubulin (γ-tubulin) in a large complex that includes other proteins (collectively known as “grips”) that forms the γ-tubulin ring complex (γ-TuRC). The pericentriolar material contains several copies of a γ-tubulin ring complex (γ-TuRC), a multiprotein complex that nucleates microtubules and

allows their elongation. In most cells, microtubules are organized into a single array with their minus ends associated with the centrosome. Nucleation plays a fundamental role in the function and intracellular dynamics of microtubules by preventing spontaneous polymerization of microtubules in the cytoplasm and thereby a random spatial organization of microtubules. This gives a cell a defined polarity, with the minus end of the microtubules located near the nucleus in the center of the cell and their plus ends toward the cell periphery near the plasma membrane.

3.2. Microtubule regulation

Microtubule dynamics can be regulated by different mechanisms *in vivo*, including the expression of different α- and β-tubulin isoforms, post-translational modifications and interaction with cellular factors that stabilize or destabilize microtubules, which operate in both spatially and temporally specific ways to generate different microtubule assemblies during the cell cycle.

3.2.1. Microtubule stabilizing proteins.

Until recently, all of the proteins known to regulate microtubule polymerization were the so-called microtubule associated proteins or MAPs. MAPs are proteins that bind to microtubules in a nucleotide-insensitive manner to the microtubule lattice and they stabilize microtubules. Classical MAPs, include MAP1, MAP2 and tau in neurons and MAP4 in non-neuronal cells. The binding of MAPs to microtubules is predominantly electrostatic involving the highly acidic carboxyl terminal domain of α- and β-tubulin. MAPs are negatively regulated by phosphorylation, as the more phosphorylated forms exhibit reduced affinity for the microtubule lattice, presumably by weakening this electrostatic interaction. Inactivation of MAPs reduces the frequency of rescue and is one of the mechanisms by which microtubule turnover can be increased *in vivo*.

Other MAPs, such as the highly conserved XMAP215/Stu2p/TOG family, may be enriched on a subset of microtubules. XMAP215 affects microtubule dynamics by strongly increasing the polymerization rate of pure tubulin, but only at the microtubule plus ends. XMAP215 also increases the rate of rapid depolymerization at the minus ends decreasing the rescue frequency, thereby increasing the microtubule turnover. Microtubule-end-binding MAPs, such as CLIP-170

and EB1, copolymerize with new tubulin subunits and selectively bind to a special conformation of the microtubule end, serving as attachments for microtubules to kinetochores or cellular membranes through interaction with adaptor proteins, such as the APC (adenomatous polyposis coli) protein and CLASPs (CLIP-associated proteins).

3.2.2. Microtubule destabilizing proteins.

Destabilizing proteins, in contrast to MAPs, reduce the net assembly and increase the microtubule turnover. The microtubule-destabilizing protein katanin, functions as a severing factor, generating new ends lacking a GTP cap. Katanin localizes at centrosomes and is responsible for the majority of M-phase severing activity in *Xenopus* while it is essential for releasing microtubules from the neuronal centrosome.

Depolymerizing kinesins of the KinI family, such as XKCM1, XKIF2 and MCAK, bind to both microtubule ends and distort the microtubule lattice, forcing protofilament peeling. These kinesin-like proteins are required for the formation and maintenance of the dynamic nature of the mitotic spindle. Op18/Stathmin increases the catasthophe rate of microtubules, presumably by either sequestering tubulin dimers or by promoting GTP hydrolysis at the E-site.

3.2.3. Isoforms of tubulin

Tubulin is encoded by a multigene family that produces distinct gene products, or isotypes, of both the alpha- and beta-tubulin subunits. In mammals, there at least six isoforms of α-tubulin and a similar number of isoforms for β-tubulin, although they are differentially expressed in cells and tissues (Table 1**)**. Although, there is evidence that the tubulin isotypes exhibit tissue specificity and play important roles during embryogenesis, their exact cellular functions are still unclear. The different isoforms of α- and β-tubulin are highly conserved and most of the sequence variations among them are found in the last 10-15 C-terminus amino acids. The C-termini of tubulin are thought to primarily participate in the binding of microtubule associated proteins (MAPs) to microtubules. While these C-terminal regions are highly variable among the isotypes within a species, the same regions are highly conserved within a single isotype, among different species as diverse as human, mouse and chicken. Variations among tubulin isoforms are expected to affect primarily the association of accessory proteins on the surface of the

microtubule rather than the microtubule polymerization *per se*. Although some tubulin isotypes have specific functions, in most cases, the isoforms seem functionally interchangeable since they freely substitute for one another and coassemble *in vitro* into microtubules [98].

	Class	Human gene	Expression
α-tubulin	1	TUBA1	Widely expressed
	1	TUBA3	Mainly in brain
	3	TUBA2	Testis-specific
	4	TUBA4	Brain; muscle
	6	TUBA6	Widely expressed
	8	TUBA8	Heart; muscle ;testis
β-tubulin	I	HM40	Constitutive; predominant isotype in many cells
	II	Hβ9	Major isotype of neurons
	III	Hβ4	Neurons; Sertoli cells of testis
	IVa	Hβ5	Brain-specific
	IVb	Hβ2	Constitutive high levels in testis
	V	ND	ND
	VI	Hβ1	Hematopoiesis-specific cell types

Table 1. Tubulin Isoforms

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In the case of cancer chemotherapy drugs, i*n vitro* studies have shown that alteration of α- and β-tubulin isotype composition has the potential to affect the sensitivity of tubulin to microtubule targeting drugs, especially taxol [99]. Furthermore, it has also been proposed that altered expression of different tubulin isotypes represents another mechanism underlying the resistance of cancer cells to microtubule-targeting drugs. However, later studies in ovarian cancer xenograft models suggested that altered expression of beta-tubulin isotypes does not influence taxol's sensitivity *in vivo*, arguing against a role for tubulin isotype composition in chemoresistance in the clinical setting [100].

In addition to the αβ-tubulin gene families, recent genetic analyses and database searches have added four new members of the tubulin superfamily, which now includes **α-, β-, γ-, δ-, ε-,** ζ-, and **η**-tubulin. **δ**- and **η**-tubulin seem to be associated mainly with flagella and cilia motility [101], whereas **ε**-tubulin and ζ-tubulin, were discovered by database searches, and their cellular

functions have yet to be established [102-104]. γ-tubulin was first identified in the filamentous fungus *Aspergillus nidulans* [105] and it is approximately 30% identical to α- and β-tubulin, and likely to be present in all eukaryotes. γ-tubulin is located in centrosomes where it plays an essential role in initiation of microtubule assembly.

3.2.4. Post-translational modifications of tubulin.

Microtubule diversity is also generated by post-translational modifications and both α-tubulin and β-tubulin can be modified. These modifications include acetylation, polyglutamylation, polyglycylation, detyrosination/tyrosination, phosphorilation and palmitoylation. All of these modifications, with the exception of acetylation, occur at the highly charged end of α- and β-tubulin, which is located on the outside of the microtubule where it is well positioned to influence interactions with other proteins. All of the post-translational modifications are ubiquitous in organisms and, with the exception of detyrosination/tyrosination, all of post-translational modifications known in higher eukaryotes are already present in the protist *Giardia lamblia*, which is considered as a representative of the oldest eukaryotes. The functionality of the post-translational modifications is still a matter of debate [106].

3.3. Microtubule Functions.

3.3.1. Cell division

A defining characteristic of living organisms is their ability to proliferate and the very fundamental level is the division of a single cell into two daughter cells with exact complements of the parental genetic material (Fig. 7).

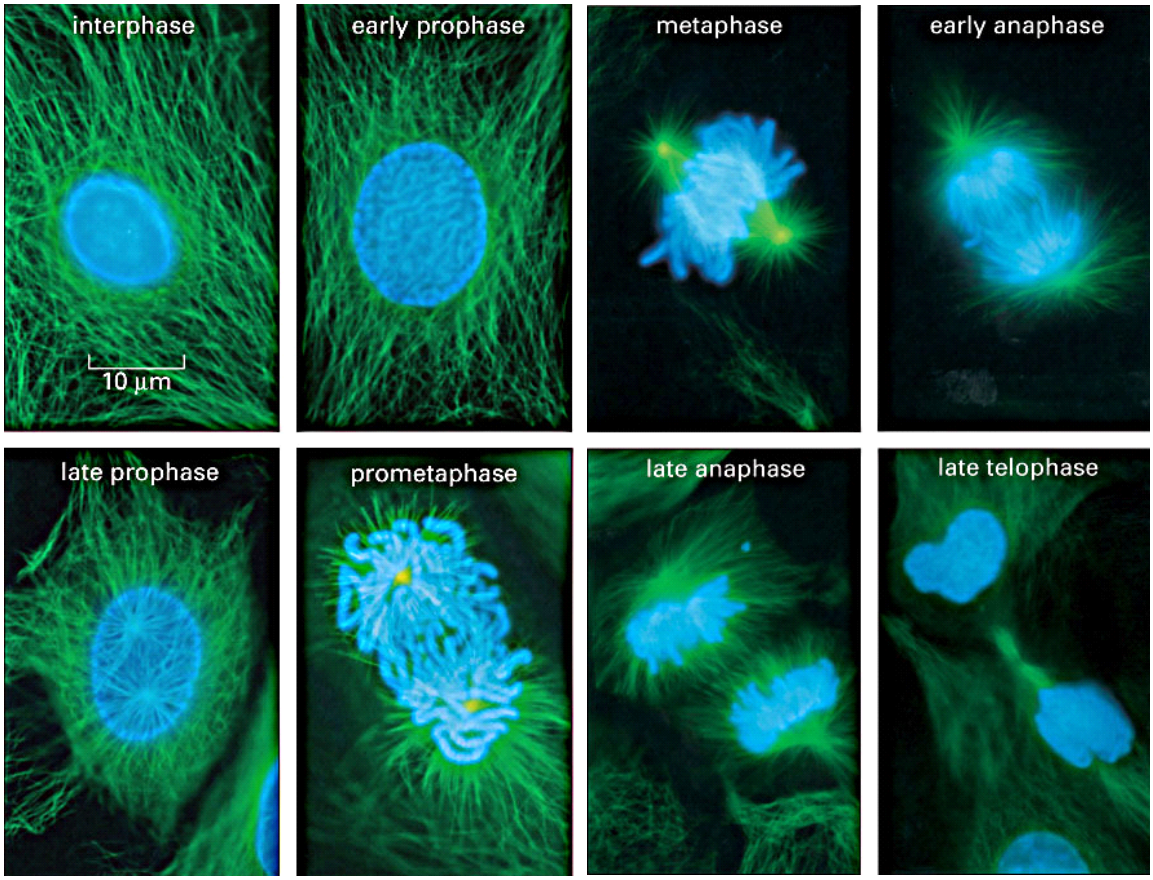

Figure 7. Phases of Mitosis In these micrographs microtubules (green) and DNA (blue) have been visualized by immunofluorescence. During the *interphase*, the centrosome (not visible) organized the interphase microtubule array. By *early prophase*, the single centrosome contains two centriole pairs (not visible). At *late prophase*, the centrosome divides and the resulting two asters can be seen to have moved apart. At *prometaphase*, the nuclear envelope breaks down, allowing the spindle microtubules to interact with the fully condensed chromosomes. At metaphase, the bipolar structure of the spindle is clear and all the chromosomes are aligned at the equator of the spindle. At *early anaphase*, the sister chromatids all separate synchronously and, under the influence of the microtubules, the daughter chromosomes begin to move towards the poles. By *late anaphase*, the spindles poles have moved farther apart, increasing the separation of the two groups of chromosomes. At *telophase*, the daughter nuclei re-form and by *late telophase*, cytokinesis is almost comlete, with the midbody persisting between the daughter cells.

At the beginning of the early prophase of the cell cycle, centrosomes are already replicated and they split into two and the two halves begin to separate. Each centrosome now nucleates its own

radial array of microtubules called an aster. By early prometaphase the two asters are completely separated and forming the bipolar mitotic spindle.

Three different kinds of microtubules are present in the mitotic spindle. Astral microtubules, radiating in all directions from the centrosomes, are though to contribute to the forces that separate the poles and orientate and situate the spindle in the cell. Polar microtubules interact in an antiparallel fashion at the equator of the spindle and stabilize the bipolarity of the spindle. Kinetochore microtubules attach end-on to the kinetochore and connect chromosomes to spindle poles. The behavior of each class is thought to be different because of the different protein complexes that are associated with their plus and minus ends.

Spindle assembly and function require localized regulation of microtubule dynamics and the activity if a variety of microtubule-based motor proteins. One of the consequences of entering cell division on microtubules is that those increase greatly their dynamic instability, driven primarily by an increase of the catastrophe rate. This increase in dynamic instability is regulated by different factors including Op18/stathmin, XKCM1 (Kin1). Counteracting the destabilizing effects of these two catastrophe factors are MAPs that bind and stabilize microtubules [107].

3.3.2. Intracellular transport

The inherent polarity of microtubules allows for the directional flow of information within the cell [108], and it is utilized by the microtubule molecular motors belonging to the dynein and kinesin family to respectively transport cargos to (minus-end directed motors) and from (plus-end directed motors) the nucleus. These “nanomachines” use ATP hydrolysis to generate force and move in a step-like manner on microtubules [109]. **Cytoplasmic dynein**, a minus-end directed microtubule-based motor, drives a wide variety of cytoplasmic motor activities ranging from movement of ER-Golgi complexes, late endosomes and lip droplets [110, 111] to viral capsids [112] and chromosomes [113, 114]. To drive such a variety of subcellular motile functions, cytoplasmic dynein works in concert with an accessory factor, dynactin. Like dynein, dynactin is a massive protein complex that directs and coordinates the activities of the dynein motor [115]. On the other hand, **kinesins** [116] constitute a large family of motor proteins that move cargos towards the microtubules’ plus-end, located in the cell periphery in most epithelial cell types. Examples of cargos that move within the cell via kinesins are the mitochondria, and the tumor suppressor protein APC.

The collective transport of intracellular particles along a specific cellular direction is a fundamental process in cell biology. Interphase microtubules are the major component of cytoskeletal systems that are responsible for the regulation of the distribution of mitochondria in mammalian cells [117], and discovery of Kif1B and Kif5B proteins belonging to the kinesin protein family, which are responsible for the movement of mitochondria along microtubules [118, 119], has accelerated the research on factors controlling the distribution of mitochondria in the cell. Furthermore, microtubules regulate the distribution of intracellular organelles besides mitochondria: endoplasmic reticulum (ER) [120], Golgi apparatus [121], peroxisomes [122] and lysosomes [123]. Overexpression of tau, a mictotubule-associated protein, not only causes aberrant distribution of mitochondria via the inhibition of kinesin-dependent movement of the organelles along MTs but also affects morphology and distribution of ER in the cell [124]. Chemical modifications of microtubule dynamics by microtubule-targeting drugs have been widely used as an experimental approach to explore the role of microtubules in various cellular functions [122, 124-126].

In addition to that, microtubules have been shown to be essential for the localization and transport of a great variety of proteins and transcriptions factors within the cells. The list of these proteins is growing very rapidly and among some of the most notorious just to mention p53 [127], Hsp90 [128], pVHL [129] or RASSF1A [130]. In a similar way, microtubules are also used by intracellular bacterias and viruses [131] and are important in the life cycle of these pathogens.

Microtubules are also involve in the localization and transport of certain species of mRNA Approximately 15-30% of cellular mRNAs and polysomes are thought to be associated with the cytoskeleton, and immunohistological and biochemical approaches suggest that translation initiation and elongation factors in certain cell types follow a microtubule pattern. Proteins that regulate translation and also associate with microtubules include elongation initiation factors. sSme mRNA species are believed to predominantly associate with cytoskeleton-bound polysomes as opposed to membrane-bound or free polysomes The fact that certain mRNAs encoding for nuclear-destined proteins preferentially associate with the cytoskeleton suggests that this interaction

provides efficient translation and trafficking of these proteins to the nucleus where they are then able to become functionally active [132].

3.3.3. Microtubules and signal transduction

Microtubules are one of the most abundant proteins present in the cell and they are unique in that they fill the entire cytoplasm from just outside the nucleus all the way to the plasma membrane, providing ample surface for protein-protein interaction. The discovery of signaling molecules that interact with microtubules, as well as the multiple effects on signaling pathways of microtubule-targeting drugs, indicate that microtubules are likely to be critical to the spatial organization and signal transduction. Based on that, microtubules and the molecules that interact with them may be contributing to signal transduction by at least three distinct mechanisms: microtubule sequestering and release, microtubule delivery and microtubule scaffolding of signaling pathways.

The binding of CI/Costal2 in the hedgehog signaling pathway and NFκB/IκB to the microtubule surface may be examples of the sequestering and release mechanism. Activation and release of microtubule -bound signaling factors could be accomplished by: modification of the factor, modification of the microtubule and/or breakdown of the microtubule. The latter two release mechanisms are specific for microtubules and there is evidence that microtubule dynamics might be regulated by signal transduction. Each of these mechanisms might also work in the reverse direction, to sequester inhibitory factors during signal transduction.

For microtubule scaffolding, microtubule surface provides a scaffold to promote the interaction of two or more factors which would otherwise not interact. It is possible that interaction of one component with the microtubule induces a binding site for a second factor or may simply bring low affinity components into proximity so they can interact. Many of the signal transduction components that interact with microtubules appear to involve large multimeric complexes (such those in the MAPK pathway) and the assembly of these complexes may be promoted by microtubules. The large size of these complexes might also limit their diffusion in the cytoplasm and this raises the possibility that without microtubules and microtubule motors to move them, they might not reach their ultimate targets [133].

Microtubule-mediated delivery could act either by delivering signaling factors to other components on the microtubule surface or to specific sites in the cell so the transduction signal is only active in a part of the cell. MLK2-KIF3 may be an example of the former, and an unidentified motor in the Wnt signaling pathway may be an example of the latter [134]. Activation of this mechanism by signal transduction could occur through enhanced motor-cargo interaction, enhanced motor-microtubule interaction, or stimulation of the motor itself.

New evidence suggests that traditional microtubule-associated proteins may also have roles in signal transduction. Microtubules themselves respond to signal transduction and this may be an important aspect of the integration and polarization of signaling pathways. Many other cellular processes are affected by drugs that interfere with microtubule dynamics and stability and one of the future challenges will be to identify the molecular basis for these effects [108].

3.4. Targeting microtubules for cancer chemotherapy.

Microtubule-targeting drugs are arguably the most effective classes of drugs in cancer therapy. For no other single target, including DNA has a more diverse group of agents successfully developed. Microtubules are crucial for a variety of functions in the cell, including the maintenance of cell shape and polarity, intracellular transport of vesicles and organelles, and signal transduction. However, microtubules are especially important during cell division, which requires an exquisite control of microtubule dynamics. During cell division microtubules form the bipolar mitotic spindle, the structure that is required for faithfully segregate sister chromatids onto the two daughter cells. The critical role that microtubules play in cell division makes them a very suitable target for the development of chemotherapeutic drugs against the rapidly dividing cancer cells. The effectiveness of microtubule-targeting drugs has been validated by the successful use of several taxanes and *Vinca* alkaloids for the treatment of a wide variety of human cancers.

Their clinical success has prompted a worldwide search for compounds with a similar mechanism of action, but improved characteristics. This search has resulted in the discovery of several novel microtubule-targeting drugs, the majority of which are natural products. Their natural sources and chemical structures are remarkably diverse, making microtubules the only

anticancer-drug target for which such a diverse group of anticancer agents has been identified. However, the effectiveness of microtubule-targeting drugs for cancer therapy has been impaired by various side effects, notably neurological [135] and hematological toxicity [136, 137]. Drug resistance is another notorious factor that thwarts the effectiveness of these agents, as with many other cancer chemotherapeutics. Therefore, continued investigation of the mechanisms of action of microtubule-targeting drugs, development and discovery of new drugs, and exploring new treatment strategies that reduce side effects and circumvent drug resistance might provide more effective therapeutic options for cancer patients.

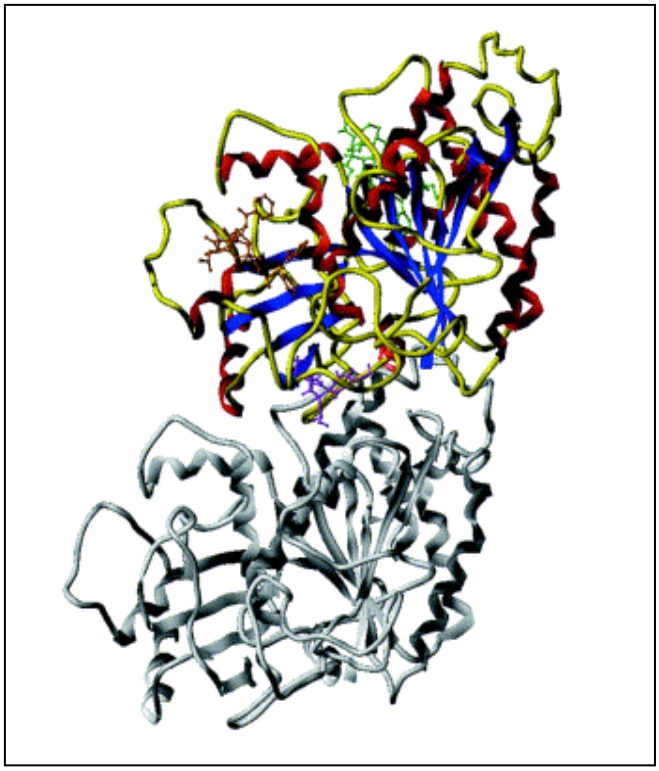

Figure 8. Microtubule drug-binding sites. A hypothetical model of three drugs bound to the tubulin dimer. The relative positions of the three major classes of microtubule-interacting drugs are shown here: vinblastine (green), paclitaxel (orange) and colchicine (magenta). There is sufficient evidence to suggest that all three drugs bind β-tubulin (colored by secondary structure) but at crucially different locations. Paclitaxel binds at the lateral interface between adjacent protofilaments from the terminal end of the microtubule. Colchicine probably binds at the intra-dimer interface between β- and α-tubulin (grey). Vinblastine probably binds at the polar, opposite side of β-tubulin at the plus end interface, adjacent to the hydrolysable nucleotide site.

3.4.1. Mechanism of action of microtubule-targeting drugs

The mechanism of action of microtubule-targeting microtubules is exerted primarily blocking mitosis at the G2/M phase, reason why these compounds are also frequently referred to as anti-mitotic drugs. In all cases, mitotic arrest takes place upon binding to tubulin (Fig. 8). Currently there are three well established drug-binding sites onto tubulin, the taxane-binding site, the vinca alkaloid-binding site and the colchicine-binding site [138]. Functionally, these antimitotic ligands can be separated in two classes, those that promote microtubule polymerization, stabilizing microtubules and increasing the polymer mass (taxanes family) and those that inhibit

microtubule assembly, destabilizing microtubules and decreasing microtubule polymer mass (the colchicine and *Vinca* alkaloid families). Despite these seemingly opposing actions on microtubules, both microtubule-destabilizing and stabilizing drugs induce mitotic arrest and subsequent apoptotic cell death.

3.4.1.1. Agents that bind to the *Vinca* alkaloid site

The *Vinca* alkaloids, vinblastine and vincristine, were isolated over 40 years ago from the leaves of the periwinkle *Catharanthus roseus*. Since then they have been widely used clinically for the treatment of leukemias, lymphomas, and some solid malignancies. Their clinical success of these two natural products together with the elucidation of their mechanism of action on cellular microtubules, have facilitated the development of several semi-synthetic derivatives notably vindesine, vinorelbine and vinflunine, which are now used in the clinic for the treatment of cancer [139]. All of the members of the *Vinca* alkaloids cause microtubule depolymerization, dissolve spindle microtubules and arrest cells at mitosis. In contrast, at low concentrations, the *Vinca* alkaloids suppress microtubule dynamics without depolymerizing spindle microtubules, but remain able to arrest mitosis and induce apoptosis.

3.4.1.2. Agents that bind to the taxane site

Isolated originally in the 1960s from the bark of the Pacific yew *Taxus brevifolia*, paclitaxel did not receive much attention until it was discovered to possess microtubule-stabilizing activity [140]. This drug is now in widespread use for the treatment of breast, ovarian, prostate and non-small-cell lung cancer, as well as Kaposi's sarcoma. Its semi-synthetic analog, docetaxel, is synthesized from a precursor isolated from the needles of the European yew *Taxus baccata*. Docetaxel is more water-soluble than paclitaxel, and is also more active than paclitaxel against cancer cell proliferation, and is now used clinically for the treatment of breast, prostate and non-small-cell lung cancer.

At relatively high concentrations, the taxanes promote microtubule polymerization and microtubule bundles in cells, although the biological significance of this phenomenon remains unclear. At lower concentrations, the taxanes suppress microtubule dynamics without affecting microtubule polymer mass, but retain their capability of inducing mitotic arrest and subsequent apoptotic cell death.

The success of paclitaxel and docetaxel in cancer therapy has inspired the discovery of new microtubule-targeting agents with similar mechanisms of action, including discodermolide (isolated from the marine sponge *Discodermia dissoluta*) [141], epothilones (isolated from the myxobacterium *Sorangium cellulosum*) [142], eleutherobin (isolated from the marine soft coral *Eleutherobia sp.*) [143], sarcodictyins (isolated from the Mediterranean stoloniferan coral *Sarcodictyon roseum*) [144], and laulimalide (isolated from the marine sponge *Cacospongia mycofijiensis*) [145]. With the exception of laulimalide, all of these compounds have been reported to bind at the taxane site [146]. These taxane-like agents block mitosis and induce cell death downstream of their binding to tubulin, and their cancer chemotherapeutic potential is also under clinical investigations.

3.4.1.3. Agents that bind to the colchicine site

Drugs binding to the colchicine site typically induce microtubule depolymerization at high concentrations, similar to the *Vinca* alkaloids, and they suppress microtubule dynamics at low concentrations similar to both the *Vinca* alkaloids and taxanes**.** Isolated from the meadow saffron *Colchicum autumnale*, colchicine is one of the earliest microtubule-targeting agents identified, whose mechanism of action has been extensively investigated. In fact, tubulin was first purified based on its high affinity binding with colchicine and was referred to as a "novel protein binding colchicine" [147, 148].

The clinical development of colchicine for cancer treatment has not been successful to date probably because of the high toxicity to normal tissues. However, development of agents binding to the colchicine site as potential cancer chemotherapeutic drugs has recently gained intense interest. This is the case of combretastatins for example, are isolated from the South African Willow *Combretum caffrum*, bind to tubulin, and exhibit both potent anti-tumor activity [149, 150] and potent antiangiogenic activity [2]. Similarly, 2-methoxyestradiol (2ME2), a naturally metabolite of estradiol, also inhibits both tumor growth and angiogenesis [1]. Taken together these results, it has raised the possibility that colchicine-site drug binding may be mechanistically distinct from the taxane- and the vinca-binding sites.

4. 2-METHOXYESTRADIOL (2ME2)

Over the past 50 years, the use of natural and synthetic estrogens and antiestrogens to regulate endocrine function and treat various disorders had been widespread and extensive. Estrogens have been effectively been use as contraceptives, for the use of breast cancer and to ameliorate cardiovascular disease and bone loss in postmenopausal women. Unfortunately chronic administration of estrogens unveiled the potential risk of cancer. It has often been argued that the metabolic conversion of estrogens to reactive metabolites is the culprit for initiating, if not promoting cancers. Such arguments have prompted investigators to search for analogs of estrogens and alternative treatment strategies. As a result of most of these studies, it appeared that among all the metabolites of estrogens, only the 2-methoxyestrogens (and in particular 2-methoxyestradiol), were devoid of any obvious physiological significance. Once largely dismissed and view as an inert metabolic end product of estradiol, 2-methoxyestradiol (2ME2) is now recognized as a potent antitumor and antiangiogenic agent [1] (Fig. 9).

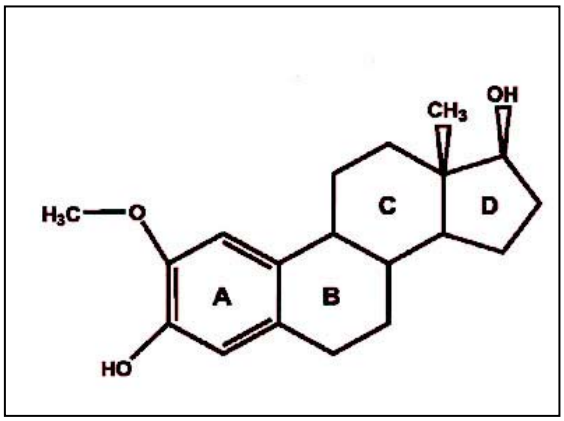

Figure 9. Structure of 2-Methoxyestradiol. 2ME2 is a natural occurring metabolite of estradiol that is arises from the hydroxylation and subsequent methylation at the 2-position

2ME2 is an antiproliferative molecule that effectively induces apoptosis in actively proliferating cells *in vitro* and *in vivo*. 2ME2 is emerging as an attractive drug candidate because of its unique characteristics: (*a*) nontoxic [151, 152]; (*b*) it is orally available [151, 153-156] and (*c*) no effect on several responses normally associated with estrogens [157-159]. The antitumor and antiangiogenic effects of 2ME2 are well reported yet there is no known physiological function for this molecule. Several mechanisms of action have been proposed for 2ME2 and, even for some of them seem to be clearly sufficient to explain its activity in certain cases there is no evidence for a common mechanism of action operative in all of the cells sensitive to 2ME2. To

date, it is not known which of the effects of 2ME2, its antiangiogenic effects or its direct effects on cancer cells are most important

4.1. Biosynthesis of 2ME2.

2ME2 is a natural metabolite of estrogen physiologically excreted with the urine. 2ME2 is formed by the sequential hydroxylation and methylation of the endogenous estradiol at the 2-position. Estradiol is hydroxylated by NADPH-dependent cytochrome P450 enzymes mainly in the liver to catechol estrogens (2-hydroxyestradiol and to less extend 4-hydroxyestradiol). These catechol estrogens are metabolically O-methylated by catechol-O-methyl transferase (COMT) in many tissues, but especially on erythrocytes where the highest enzymatic activity is found. In the blood 2ME2 is present conjugated or unconjugated form and serum concentrations range from 10.3pg/ml in men to 3768pg/ml in pregnant women.

4.2. Antiproliferative effects of 2ME2

2ME2 has been shown to inhibit tumor growth in a variety of different cell lines and solid tumors. A wide diversity of cell types are sensitive to 2ME2 including both tumor and non-tumor cell lines and various primary cultures. The ability of this compound to target endothelial cells was first identified by its antiproliferative activity against microvascular and large vessel endothelial cells [151]. Additional studies showed that 2ME2 inhibits the migration and invasion of capillary endothelial cells and their ability to form capillary-like structures on collagen gels [151, 160].

2ME2 induces apoptosis independently of p53. 2ME2 induces apoptosis through both the intrinsic and extrinsic pathway. In multiple myeloma, 2ME2 has been reported to cause apoptosis through activation of JNK and its transport to the mitochondria, where it cause a decrease in mitochondrial membrane potential and release of the cytochrome-c and second mitochondria activator of caspase (Smac) [161]. On the other hand, addition of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), the ligand of the death receptor 5 (DR5) sensitize breast cancer and endothelial cells to 2ME2 [162].

Importantly, the induction of apotosis and antiprolifetative activity of 2ME2 is independent of estrogen receptors α and β for which 2ME2 has very low affinity. This is particularly important since 2ME2 is emerging as a promising drug candidate [163].

4.3. Antiangiogenic effects of 2ME2

As predicted from the *in vitro* studies, 2ME2 has antiangiogenic activity in several models. Angiogenesis induced by basic fibroblast growth factor or vascular endothelial growth factor was inhibited by the oral administration of 2-ME2 in the murine corneal neovascularization assay [164]. The oral administration of 2ME2 reduced tumor burden and neovascularization with little evidence of toxicity [151]. In a mouse xenograft model using MDA-MB-435 cells, 2ME2 reduced tumor burden by 60% [164] , and in a xenograft model with H460 non-small cell lung carcinoma, 2ME2 inhibited tumor growth by 70% [155]. 2ME2 inhibited tumor burden approximately 39% in a transgenic murine model of androgen-independent prostate cancer [165]. Because of its efficacy in murine tumor models, 2ME2 is being evaluated in Phase I and Phase II clinical trials against a variety of human tumors.

4.4.Antitubulin effects of 2ME2

2ME2 has been shown *in vitro* to bind at the colchicine-binding site of microtubules (Figure 10) and inhibit tubulin polymerization by interacting at the colchicine binding site [166-168]. Although 2ME2 have some structural similarities with colchichine, it has been shown that 2ME2 is relatively weak competitive inhibitor of the binding of ^{3H}colchicine to tubulin. 2ME2 has also been shown to bind both to unpolymerized tubulin (a reaction strongly inhibited by colchicine site drugs) and to polymerize tubulin in a reaction minimally inhibited by colchicine-site drugs. The latest observation suggest a distinct binding site for 2ME2 on polymer or that 2ME2 binds in a site inaccessible to colchicine in polymer [169].

In vivo, 2ME2 has been shown to depolymerized endothelial [151] as well as in tumor cells [170]. One of the hallmarks of microtubule-disrupting drugs is their ability to induce G2/M arrest. In agreement with that, 2ME2 was shown in the earliest studies to arrest MCF-7 breast cancer cells in G2/M arrest with malformed spindles [171]. In contrast, G2/M arrest was also observed in Jurkat cells [172] comparable to the effects produced by colcemid, but authors

claimed this mitotic arrest was disconnected from the effects of 2ME2 on the microtubules and instead, mediated thorough inhibition of calmodulin. However, the effects of 2ME2 on microtubules were analyzed in confluent A431, even though it has been reported that 2ME2 is non-cytotoxic in non-proliferating cells [151]. Therefore, the evidences presented on hat study were not conclusive and will require further investigation.

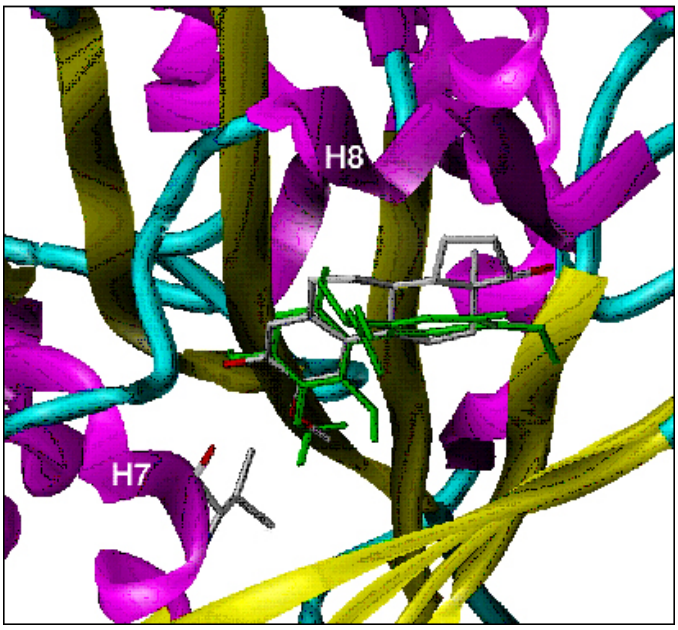

Figure 10. Model of 2ME2 binding to tubulin in relation to colchicine. This figure illustrates the close spatial relation (<4Å) of the colchicine-binding site in β- tubulin. The colchicine structure is colored green and 2ME2 is colored by atom type with carbons white and oxygens red. Tubulin backbone is shown as ribbons (green) and helices (magenta).