UNIVERSITY OF NAPLES FEDERICO II

Department of Pharmacy

ROLE OF ENDOCANNABINOID SYSTEM AND ITS EFFECT ON THE CONTROL OF ANGIOGENESIS IN A MURINE MODEL OF LUNG CARCINOMA

Ph.D. Thesis in Pharmaceutical Science

XXVII Cycle

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Abstract

The present work aims at evaluating the possible role of the endocannabinoid system (ECS) and its effects on the control of angiogenesis in lung carcinoma.

Worldwide, lung cancer is the most common cause of cancer-related deaths. The target of angiogenesis in solid tumours, including lung cancer, represents a new promising strategy (Giaccone, 2007), since tumour angiogenesis is a process necessary for the growth of the solid tumours. The implant of the tumoural cells in a tissue leads to a release of pro-angiogenic factors that activate endothelial cells, allowing the formation of new capillaries and their subsequent stabilization. Therefore, angiogenesis supplies oxygen and nutrients to the tumour mass. The therapeutic efficacy of anti-angiogenic drugs has been demonstrated in clinical studies and anti-angiogenic compounds currently represent an important coadjuvant approach to the standard anti-cancer therapies. A promising class of anti-angiogenic compounds is represented by Cannabinoids (CBs), all the natural compounds, synthetic and endogenous factors exhibiting the same biological activity of the derivatives of *Cannabis Sativa L.*, Marijuana (De Filippis and Iuvone 2009). ECS is formed by receptors, CB_1 and CB_2, their ligands mainly anandamide (AEA) and 2-arachidonylglycerol (2-AG) and the main enzymes involved in the metabolism and transport of these endogenous ligands.

On the basis of these evidences this Ph.D. study focused attention on the deregulation of ECS associated with the tumoral angiogenesis in a murine model of lung carcinoma to strengthen the numerous evidences indicating CBs as possible new coadjuvant molecules to the classic anti-cancer therapy.

In this study, a model of lung cancer induced in C57BL/6 mice injected with Lewis Lung Carcinoma cells was used. The deregulation of the ECS was evaluated during the various

stages of tumor growth and correlate to the process of angiogenesis. The experiments show, for the first time, a strong deregulation of ECS in lung cancer; in fact, there is a significant increase in the levels of the MAGL enzyme, involved in the degradation of the main endogenous ligand for these receptors, 2-AG, and the expression of the CB_2 receptor. This deregulation is, closely, associated with the angiogenic process, given that it is highlighted in the second phase of tumor progression, a phase in which the tumor mass requires oxygen and nutrients for its growth, and the CB_2 receptor seems to be the protagonist of this regulation. Considering these results, we tried a therapeutic approach with a selective agonist of the CB_2 receptor, JWH133. The treatment with JWH133 improved tumor parameters reducing the principal angiogenic markers, such as VEGF, MMP-9, MMP-3, CXCL16, Endoglin, angiopoietin, aFGF, bFGF, SDF-1. The CB_2-mediated activity of JWH133 was confirmed through the use of CB_2 knockout animals in which the tumor was induced in the same experimental conditions. An alternative strategy was also used in the agonist treatment, to have a pharmacological intervention only in the tumor mass, the area where the ECS appears deregulated. This alternative approach provided for the use of an inhibitor of MAGL, JZL184. The data demonstrates that JZL184 reduces the tumor mass and the angiogenic process, similarly to the JWH133.

In conclusion, the data here obtained strengthen the possibility to use cannabinomimetics molecules as adjuvants in standard anti-tumor therapies n order to control the ECS deregulation, to reduce angiogenesis associated with tumoral conditions and, consequently, to improve the pathological condition. Moreover, these compounds have lower price and less side effects compared to currently use anti-angiogenic agents.

Abbreviations

AA	*Arachidonic acid*
AEA	*Anandamide*
aFGF	*Acidic Fibroblast Growth Factor*
AMT	*Anandamide membrane transporter*
Angs	*Angiopoietins*
bFGF (FGF-2)	*basic Fibroblast Growth Factor*
ATP	*Adenosine triphosphate*
BBB	*Blood-brain barrier*
BM	*Basement Membrane*
BrdU	*BromodeoxyUridine*
CBs	*Cannabinoids*
CBD	*Cannabidiol*
CNS	*Central Nervous System*
COX-2	*CycloOXygenase-2*
CD105	*Endoglin*
CXC	*Chemokines*
DAGL-α	*Diacil glycerol lipase*
DMEM	*Dulbecco's Modified Eagle Medium*
ECM	*ExtraCellular Matrix*
EC	*Endothelial Cell*
eCBs	*Endocannabinoids*
ECS	*Endocannabinoid System*
EGF	*Epidermal Growth Factor*

EGFR	*Epidermal Growth Factor Receptor*
ELISA	*Enzyme-Linked ImmunoSorbent Assay*
EPCs	*Endothelial Progenitor Cells*
ET	*Endothelin*
FAAH	*Fatty acid hydrolase amide*
FAK	*focal adhesion kinase*
FBS	*Foetal Bovine Serum*
FDA	*Food and Drug Administration*
GCSF	*Granulocyte-colony stimulating factor*
GM-CSF	*Granulocyte-macrophage colony-stimulating factor*
HIF1-α	*Inducible Hypoxic factor*
HDL	*High Density Lipoprotein*
HSPG	*Heparin Sulphate ProteoGlycans*
IAR	*Intussusceptive arborization* intussusceptive
IBR	*Intussusceptive branching*
IMG	*Remodeling microvascular growth*
IFN	*Interferon*
ILs	*Interleukins*
JNK	*c-Jun N-terminal kinase*
LDL	*Low Density Lipoprotein*
MAGL	*Mono-acil glycerol lipase*
MAPK	*Mitogen-activated protein kinases*
MMPs	*MatrixMetalloProteases*

MTT	*3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenil*tetrazoliumbromide
mTOR	*Mechanistic target of rapamycin*
NAPE-Phospholipase D	*N-acyl phosphatidylethanolamine* phospholipase *D*
NO	*Nitric Oxide*
NOs	*Nitric Oxide syntase*
OPN	*Osteopontin*
PAI-1	*Plasminogen activator inhibitor-1*
PECAM-1	*Platelet endothelial cell adhesion molecule*
PlGF	*Placental growth factors*
PBS	*Phosphate Buffer Solution*
PDGF	*Platelet-Derived Growth Factor*
PI3K	*Phosphatidyl-Inositol 3 Kinase*
TAF	*Tumor Angiogenesis Factor*
THC	*Tetrahydrocannabinol*
TIMPs	*Tissue inhibitor of metalloproteinases*
TKI	*Tyrosine kinase inhibitor*
TRPV1	*Transient receptor potential vanilloid-1*
TSP-1/-2	*Trombospondin-1/-2*
TGF-α	*Transforming Growth Factor-α*
TGF-β	*Transforming Growth Factor-β*
TNF-α	*Tumor Necrosis Factor-α*
TKI	*Tyrosine kynase inhibitors*
uPA	*urokinase-type PA*

VEGF	*Vascular Endothelial Growth Factor*
VEGFR	*Vascular Endothelial Growth Factor Receptor*
VPF	*Vascular permeability factor*
VR1	*Type 1 vanilloid receptor*
2-AG	*2-Arachidonyl glycerol*
2-AGE	*2-Arachidonyl glyceryl ether*

1. Introduction

1.1 Angiogenesis

1.1.1 Angiogenesis: an overview

Angiogenesis refers to the building of new capillary branches from existing blood vessels; in physiological conditions, angiogenesis occurs primarily in embryo development, during wound healing and in response to ovulation. However, pathological angiogenesis, or the abnormal rapid proliferation of blood vessels, is implicated in over 20 diseases, including cancer, psoriasis and age-related macular degeneration (Bouïs et al., 2006).

The angiogenic process is made up of different steps:

- ✓ Start of angiogenic reaction;
- ✓ proliferation and migration of endothelial cells;
- ✓ lumen acquisition, stabilization, maintenance, differentiation and remodeling of nascent vessel. (Ucuzian et al.,2007; Carmeliet et al.,2009; Pugh and Ratcliffe, 2003).

Start of angiogenic process

The beginning of angiogenic cascade is due to hypoxic events, inflammation causes or stress; all these *stimuli* promote the destabilization of pre-existing vessels and loss of junctions between endothelial cells. Endothelial cells are activated to produce cytokines and growth factors that cause modification of pre-existing vessels (Milkiewicz *et al.*, 2006), increase of vascular permeability, a process involving nitric oxide (NO) and allow of the extra-vasation of plasma proteins that lay down a provisional scaffold for migrating endothelial cells. At this point, plasminogen activator, matrix metallo-proteases (MMPs), chymase or heparanase families influence angiogenesis by degrading basal membrane and extracellular matrix molecules as well as by activating or liberating growth factors sequestered within the extracellular matrix (Coussens et al. 1999). Each stage is regulated in physiological

condition, while this regulation is lost in pathological condition, such as, tumor growth and metastasis (Liekens et al., 2001)

Proliferation and migration of endothelial cells

Cell adhesion to many matrix components and intra-cellular remodulation of cytoskeletal filaments, bring cells to migrate. In this phase some receptors placed on the cell surface membrane, such as integrines, in particular the classes of $\alpha v\beta 3$ $\alpha v\beta 5$, over-expressed on proliferant endothelial cells play an important role (Nisato et al., 2003). The migration can be promoted by a series of mechanical perturbations on matrix-cell contact. During angiogenesis proteins proteolysis of basal membrane takes a local thinning of the structure that results at certain points in a distortion of cell membrane such as the alteration of tractional forces (Ingber et al., 2002). The mechanical signal so originated, is converted intracellularly in biochemical stimuli that trigger the movement (Shin et al., 2001). Endothelial cells "guide" then proceed in interstitial space, followed by proliferating cells. The collapse of the extra-cellular matrix (ECM) produces an increase in the extracellular concentration of various soluble pro-angiogenic mediators, which feed and strengthen the signaling pathways underlying the chemotactic and proliferative process.

Lumen acquisition, stabilization, maintenance, differentiation and remodeling of nascent vessel

The proliferation of endothelial cells, already, occurs in the early stages of the angiogenic process and continues throughout the period of elongation of the neo- capillary. Immediately, along sketch vascular cells are organized in order to create the lumen of the vessel. The appearance of this structure may occur through a process of fusion of cytoplasmic vacuoles (channeling intracellular) or by appending more cells around a central tubule (channeling intercellular) (Egginton & Gerritsen, 2003).

The tissue perfusion is restored at the expense of anastomosis between the vascular endothelium that advances, and pre-existing vessels; thus, the merger of two capillaries branches occur to create a new channel through blood can flow.

The final phase of maturation and stabilization of the capillary tube involves the deposition of a new basement membrane and the re-establishment of the contact between the adherent cells. In order to delineate the vessel lumen, distribution and type, adhesion factors determine the polarity of the endothelial cells, by discriminating the side luminal from the not-luminal (Liekens, 2001).

The stabilization of new capillary is the last phase of angiogenic process; several factors, as Angiopoietin 1 (Ang1), Angiopoietin 2 (Ang2) and placental growth factor (PlGF), play a pivotal role in this events (Milkiewicz, 2006). When the nascent vessel is formed, the proliferation and migration of endothelial cells are inhibited and a new basal membrane is secreted (Bouïs, 2006). To obtain a functional vascular network, the new vessels are remodeling to become mature; this maturation is due to Platelet-Derived-Growth Factor (PDGF), that also stimulates the recruitment of perycites and smooth muscle cells that stabilize vessels (D'Andrea 2006).

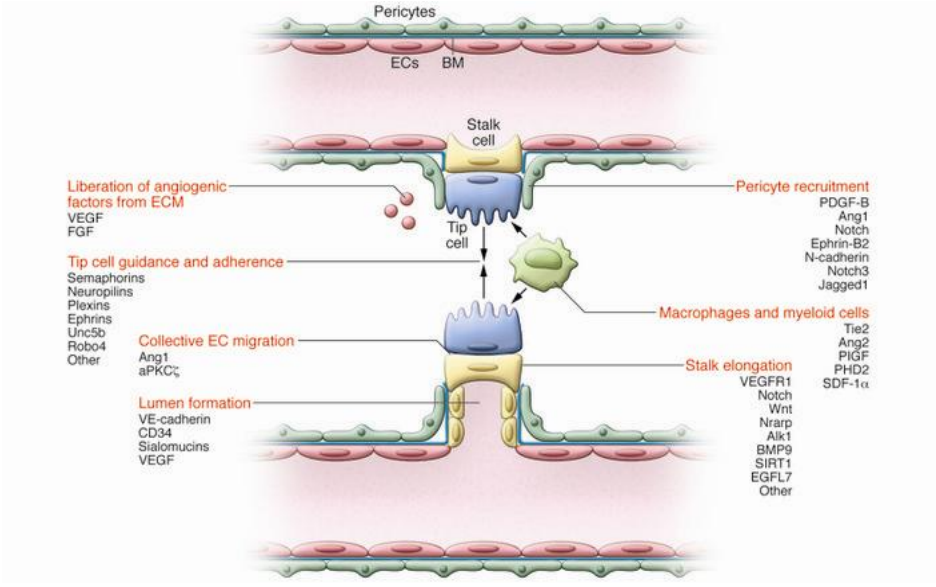


Fig.1: Angiogenic cascade scheme

1.1.2 Formation of new blood vessels

Angiogenic process occurs not only as previously described, but also by another two different mechanisms:

- ✓ intussusception,
- ✓ starting from endothelial precursors which come from bone marrow.

Intussusception

Intussusception is a process in four phases; it starts with the invagination of the capillary wall in two opposite points. In this way, an inter-endothelial contact-zone, into the lumen vessel is formed, which will be perforated centrally. The area between the new two blood vessels is rich in perycite and fibroblaste responsible for the deposition of collagen fibers as the main component of the extracellular matrix (Djonov et al., 2002).

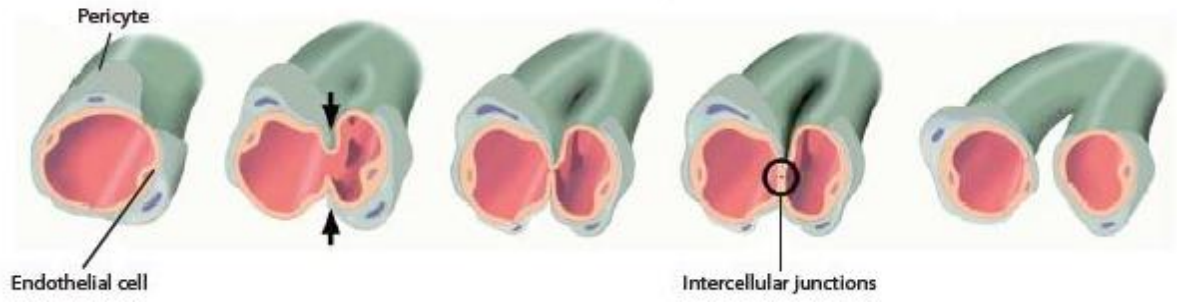


Fig.2: Synthetic representation of new blood vessels formation by intussusceptions mechanism

Different aspects of angiogenesis and remodeling in perfused microvascular systems are accomplished via three modes of intussusceptions: intussusceptive microvascular growth (IMG), intussusceptive arborization (IAR), and intussusceptive branching remodeling (IBR). IMG can rapidly expand a capillary plexus and produce a large endothelial surface without altering capillary dimensions. IAR can generate feed vessels from the capillary plexus by changing their dimensions, position, and identity and thus, influence "endpoint density" of the

supplying network. Finally, IBR can modulate the position of and diameters at branching points in larger microvessels and can, therefore, influence pre- and postcapillary transport properties. Moreover, IBR can lead to the removal of branches, and this process of vascular pruning is of considerable importance to achieving transport efficiency (Kurz et al., 2003).

Endothelial precursors

The process about the formation of new blood vessels is, also, due to the recruitment of endothelial precursors: endothelial progenitors cells (EPCs). These cells come from emathopoiteic staminal cells and they are able to migrate from bone marrow to the damaged area. Neovascularization is due to both the capacity of these precursors to differentiated themself in endothelial cells, incorporated then in newly formed vessels, and the secretion of growth factors. EPCs are, in fact, able to release growth factors, as vascular endothelial growth factors (VEGF), contributing to the growth of the vessel. VEGF promotes vasculogenesis by promoting EPC chemotaxis, recruitment (Li et al., 2006), and vessel formation. The cells received at the point of sprouting take advantage of the increase in local permeability, to escape into the interstitial space and clustering; some of them will be integrated in the neo-formed endothelial monolayer while a part of the EPC can be traced in the perivascular structure layers (Murasawa & Asahara, 2005; Schatteman et al., 2004).

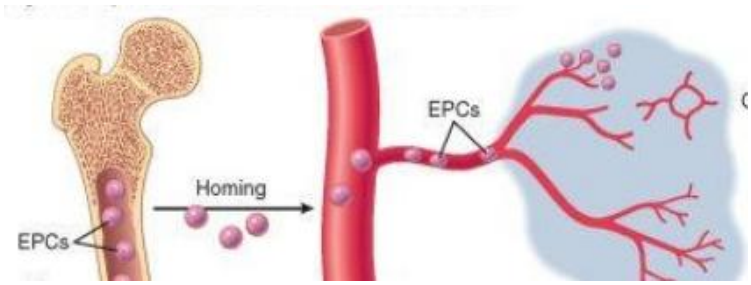


Fig. 3: Angiogenesis by mobilization of EPCs from the bone marrow

1.2 Angiogenic switch

The angiogenic process is a fine regulated process, strictly controlled by a balance between stimulatory and inhibitory factors; a change of this harmony can caused an "angiogenic switch" (Bergers and Benjamin, 2003; Ribatti et al., 2007) allowing the formation or the non-formation of new blood vessels (Mandriota and Pepper, 1997), causing damage (Hanahan and Folkman, 1996). Many of the intra-cellular *stimuli* associated with the "angiogenic switch" shift the balance to the activation of angiogenesis; it is possible to identify these factors as **pro-angiogenic** or, those inhibiting them (**anti-angiogenic** mediators).

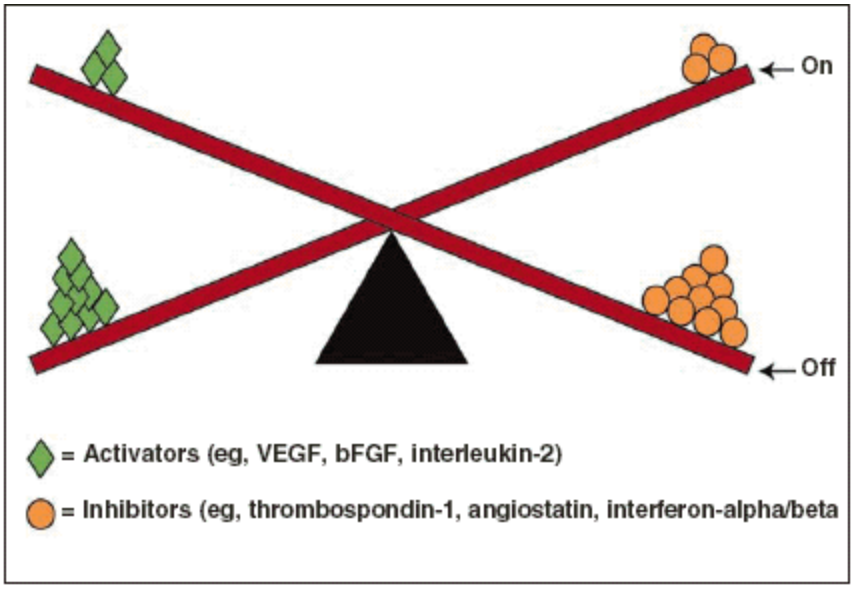


Fig. 4: A ngiogenic switch

Pro-angiogenic factors can be divided in two groups: direct and indirect factors; the first directly stimulate the proliferation and migration of endothelial cells; the second, promote the activation of endothelial cells by other factors (Folkman and Shing, 1992).

The main pro-angiogenic factors (table 1) are:

VEGF (Vascular Endothelial Growth Factor)	increase the permeability and migration of endothelial cells, stimulate and promote angiogenesis
Ang1 (Angiopoietin1)	stimulate the formation of endothelial cells and increase the stability of endothelium
Ang2 (Angiopoietin2)	destabilizes endothelium in presence of VEGF
aFGF, bFGF	stimulate angiogenesis *in vivo,* the proliferation and formation of tubes and the production of collagenase in endothelial cells; also, increase the migration of endothelial cells
PDGF	increases the formation of perycite and smooth muscle cells and augments the stability of capillary walls; also, induces the expression of VEGF and VEGFR-2

TGF-β	supports the fibroblaste growth and inhibits the proliferation and migration of endothelial cells; supports the stability of the capillary wall
TNF-α	Stimulates angiogenesis *in vivo* and the formation of endothelial cells *in vitro*
EGF, TGF-α	Stimulate the proliferation and migration of endothelial cells
G-CSF, GMCSF	Stimulate the proliferation and migration of endothelial cells
Angiogenin	Supports the link and extension between endothelial cells
Tissue factor	Contributes to the formation of the vasculature of the yolk sac

Table 1: The main pro-angiogenic factors

On the other hand, there main anti-angiogenic factors may be classified as follows (Table 2):

ANG2	Antagonist of Ang1
TSP-1 e -2	Inhibits migration, growth, survivor and adhesion of endothelial cells
Angiostatin	Inhibits tumoral angiogenesis and proliferation
Endostatin	Inhibits the activation of endothelial cells; promotes apoptosis; inhibits the activity of metallo-proteases
Anti-trombin	Inhibits angiogenesis and tumoral growth
IFN-α,-β,-γ; IP-10; IL-4, IL-12, IL-18	Inhibits the migration of endothelial cells and down-regulate bFGF
TIMPs	Inhibit metallo-proteases
Tumastatin	Inhibits angiogenesis and promotes apoptosis

Table2: The main anti-angiogenic factors

1.2.1 Vascular Endothelium Growth Factor

VEGF, also known as Vascular Permeability Factor (VPF), is the most important mediator in angiogenic process since it is able to regulate all events in angiogenesis. VEGF is a survival factor for ECs, both *in vitro* and *in vivo* (Plate et al., 1992; Shweiki et al., 1992). *In vitro*, it prevents apoptosis and its activity is mediated by the phosphatidylinositol (PI)-3Akt pathway (Gerber et al., 2002). *In vivo*, VEGF induces angiogenesis as well as permeabilization of blood vessels, and plays a central role in the regulation of vasculogenesis. This factor has, also, an effect on bone-marrow-derived cells; it promotes monocyte chemotaxis (Ferrara et al., 2003), inhibits antigen presenting cells, inducing a decrease of immunity response (Ferrara et al., 1997).

Hypoxic signal is the first event that induces an increase of VEGF expression and this causes an increase of its mRNA. The transcriptional regulation of VEGF is mediated by hypoxia inducible factor-1 (HIF-1α); so when HIF-1α is activated a cascade of events starts; in particular, HIF-1α is involved in an iper-activation of VEGF in pathological conditions, such as cancer. In fact, the proliferation, the migration and the permeability of endothelial cells, inducted by VEGF, carry out a critical role in angiogenic process (Keyt et al., 1996; Bernatchez et al., 1999; Waltenberger et al., 1994). HIF-1α, remarkably, produces VEGF and contributes to the formation of vascular tubes in embryogenesis as well as in adults.

VEGF is not a single protein, but a small group of several peptide growth factors of different amino-acids in length and type. It was firstly discovered in 1983 as vascular permeability factor (Senger et al., 1983) and in 1989 it was characterized as an endothelial cell mitogen and called vascular endothelial growth factor (Gerber et al., 2002). The VEGF family's is made up of six members: VEGF-A (o VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E e PlGF.

All of these type of VEGF due their function to the bind with three tyrosine-kinase receptors: VEGFR-1 (FLT-1), VEGFR-2 (KDR/FLK-1) and VEGFR-3 (or Flt-4), which are expressed

almost exclusively in endothelial cells. The bind to the ligand allows a conformational change, causing dimerization that triggers a process of autophosphorylation. The receptor, thus, activatd can promote the release of a several second messenger that causes a biological response.

VEGF-A act, principally, on the angiogenic control; alternately spliced isoforms of 121, 145, 165, 189 and 206 aa have been identified in humans (Shima et al., 1996; Robinson et al., 2001). $VEGF_{165}$ is the most abundant isoform, followed by $VEGF_{121}$; $VEGF_{206}$ is expressed during embryonic progress (Houck et al., 1991), while isoform 145 has been observed in cell lines derived from cancers of the female reproductive organs (Poltorak et al., 1997) and its expression seems to be more restricted. $VEGF_{189}$ displays a higher affinity to heparin and heparin-sulfates than $VEGF_{145}$ or $VEGF_{165}$; it is sequestered on heparin-sulfate proteoglycans of cell surface in the extracellular matrix protecting VEGF to enzymatic degradation (Robinson et al., 2001). VEGF-A binds with high affinity VEGFR-1 and -2 and plays an essential role in vasculogenesis and angiogenesis. It has also been shown to induce lymphangiogenesis through VEGFR-2. **VEGF-B** is, principally, involved in monocyte chemotaxic process (Ferrrara et al., 2001); it is expressed in several tissues and most abundantly in the heart, brain, skeletal muscle, and kidney, and it occurs as two splice isoforms of 167 and 186 amino acids, respectively (Olofsson et al., 1996). Because VEGF-B binds VEGFR-1, it may have a role in the regulation of blood vessel physiology. The ability of VEGF-B to heterodimerize with VEGF is consistent with the conservation of the eight cysteine residues involved in inter- and intramolecular disulfide bonding. Furthermore, the coexpression of VEGF-B and VEGF in many tissues suggests that VEGF-B/VEGF heterodimers occur naturally. The formation and cell association of such heterodimers may affect the formation of VEGF homodimers and thus indirectly control release and bioavailability of VEGF. VEGF-B binds, selectively, VEGFR-1 and induces an increase of

urokinase-type PA (uPA) and plasminogen activator inhibitor-1(PAI-1) expression, suggesting an its role in the degradation of the matrix process and in the migration of endothelial cells (Olofsson et al., 1998); it, also, overlaps VEGF-A activities by activating VEGFR-1 (Olofsson et al.,1999). **VEGF-C** and **VEGF-D** are involved in lymphangiogenesis, its binds to and induces tyrosine phosphorylation of VEGFR-3 (Makinen et al., 2001). A correlation between VEGF-C and VEGF-D expression, tumor lymphangiogenesis and the formation of metastases in regional lymph nodes has been described in a range of human tumors, including malignant melanoma and lung, breast, colrectal and gastric carcinomas (Stacker et al., 2002). VEGF-C and VEGF-D are both angiogenic via VEGFR-2 and VEGFR-3, and lymphangiogenic (primarily VEGF-D) via VEGFR- 3 (Hamada et al., 2000; Achen et al., 2002). **VEGF-E** is codificated by parapox-Orf-virus gene and act, only, on VEGFR-2. **PlGF** induces angiogenesis *in vivo* and it is, principally, express in placenta and tumors.

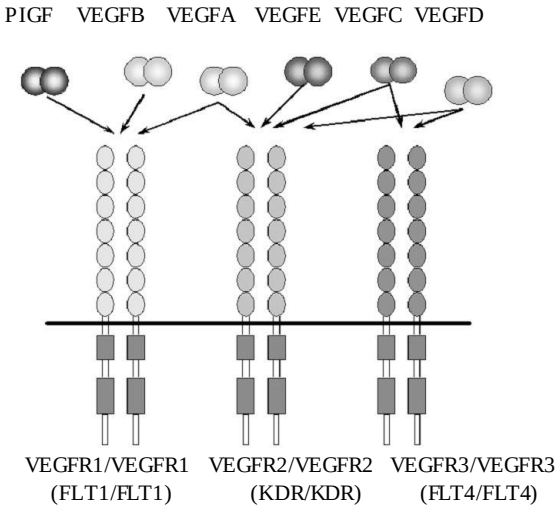


Fig 5: VEGF receptors and its ligands

1.2.2 Other pro-angiogenic factors

There are several other growth factors thought to be involved in angiogenesis. Many factors act directly on endothelial cells as cytochine, chemokine and angiogenic enzymes.

Fibroblast Growth Factor

Currently, the family of fibroblast growth factors (FGF), has at least 22 members, all capable of acting as a pleiotropic molecules on different cell types, including endothelial cells.

Gospodarowicz, in 1974, isolated two distinct protein forms: the acidic fibroblast growth factor, FGF-1, and the basic fibroblast growth factor, FGF-2. Although it showed a high degree of sequence similarity, possessed a distinct act as mitogen (Gospodarowicz, 1974). The activity of these polypeptides is manifested as a result of the interaction with 2 receptors: a high affinity tyrosine kinase receptor (FGFR) and a low affinity one represented by proteoglycan heparan sulfate (HSPG) (Presta et al., 2005).

FGF-2 attends angiogenic process stimulating, principally, the proliferation of endothelial cells. Moreover, this factor can stimulate the migration of macrophages and fibroblasts to the damaged area.

Many solid tumours demonstrate increased expression of FGF-2 and its receptor including malignant melanoma (Becker et al.,1989), ovarian carcinoma (Crickard et al., 1994) and NSCLC (Takanami e al., 1996). bFGF and FGFR-1 expression in adenocarcinoma has been shown to correlate with microvessel density (Takanami et al.1997) although a more recent study has contradicted these findings (Gubbo et al., 1999).

Angiopoietins

Ang-1 and Ang-2 are secreted factors that mediate their effects by binding to the endothelial-specific Tie-2 tyrosine kinase receptor (Davis et al., 1996). *In vivo* analysis has revealed that Ang-1 recruits periendothelial cells, whereas Ang-2 is presumed to destabilize blood vessels by interfering with constitutive Ang-1/Tie-2 signals in the vessel wall, leading to the

detachment of the perivascular cells and allowing the vessel endothelium to revert to a more plastic state of angiogenesis. Apparently conflicting publications report increased, decreased or unchanged expression levels of both Ang-1 and Ang-2 in a wide range of tumors (Tail et al., 2004). Angiopoietins are growth factors, that have a high affinity to the vascular endothelium (Gale et al., 1999). Although absolute levels of either Angs may increase or decrease, the ratio Ang-1:Ang-2 shift in favor of Ang-2. This implicates Ang-2 as a candidate for the angiogenic switch.

Trasforming Growth Factor

Trasforming Growth Factor is used to identify two classes of growth factors: TGF-α and TGF-β. TGF-α polypeptides are produced by macrophages and cerebral cells; they are over-expressed in some tumoral forms, they induce epithelium development and contribute to the recovery of nervous tissue functionality after damage or neurodegenerative disorders (Fallon et al., 1990)

Transforming growth factor beta form, instead, consists of three multifunctional isoforms which are able to regulate the proliferation and cell differentiation. Produced and released by various cell types, it plays a central role in tissue regeneration, in cell differentiation, in embryonic development and in the regulation of the inflammatory system. TGF-α and TGF-β act, synergistically, to activate transforming cellular processes and to promote angiogenesis (Pepper,1997; Schreiber et al., 1986). TGF-β effect on angiogenic process is due to recruitment and stimulation of macrophages and fibroblasts (Pepper, 1997); moreover, they stabilize neo-formed vessels, recalling smooth muscle cells and perycites (Darland et al., 1999).

Platelet-derived growth factors

PDGFs are growth factor that act by tyrosine kinase receptor (PDGFRs). Studies of PDGFs and PDGFRs in animal development have revealed roles for PDGFR-α signaling in gastrulation and in the development of the cranial and cardiac neural crest, gonads, lung, intestine, skin, CNS, and skeleton. Similarly, roles for PDGFR-β signaling have been established in blood vessel formation and early hematopoiesis.

Endothelin

There are, also, other endogenous factors which are able to regulate angiogenic process. Endothelin (ET) is part of the family of peptide, principally, secreted by endothelial cells; these molecules act through the interaction with two classes of receptors GRP, ETA-R and ETB-R (Nussdorfer et al.; 1999). ETs are formed by three isoform: ET-1, ET-2, ET-3; it was demonstrated that ET, in association with VEGF, have a clear pro-angiogenic activity (Matsuura et al.; 1998; Pedram et al.; 1997).

Matrix Metallo-proteases

MMPs consist in a family of at least 20 zinc-dependent endopeptidases, capable of degrading the extracellular matrix. They are known for their involvement in the release of apoptosis inducer and in the regulation of chemokines activity. MMPs, also, take part in various cellular events, the proliferation, migration, differentiation and survival. As modulators of extracellular microenvironment, they are essential for the various angiogenic stages: from the deposition to the destabilization of basal membrane, for direct effect on the components of the ECM (Chang et Werb, 2001).

Produced by epithelial cells, by fibroblast and by inflammatory mediators, MMPs are secreted in an inactive form and subsequently converted into the active form by proteases present in extracellular environment (Westermarck et al., 1999).

To guarantee tissue homeostasis, there is a control system of the proteolytic activity of MMP, which acts at the level of gene expression and producing endogenous tissue inhibitors (tissue inhibitors metallo-proteases, TIMP). MMPs are involved in tissue remodeling not only in physiological processes, but there are several pathological conditions that present abnormal proteolytic system: cirrhosis, arthritis, development and tumor angiogenesis.

Chemokines

Chemokines are broad-range regulators that play important roles in inflammation and cancer (Keeley et al., 2008). They are divided into four families, based on structural properties and primary amino acid sequence, as CXC, CC, C or CX3C. CXC chemokines represent a family of homologue peptides exhibiting positive or negative activity on the control of angiogenesis. Angiostatic CXC chemokines have an important role against tumour development and diffusion. As these molecules also directly affect the tumour cells themselves, they may be, in addition, involved in adaptive/evasive resistance. Overexpression of CXCL4 (CXC chemokine ligand 4) and IP-10 (interferon-γ -inducible protein 10) blocks tumour progression and can also induce regression of metastasis.

CXCL-8 and its receptors, CXCR1 and CXCR2, also, play an important role in angiogenic phenomenous and in tumoral development (Salcedo et al., 2000).

1.2.3 Inhibitors factors of angiogenesis

The existence of angiogenic inhibitors was postulated by Folkman in 1971, but the real discovery of the first angiogenic inhibitor was due to Zetter and Folkman, in 1980; in these years they demonstrated that interferon–α was able to inhibit endothelial cells migration, in reversible and dose-dependent manner. Since 1980 to 2005 Folkman and his colleagues, identified twelve angiogenic inhibitors:

interferon α–β, platelet factor 4, angiostatic steroids, fumagallin, angiostatin, thalidomide, 2-metoxyestradiol; endostatin, ATC (Cleved Antithrombin III), 3-aminothalidomide, BDF-maf, caplostatin.

Endogenous inhibitors of activated endothelial cell can be divided in:

- Soluble mediators, as Trombospondin-1 (TPS-1), which is considerate the principal physiological inhibitor of vascular genesis being produced by normal cells. The over-expression of TSP-1 causes the inhibition of angiogenic process (Streit et al.; 1999). Other same inhibitors are Troponin I, INF-a, IL-12 and IL-4, retinoic acid;
- Proteic fragments as angiostatin and endostatin; the first, binds ATP synthetase interfering, in this way, with ATP production and inhibiting, consequently, cell growth (Moser et al.; 1999). It seems that this fragment is involved in the inhibition endothelial cell migration and proliferation (Claesson-Welsh et al.; 1998). Endostatin inhibits endothelial cell proliferation *in vitro* and increases tumoral apoptosis (Liekens et al.; 2001).
- Genetic factors as p53, which belonged to onco-suppressor family, molecules that encode for gene which block the uncontrolled proliferation of cells. Some studies have highlighted its involvement in the degradation process of inducible hypoxic factor (HIF-1), that favorites expression angiogenic activators (Ravi et al.; 2000).

Endogenous non- classical inhibitors are represented by: samatostatin, which inhibits cell growth and angiogenic process (Patel et al., 1999; Garcia de la Tore et al., 2002). Somatostatin receptor, SST2-R, mediates anti-angiogenic action of somatostatin directly involving the inhibition of cell proliferation (Danesi et al., 1997) and, indirectly, blocking the production of growth factors (Cascinu et al., 2001; Mentlein et al., 2001).

1.3 *Tumoral angiogenesis*

When the angiogenic balance shifts to pro-angiogenic factors, pathological angiogenesis, a process in which there is an uncontrolled growth of new blood vessels, starts. This event is typical of tumoral process/progression; in fact, when cancerous cells are established, they need oxygen and nutrients to allow the mass growth. Classic studies by Folkman showed that tumours cannot grow beyond 1-2 mm without the new blood vessels (Folkman, 1971). Oxygen and nutrients come from vessels and, for this reason, tumoral cells induce an environment in which there is a low concentration of oxygen and this event causes an increase, in this site, of HIF-1α that transcript for some pro-angiogenic factors, such as VEGF, to unleash angiogenic process and formation of new blood vessels. Tumoral nascent blood vessels are structurally and functionally abnormal; they are not organized like normal blood vessels and have an irregular diameter. Furthemore, the wall of these vessels is not formed by an uniform layer of endothelial cells, but by a mix of endothelial and tumoral cells. The abnormality of tumor blood vessels (high disorder, tortuousness, swelling, and excessive branching) leads to chaos of blood flow, hypoxia, and accumulation of acidic materials (Peng and Chen, 2009). The vascular wall presents numerous gaps, dilated inter-endothelial junctions and a discontinuous or absent basal membrane; all these features make the vessels particularly permeable.

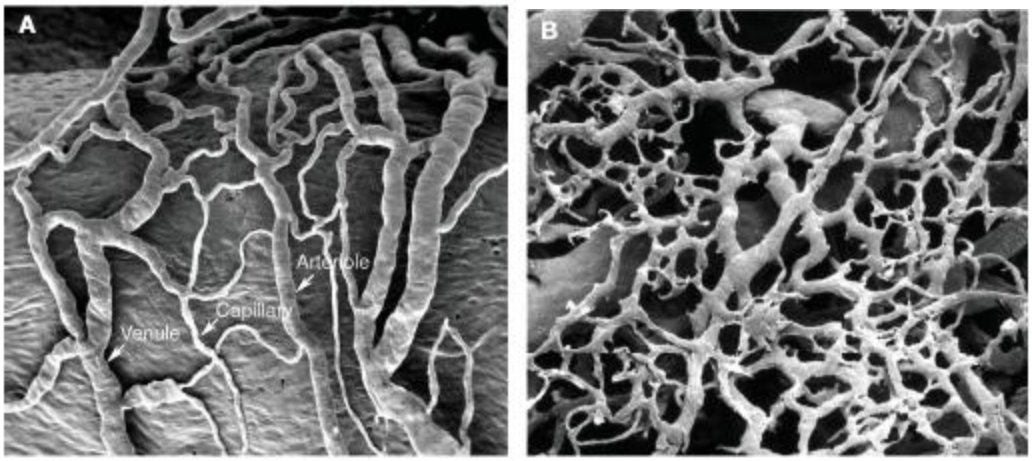


Fig.6: Structure of physiological vessel (A) and tumaral vessel (B)

This idea of tumoral angiogenesis comes from the experiments of Folkman (1971); he implanted tumoral cells in the eyes of rabbits, and showed a production, by the side of tumoral cells, of growth factors able to induce the formation of a new vascular network, indispensable for the growth of tumors. Folkman tried to implant tumoral cells, also, in corneas, which, lacking in blood vessels, received nutrients by *aqueous fluid* contained in the anterior chamber. In these conditions, after a week, the tumor arose as a sleeper. Then, he implanted the same tumoural cells around the iris, a region rich in blood vessels, and he showed the trigger of angiogenic process. After these results Folkman believed that at the basis of angiogenic process there was the production of Tumor Angiogenesis Factor (TAF). He, also, found a way to explicate why tumoral implants, in cornea and in iris, were so different. He concluded that, to have angiogenic process the presence of preexisted vessels in the proximity of the tumoral implant was necessary; in fact, tumoral cells implanting at distance major of 3 mm of blood preexisting vessels there was no presence of angiogenesis. On the basis of these results, it was supposed that tumoral cells producing TAF, were able to arrive at blood preexisting vessels and then got off angiogenic process. Folkman wanted to, also, demonstrate that tumoral cell in order to growth needed besides oxygen and nutrients,

also blood vessels, and in this way confirming his hypothesis. For this reason, he did an experiment *in vitro* in which tumoral cells had oxygen and all nutrients for their growth and survival. This proof highlighted the death of internal cells of tumor; this death was understandable as tumoral internal cells did not receive nutrients.

On the basis of these data, tumor progression is characterized by two phases:

- Avascular phase: oxygen and nutrients are guaranteed to the mass by the simple process of passive diffusion;
- Vascular phase: hypoxic state of cells more distant from blood vessels stimulates the production of pro-angiogenic factors, responsible of the formation of new blood vessels.

Following his studies, Folkman published in the ‘‘New England Journal of Medicine’’ a hypothesis that tumor growth is angiogenesis dependent and that inhibition of angiogenesis could be therapeutic (Folkman, 1971).

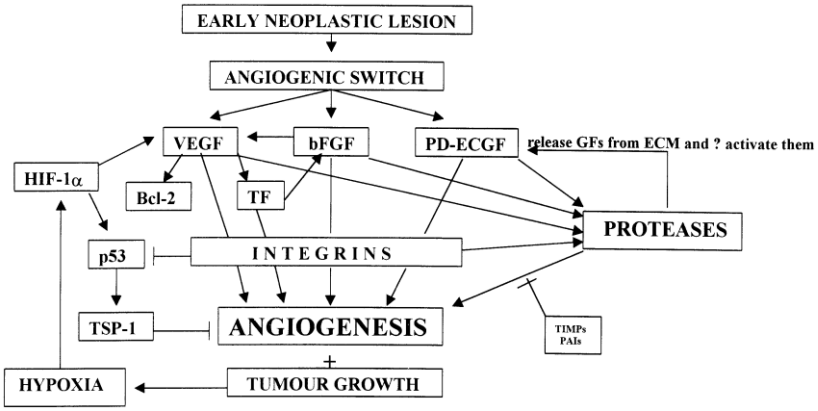


Fig. 7: Scheme of the all events that occurs in tumoral angiogenesis

1.3.1 Antiangiogenic therapy in cancer treatment

Knowledge about molecular mechanism of formation, growth and progression of cancer, had allowed "focused therapy" to be produced. These therapies are based on the use of "intelligent drugs" that, unlike the standard anti-cancer drugs, act, preferentially on tumoral cells.

Standard chemotherapy, in fact, uses substances that act inducing cell death interfering with replication mechanism of the same cells. One of the negative aspects of the use of classical chemotherapic drug is their capacity to act not only on tumoral cells, but, also, on normal cells such as blood cells or the cells of gastric mucosa or cells of hair bulbs. In fact, for this side effect, patients are affected by hair loss, nausea and vomit, blood disorder like anemia.

By using "intelligent drugs" many side effects are reduced, given that these drugs act with a focused approach on one or more events that promote tumoral progression. In particular, these molecules are able to act reducing tumoral cells proliferation, obstructing angiogenic process, promoting tumoral cells apoptosis, stimulating immune system to attack tumoral cells.

Anti-angiogenic therapy shoots tumoral cell death for the absence of oxygen and nutrients, normally transported by blood vessels. Moreover, limiting supply of vessels in tumoral mass, the migration of tumoral cells to other organs (tumoral metastasis) it is, also, limited.

Anti-angiogenic treatments, actually approved in cancer therapy, are based on the use of:

- Monoclonal antibodies;
- Tyrosine kynase inhibitors (TKI);
- mTOR (mechanistic target of ripamycin) inhibitors

Monoclonal antibodies

Monoclonal antibody therapy is based on its interaction with specific growth factors and their related receptors. One of the first drugs used with this mechanism was Avastin®. Avastin®, also known as Bevacizumab, is able to bind VEGF blocking its interaction with receptors presents on the surface of endothelial cells. The absence of VEGF is translated in a decrease

of endothelial cells migration and proliferation. The major success of this therapy was obtained in the treatment of lung cancer, which is the most common malignancy and the leading cause of cancer death worldwide (Parkin et al., 2002; Jemal et al., 2005). It was, in fact, estimated that in 2011 over 221.130 new cases of lung cancer were diagnosed and over of 156.940 people had died due to this pathology.

FDA has approved the use of Avastin® for the treatment of non small cell lung carcinoma (NSCLC). Clinical studies have demonstrated that, in patients affected of NSCLC, the treatment with Avastin®, associated with common chemotherapics drugs, has prolonged the period of survival of patients by about two months. It has gone from a time of 10.3 months in case of treatment with only chemotherapic drugs, to 12.3 months in patients treated with chemotherapy associated to anti-angiogenic agents.

The most common side effect of all monoclonal antibodies is an allergic reaction to the drug: fever, changes in blood pressure, feeling sick and breathlessness, nausea and diarrhea.

Tyrosine kinase inhibitors

Another therapeutic strategy in phase of study, is proposed by tyrosine kinase inhibitors. This strategy acts not only on VEGF, but also on epidermal growth factor (EGF), that linking a tyrosine kinase receptor (EGFR), stimulates endothelial cell proliferation, too. Tyrosine kinase receptors are membrane receptors, that have extracellular domain, through which link the ligand, and citoplasmatic domain, that has kinasic activity. This receptor is presented, initially, as a monomer having a inactive tyrosine kinase domain; as a result of ligand binding, the receptor occurs as a dimer in which the cytosplasmatic domain is active. Each monomer phosphorylates the other on a tyrosine residue and the kinase is able to phosphorylate other intracellular proteins thereby initiating the signal transduction.

Tarceva® (Erlotinib) inhibits EGFR binding intracellular portion, that normally, interact with ATP; in this way is blocked kinase activity, due to the lack of signal, and cell proliferation stops.

mTOR inhibitors

mTOR is a serine/threonine kinase-protein that regulates protein synthesis, cell proliferation and survival in response to hormones, stress and growth factors. mTOR is a catalytic subunit of two complex as mTORC1 and mTORC2. Activated mTORC1 up-regulates protein synthesis by phosphorylating key regulators of mRNA translation and ribosome synthesis. This includes phosphorylation of EIF4 EBP1 and release of its inhibition toward the elongation of initiation factor 4E (eiF4E). These kinases mediate cellular responses to stresses such as DNA damage and nutrient deprivation. This protein acts as the target for the cell-cycle arrest and immunosuppressive effects of the FKBP12-rapamycin complex.

Afinitor® (Everolimus) is an mTOR inhibitor; it acts by a type of targeted therapy that blocks FKBP12, blocking, consequently, mTOR activity. By blocking this protein, Everolimus can stop cancer cells from growing. Everolimus may also prevent tumors from developing new blood vessels, which would help to limit their growth. This drug is, actually, used to treat advanced kidney cancer, advanced breast cancer, a rare type of brain tumor (subependymal giant cell astrocytoma), and a rare type of pancreatic cancer (pancreatic neuroendocrine tumor).

1.4 FDA approved drugs in antiangiogenic therapy

Recently, many drugs, which explicate their action by an anti-angiogenic mechanism, used to treat cancer were approved. In particular, FDA approved many monoclonal antibodies which have different target as reported in table 3.

Table 3 FDA Approved Monoclonal Antibodies Used to Treat Cancer.

Drug	Trade Name	Target	Cancer Type
Trastuzumab	Herceptin	HER2	Breast, gastric
Pertuzumab	Parjeta	HER2	Breast
Cetuximab	Erbitux	HER1	Squamous cell carcinoma
Panitumumab	Vectibix	HER1	Colon
Bevacizumab	Avastin	VEGF	Glioblastoma, NSCLC, colorectal, kidney
Rituximab	Rituxan	CD20	B-cell non-Hodgkin lymphoma, chronic lymphocytic leukemia
Alemtuzumab	Campath	CD52	B-cell chronic lymphocytic leukemia
Ofatumumab	Arzerra	CD20	Chronic lymphocytic leukemia
Ipilimumab	Yervoy	CTLA-4	Melanoma

Abbreviations: HER, human epidermal growth factor receptor; VEGF, vascular endothelial growth factor; CD, cluster of differentiation; CTLA, cytotoxic T-lymphocyte antigen.

Table 3: List of approved monoclonal antibodies by FDA

Moreover, other small molecules drug were approved by FDA in anti-angiogenic cancer therapy as reported belowe:

Targets for small-molecule drugs in anti-angiogenic therapy

Molecular targets	Small-molecule drugs	Current status
Growth factor receptors	Gefitinib/Iressa®	FDA approved (Cohen et al.; 2003)
	Lapatinib/Tykerb®	FDA approved (Moy et al.; 2007)
	Erlotinib/Tarceva®	FDA approved (Cohen et al.; 2005)
Multiple growth factor receptors	Imatinib/Glivec®	FDA approved (Habeck et al.; 2002)
	Sunitinib/Sutent®	FDA approved (Rock et al.; 2007)
	Sorafenib/Nexavar®	FDA approved (Land et al.; 2008)

(Wu et al, 2008)

Although these drugs have reported satisfying effects in cancer treatment, the principal limitation about the large use of these molecules is linked to its higher costs and its high immunization. Therefore, several researchers are focused on the identification of other molecules able to treat angiogenesis, but at lower costs.

In this contest, the idea of the present thesis was to test, in cancer treatment, marijuana derivates for its well know anti-tumoral and anti-angiogenic proprieties.

2. Cannabinoids

2.1 Cannabis Sativa L.: a brief history

Cannabis is a flower plant and its history, in pharmacological environment, goes back to ancient times; documents that certify its use only date back to later times such as that of the great Chinese empire Chen Nung, about 5000 years ago. In this period, in fact, a herbarium highlighting the medicinal properties of many plants even including hemp was published. It was recommended for malaria, constipation, pain, "female problems" and in association with wine and resin was indicated as an analgesic during surgery. Even though other civilizations have released evidence on the use of this plant , such as the Egyptians, the Assyrians and the Indians. The most important text dated back to the first century BC and belongs to the Greeks and Romans communites. In fact, a Greek doctor, described in Pedanius Dioscorides approximately 600 medicinal plants of the Middle East including the *Cannabis* which was reported to have anti-inflammatory and analgesic effect.

The structure of the main psychoactive ingredient Δ^9-tetrahydrocannabinol (Δ^9-THC) was not elucidated until 1964 (Gaoni, Mechoulam, 1964) and it was generally assumed that the high lipophilicity of this compound was the cause of a lot of its pharmacological effects. In 1988, Howlett (Howlett, 2004) and his colleagues identified the specific Δ^9-THC binding site in the brain and then, Matsuda (Matsuda et al., 1990) characterized the first cannabinoid receptor, CB_1, from a series of orphan G-protein coupled receptor. Mechoulam's (Devane et al., 1992) hypothesis was that it was not possible that THC binds a receptor free from endogen biological activity; in fact, he supposed that exists necessarily an endogenous compound able to act and to promote a biological response of CB_1. Devane and Mechoulam's work led up to the discovery of an endogenous THC-like molecule, called Anandamide (N-arachidonoylethanolamine; AEA) from 'ananda', the sanskrit word for 'bliss'(Devane et al.,

1992), was isolated and found to activate CB receptors, thus mimicking the psychotropic effects of THC (Mechoulam et al.,2002). In a few years other endogenous agonists of CB_1 receptors were characterized, and were collectively called 'endocannabinoids' (Di Marzo, 1998). Munro (Munro et al., 1993), in 1993, identified a second cannabinoid receptor, CB_2, in HL60 (*human promyelocyties leuchemic 60*) cells line; this receptor had the ability to interact with anandamide in a manner minor compared to the CB_1 receptors, and its localization was identified prevailing at the level of peripheral organs as spleen and thymus. These informationled to the identification, by a Japanese group, in 1995, of a second cannabinoid ligand, 2-arachydonoil glycerol (2-AG) (Mechoulam et al., 1995) and, also, this compound had major affinity to CB_1 and CB_2 receptor then AEA.

Fig. 8: ***Cannabis Sativa L.***

2.2 Endocannabinoids System (ECS)

The Endocannabinoid System (ECS) is a complex lipidic transmission net localized in human system both in central and peripheral system. To this day, five endogenous ligands, able to interact with cannabinoid's receptors, have been discovered: AEA, 2-AG, 2-Arachidonoilglycerol ether (2-AGE), virodamin and N-arachidonoildopamin (Rodriguez de Fonseca et al., 2005). All of these compounds have a common structure, a portion of arachidonyl glycerol, that is condenced with an ethanol-amine, glycerol or dopamine.

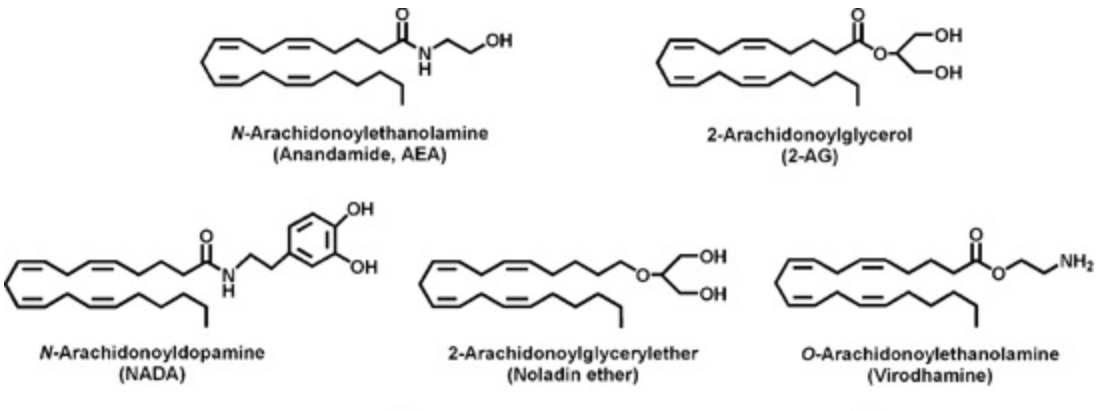


Fig 9: Chemical structure of endocannabinoids

Other ligands of cannabinoid receptors can be divided into: classical, non-classical and aminoalchylindols (Howlett, 2002). **Classic derivatives** includes benzopyraminic derivates, that are both compounds derives by Cannbis Sativa (fitocannabinoids) and their synthetic analogues. An example is Δ^9-THC, Δ^8-THC, and cannabidiol. Moreover, synthetic anologues are: 11-hydroxy-Δ^8 - THC-dimethylheptyl (HU-210), JWH-133, L-759633, L-nantradol and desacetyl-l-nantradol. **Non classical compounds** consist of bicycles and tricycles analogues of Δ^9-THC that link a pyranic ring; examples of these are: CP55940, CP47497 and HU-308. Class of **aminoalchylindols** includes compounds with a different structures of other

cannabinoids: WIN5521-2, AM1241, JWH- 015, BML190. (Howlett 2002; Pertwee, 1999; Porter, 2002).

AEA and 2-AG are the most important endocannabinoids both for their abundance in human body and for their capacity to bind cannabinoid receptor (AEA Ki CB_1 = 32 nM, Ki CB_2 = 1930; 2-AG Ki CB_1 = 472 nM, Ki CB_2 = 1400).

These two mediators are biosynthesized by different process (Sugiura et al., 2002), but both need Ca^{++} for their synthesis.

For the synthesis of AEA there are two different and independent strategy:

- formation of AEA started by arachidonyl acid and ethanolamine; this reaction is not specific and the ethanolamine react with several fatty acid presents at cellular level (Sugiura et al., 1996);
- formation of AEA started with the formation of *N*-arachidonoylphosphatydilethanolamine (NAPE) by a mechanism via transacylase, that needs Ca^{++}, and, then, by a NAPE-phospholipase D there are a release of *N*-acylethanolamine (NAE) and phosphatidic acid. NAPE synthesis is thought to occur through the action of an *N*-acyl transferase that catalyzes an exchange reaction between the *sn*-1 position of donor phospholipids and the primary amine of phosphatidylethanolamine (PE).

The biosynthesis of 2-AG is more complicated; several pathways are identified for the synthesis of this compound but no one is favoured over the others. This endocannabinoid is most frequently synthesized through the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) with arachidonic acid (AA) on the *sn*-2 position to diacylglycerol (DAG) by phospholipase C-β (PLC-β). The DAG is then hydrolyzed to 2-AG by diacylglycerol lipase (DAGL) (Stella et al., 1997; Bisogno et al., 1999; Bisogno et al., 2003).

AEA and 2-AG are transported by a common molecule: AMT, Anandamide member transporter, while their degradation is mediated by different enzyme.

AEA is metabolized by *fatty acid amide hydrolase* (FAAH), that is an enzyme which divide AEA in AA and ethanolamide. FAAH is an enzyme of serin-hydrolase family and it is composed by 579 amino acids of which Ser-241 is the catalytic nucleophilic site (Cravatt et al., 1996). This hydrolase metabolizes AEA, but it can, also, promote the synthesis of AEA despite this reaction needs a high quantity of that endocannabinoids (Schmid et al., 1998). AEA is, also, metabolized by another amino-hydrolase that acts in an acid environment instead of an alcalin environment in which acts FAAH (Ueda et al., 1999).

The main enzyme responsible for 2-AG hydrolysis is believed to be *monoglyceride lipase* (MAGL; also called *monoacylglycerol lipase*) (Drin et al., 2002; Saario et al., 2004). MAGL is a serine hydrolase with three catalytic essential amino acid residues: Ser-122, Asp-239 and His-269 (Karlsson et al., 1997). MAGL has been cloned from both human adipocytes and rat brain, it consists of 303 amino acids and has a molecular weight of ~33 kDa (Karlsson et al., 2001). This enzyme degrades 2-AG in AA and glycerol; MAGL is not the only enzyme degrading 2-AG, in fact, other metabolic pathways have been discovered. One of these, is the pathway that consisting in the degradation of 2-AG in 2-arachidonyl LPA; this way allows the recycling of 2-AG to form glycerol phospholipids, as phosphor-inositols (Shire et al., 1999). Another pathway outlook the degradation of both endocannabinoid by COX-2 in prostaglandins due to the fragment of arachydonoyl acid in their structure (Kozak et al., 2003).

After AEA and 2-AG production, these mediators are not preserved in vesicles but they are synthesized “on demand” by post-synaptic cells and function as retrograde signaling molecules, diffusing back across the synapse to bind with pre-synaptic CB_1 receptors, which reduces synaptic transmitter release (Stella et al., 1997). In the central nervous system,

endocannabinoids act as neuromodulators or retrograde messengers (MacDonald and Vaughan, 2001) which inhibit the release of various neurotransmitters (Schlicker and Kathmann, 2001); in the peripheral and neural tissues, they modulated the effects of proteins and nuclear factors involved in cell proliferation, differentiation and apoptosis, as paracrine or autocrine mediators.

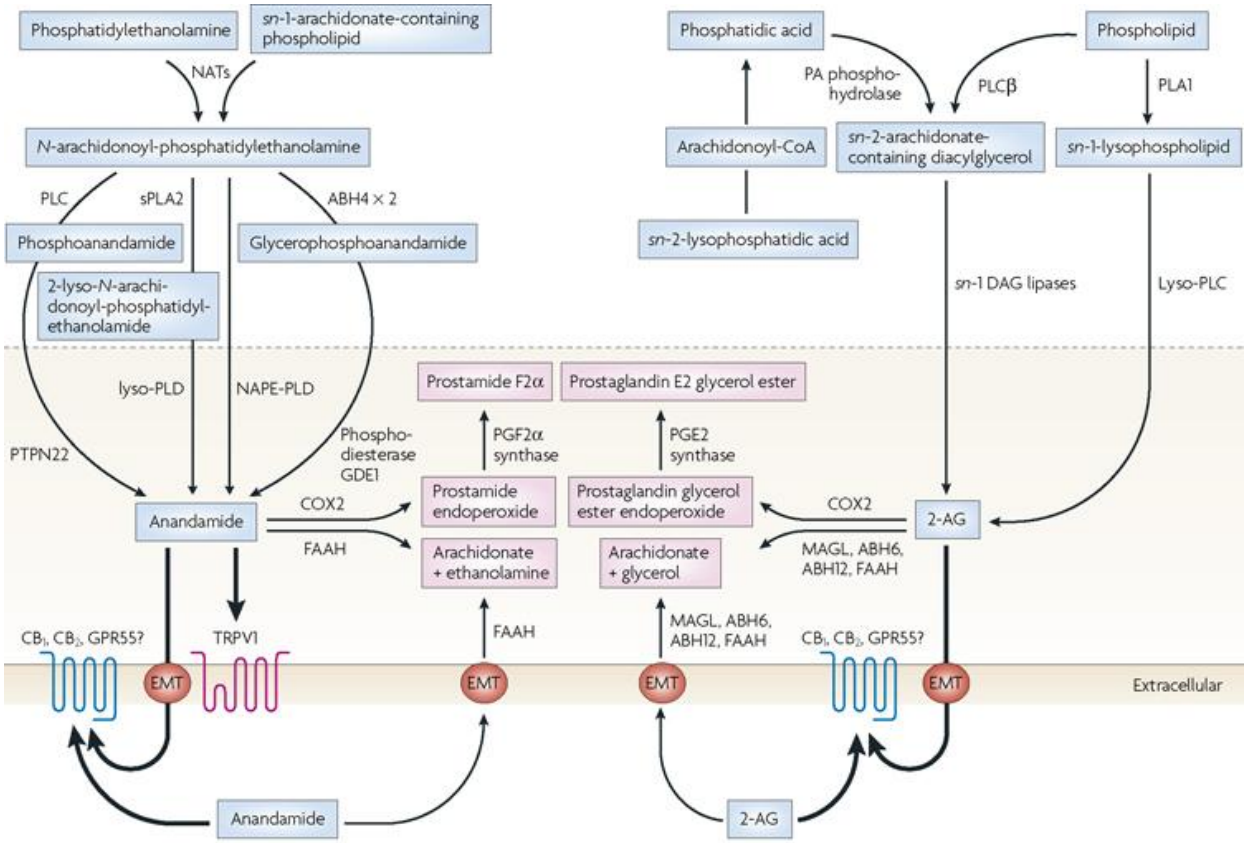


Fig.10: Biosynthesis, action and degradation of AEA and 2-AG.

Two receptor were cloned for cannabinoids: CB_1 and CB_2; both receptors belong to the family of the 'seven trans-membrane spanning receptors', and are coupled to G proteins, particularly those of the Gi/Go family (McAllister and Glass 2002; Mechoulam et al., 2002; Di Marzo, 1998).

CB_1 receptors are, mainly, localized in nervous system, bone marrow, but they are, also, in peripheral system as in endocrine and salivary glands, heart, gastrointestinal system. They are expressed in nervous system, principally, in that area responsible of movement and modulation of pain. Signal transduction pathways regulated by CB receptor-coupled G proteins include the inhibition of adenylyl cyclase, the regulation of ionic currents (inhibition of voltage-gated L-, N- and P/Q-type Ca^{++} channels, activation of K^+ channels), the activation of focal adhesion kinase (FAK), of mitogen-activated protein kinase (MAPK), of cytosolic phospholipase A2 and of nitric oxide synthase (NOS). Central and peripheral CB_1 receptors modulate appetite and energy metabolism, respectively. CNS receptors are expressed on hypothalamic and limbic neurons; those in the periphery exist on adipocytes, skeletal muscle cells and hepatocytes. Activation of peripheral CB_1 receptors promotes fat deposition and insulin resistance (Tibirica, 2010).

CB_2 receptors are, principally, localized on immune cells (mast cells, macrophages, B cells, T lymphocytes), spleen and tonsils. CB_2 is implicated in immune regulation Unlike CB_1 receptor, there is a considerable level of sequence variation CB_2 receptor among human, rat and mouse species, particularly when comparing rat and human sequences. There is 81% amino acid identity between rat and human CB_2, as compared to 93% amino acid identity between rat and mouse CB_2 (Griffin et al., 2000). Located on antigen-presenting cells, it influences their cytokine profile (Tanasescu, 2010). Its expression on microglia is upregulated in the dorsal root ganglia and spinal cord following sciatic nerve injury. It also may be expressed on neurons (Atwood et Mackie, 2010).

Recent studies have demonstrated that CB_2 is expressed also within the CNS and that this expression occurs during various states of inflammation (Nunezet al., 2004; Cabral et al., 2005; Fernandez-Ruizvet al., 2007). This expression of CB_2 has been localized primarily to microglia, the resident macrophages of the CNS. CB_2 expression is detected in these cells upon activation by various insults and stimuli, but measurable levels of CB_2 expression cannot be detected in resident, un-stimulated microglia. In addition, during neuro-inflammation, infiltrating immunocytes from peripheral non-neuronal sites that influx into the brain as a result of breakdown of the blood-brain barrier (BBB), contribute to the overall expression of CB_2 (Schatz et al., 1997; Ramirez et al., 2005).

Evidence has emerged that in addition to CB_1 and CB_2 receptors there are other molecular targets through which the endocannabinoids might induce a biological activity. In particular, a target of AEA which is attracting great interest is the type 1 vanilloid receptor (VR1), a 'six trans-membrane spanning protein' with intracellular N- and C-terminals and a pore loop between the fifth and sixth transmembrane helices (Jordt and Julius, 2002). It is, also, demonstrated that AEA might function as a modulator of orphan receptor, GPR55.

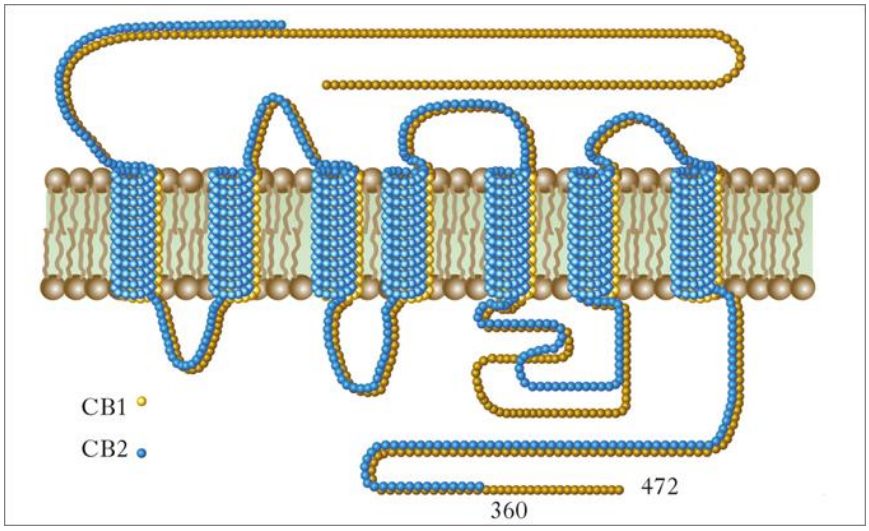


Fig. 11:Molecular structure of CB_1 and CB_2 receptors

2.3 Patho-physiological action of CBs

Several scientific works show the involvement of endocannabinoid system in various pathological conditions (Di Marzo, 2006). One of the first uses of Cannabis was as a painkiller; in fact, cannabinoid receptors are, abundantly, expressed in areas that are involved in the transmission and modulation of nociceptive stimuli, such as rostral ventromedial medulla (RVM), spinal cord dorsal horn, periaqueductal grey (PAG) (Lichtman et al., 1996). Cannabinoids, either natural, endogenous or synthetic, produce anti-nociceptive effects primarily through the activation of CB_1 receptors which, in these sites, are generally located presynaptically and so their activation inhibit the release of neurotrasmitters (Millan, 2002).

On the other hand, it is, also, conceivable that the analgesic/antinociceptive effect is due to the inhibition of the release of several mediators of pain or inflammation through the activation of CB_2 peripheral receptors (Walker et al., 2002; Calignano et al., 1998). CB_2 receptors have been related, traditionally, to the peripheral effects of cannabinoids (mainly related to modulation of the immunologic responses), but recent findings suggest that they also contribute to antinociception by inhibiting the release of proinflammatory factors by non-neuronal cells located near nociceptive neuron terminals. In fact, CB_2 receptors are expressed in several types of inflammatory and immunocompetent cells and their activation generates an antinociceptive response in situations of inflammatory hyperalgesia and neuropathic pain (Ibrahim et al., 2003; Valenzano et al., 2004). On the other hand, CB_1 receptors are also present in mast cells and may participate in some anti-inflammatory effects through inibition of their degranulation (Small-Howard et al., 2005).

It is, also, well know, that the use of cannabis increases the appetite. Several studies, have shown that both AEA and 2-AG, binding CB_1 receptor, directly, induce an increase in food intake (Kirkham et al., 2002). Treatment with CB_1 antagonists has shown that reduce body weight, and also it lowers the triglyceride concentration in the blood, increasing the

concentration of HDL in spite of LDL, decreased glucose tolerance by reducing the incidence of the metabolic syndrome (Despreset al., 2005).

Cannabinoids may also modulate intestinal motility *in vivo* through the activation of CB_1 receptors, thus its pharmacological modulation through the use of agonists could be useful for decreasing gut motility in intestinal bowel diseases, whereas its inhibition by antagonists can increase motility, acting so as a prokinetic (Aviello et al., 2008).

Phytocannabinoids resulted neuroprotective in several models of neurodegeneration and this effect has been correlated with their ability to reduce gliosis and neuroinflammation (Iuvone et al., 2004; Esposito et al., 2011) effects followed by an amelioration of mnemonic and motor performances in behavioural studies executed on *in vivo* models of Alzheimer's disease and Parkinson's disease.

2.4 Cannabinoid drugs in therapy

Medicines that activate cannabinoid CB_1 and CB_2 receptor are already in the clinic. These are Cesamet® (Nabilone), Marinol® (Dronabinol; Δ^9-tetrahydrocannabinol) and Sativex® (Δ^9-tetrahydrocannabinol with cannabidiol).

The first two of these medicines can be prescribed to reduce chemotherapy-induced nausea and vomiting. Marinol® can also be prescribed to stimulate appetite for AIDS patients, while Sativex® is prescribed for the symptomatic relief of neuropathic pain in adults with multiple sclerosis and as an adjunctive analgesic treatment for adult patients with advanced cancer (Pertwee,2009). Sativex® is a cannabis extract and contains about the same amount of THC and cannabidiol (CBD).

Another drug, based on cannabinoid ingredients, is Rimonabant,Acomplia®; it was the first in a new class of therapeutic agents called CB_1 blockers. Acomplia® was studied for use in the treatment of obesity and related conditions and it acts by selectively blocking CB_1 receptors

found in the brain and in peripheral organs important in glucose and lipid (or fat) metabolism, including adipose tissue, the liver, gastrointestinal tract and muscle (Pagotto et al., 2005). However, in clinical studies, Acomplia® was has been shown to improve a wide array of cardiometabolic risk factors as well as promoting sustained weight loss (Di Marzo, 2001; Despres et al., 2005), and, in 2009, Acomplia® was officially withdrawn by the European Medicines Agency (EMEA) due to the risks of dangerous psychological side effects, induce to suicid.

2.5 Cannabinoids and cancer

Many studies produced information about anticancer effect of cannabinoid-related drugs. Cannabinoids can play an important role in the palliation of pain, nausea, vomiting, and appetite for cancer patients. Plant-derived, synthetic, and endogenous cannabinoids, also, modulate tumour growth, apoptosis, migration and neo angiogenesis in various types of cancer (Bifulco and Di Marzo, 2002; Guzman et al., 2002) as prostate, breast, tyrode, leukemia and colon carcinoma.

Initially, it was shown a different expression of CB_1 and CB_2 receptors in normal and malignant cells. In fact, CB_1 is up-regulated in mantle cell lymphoma and in prostate cancer cells, while, CB_2 expression is increased in breast cancer (Caffarel et al., 2006). It was, also, found that CB_2 receptors increased in gliomas and the expression levels positively correlated with malignancy. Also, AEA and 2-AG were up-regulated in cancer; both these endocannabinoids were increased in their expression in human colorectal adenomatous polyps compared to normal mucosa (Ligresti et al., 2005). AEA was, also, overexpressed in prostate cancer, endometrial sarcoma, thigh histiocytoma (Schim et al.,2002) and in glioblastoma (Peterson et al., 2005). 2-AG was, on the other hands, increased in its expression in human

pituitary adenomas compared to normal pituitary glands (Pagotto et al.,2001), in meningiomas and in lung cancer (Peterson et al., 2005).

Despite that, the use of cannabinoid has been limited for its psychotropic effects; in 1975, for the first time, Munson and his collaborators showed anti-tumor effect of cannabinoids. They showed that Δ^9-THC and Δ^8-THC inhibited Lewis Lung adenocarcinoma cells growth both *in vitro* and *in vivo*. Then, other studies demonstrated anti-proliferative, anti-metastatic, anti-angiogenic and anti-apoptosic effect of cannabinoids in various cancer type (lung, glioma,thyrod, lymphoma, skin, pancreas, uterus, breast and prostate cancer) using *in vitro* and *in vivo* tumor models (Bergman et al., 2008; Godlewski et al., 2013). In 2002, a clinical study about Δ^9- THC, its safety profile and its anti-tumoral activity in nine patients affect of neuroblastomas, an aggressive brain tumor, was approved in Spain. This study demonstrated the safety of intracranic administration of Δ^9-THC and the reduction of tumoural growth; this effect was due to a reduction of proliferation and an increase of apoptosis, inducted by Δ^9-THC, in cancer cells. On the basis of these results, a new approach of cancer treatment is evaluated. In fact, in addition to this information, many scientific papers have been published about the involvement of Cannabinoid system in cancer disease.

Various cannabinoids, especially anandamide and THC, promote apoptosis of astrocytoma, glioma, neuroblastoma and pheochromocytoma cultured cells with a pathway involving cannabinoid receptors (Guzman, 2003; Goncharov et al, 2005; Velasco et al, 2007).

After interaction with the cannabinoid receptors, their ligand activate different signaling pathways, which can be involved in their anti-proliferative effects as p38, MAPK and c-Jun N-terminal kinase (JNK) activation, increased synthesis of pro-apoptotic sphingolipid ceramide and other stress-related genes expressed in the endoplasmic reticulum (Diaz-Laviada e Ruiz-Llorente, 2005). Ceramide, the central molecule of sphingolipid metabolism, generally average anti-proliferative responses, such as inhibition of cell growth, induction of

apoptosis and/or modulation of senescence (Saddoughi et al, 2008). Several therapeutic agents that induce apoptosis in ceramide-dependent cancer cells existed, and a number of enzymes involved in the metabolism of ceramide are starting to be recognized as potential targets for cancer therapy (Savtchouk et al, 2007; Carpinteiro et al, 2008). Recently, researches have shown that cannabinoids induce the accumulation of ceramide in tumor cells, that is linked to the pro-apoptotic effect of cannabinoids in cancer cells (Guzman et al, 2001; Velasco et al, 2005).

Finally, cannabinoids regulate cell survival pathways, modifying several signaling that are involved in tumoral cell growth and survival (Bifulco et al., 2008). The suggested mechanism underlying these effects, are complex specific, dependent by cancer type and include:

- induction of apoptosis in tumoural cells;
- anti-proliferative action by suppression of many mitogen signal;
- anti-metastatic and anti-proliferative action by inhibition of angiogenic process.

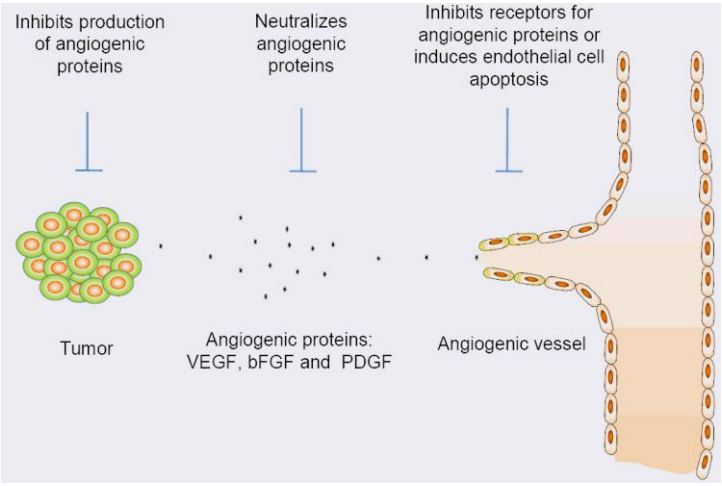


Fig. 12: Inhibition mechanism of tumoral growth by cannabinoids

2.6 Cannabinoids and angiogenesis

Recently, in addition to their anti-proliferative and pro-apoptotic effects, it has been shown that cannabinoids can affect other important processes in tumourigenesis, in particular angiogenesis.

In fact, another mechanisms involved in the pharmacological actions of cannabinoids during cancer is related to the inhibition of angiogenic process, which plays a key role in tumor growth. Numerous evidence suggests that antitumour effect of cannabinoid-related drugs could be, at least in part, ascribed to inhibition of tumour neoangiogenesis in animal models. Many cannabinoids, such as WIN-55,212-2, HU-210, JWH133 and THC, inhibit, *in vitro*, the survival of human umbilical vein endothelial cells (HUVEC) and their migration during angiogenetic process (Blazquez et al., 2003). Treatment with these cannabinoids reduces vascular density in experimental tumors (Blázquez et al., 2003; 2006; Casanova et al., 2003; Portella et al., 2003; Preet et al., 2008). It is ,also, suggested, that, anti-angigenic propriety of cannabinoids might be due to the reduction of the expression of several pro-angiogenic factors. Particularly, a lot of study demonstrated that VEGF expression was strongly influenced by cannabinoids; in fact, nude mice treated with tyroide cell Kras-transformed, have demonstrated a decrease of VEGF and VEGFR1 levels (Rak et al., 1995; Casanova et al., 2002) after treatment with Met-fluoro-anandamide (Met-F-AEA), a metabolic analogue of AEA. Anti-metastatic and anti-angiogenic effects are, also, ascribed to THC in non small cells lung cancer (NSCLC); in fact, after treatment with THC, lung cancer cells showed a minor expression of VEGF. In 2004, Blazquez, through cDNA arrays, demonstrated that JWH133, a full agonist of CB_2 receptor, was responsible of a reduced expression of VEGFA, VEGFB and hypoxic inducible factor 1 (HIF-1) in glioma in mice. In 2003, Casanova showed that JWH133 and WIN-55,212-22 were able to reduce mRNA levels and EGFR auto-phosphorilations, in skin tumors. In the same study, cannabinoids reduced the expression of

Ang-2 and PlGF. Treatment with WIN-55,212-2 or JWH- 133 caused impairment of tumor vascularization in skin cancer (Blazquez et al.; 2006). JWH133 is, also, able to reduce Ang-2 expression in gliomas and astrocytomas.

Cannbinoids exerts their anti-angiogenic effect not only on growth factors but also on proteolytic enzymes involved into matrix remodelling, such as MMPs. It is demonstrated that MMP-2 is down-regulated by THC in human tumoral samples of glioblastoma. The expression of this enzyme is also decrease, *in vitro,* by THC and met-AEA in uterus cancer and, *in vivo,* by JWH133 in xenograph models of gliomas. CBD induced endothelial cell cytostasis without inducing apoptosis, inhibited endothelial cell migration, invasion and sprouting *in vitro* and inhibited angiogenesis *in vivo*. These effects were associated with a down-modulation of several molecules associated with angiogenesis, including MMP-2 and MMP-9, ET-1, platelet-derived growth factor-AA (PDGF-AA) and chemokine (c-x-c motif) ligand 16 (CXCL16) (Massi et al., 2008). Finally, Pisanti and his colleagues, in 2007, demonstrated an inhibition of MMP-2 in endothelial cells incubated with Met-F-AEA. On the contrary, the influence that cannabinoids have on anti-angiogenic factors, such as tissutal inhibitors of MMPs (TIMPs) is, rather controversial. There are few evidences that the treatment with THC reduces the expression of TIMP in human cell line of glioma (Blazquez et al., 2008), while CBD inhibits invasion of A549 cells both *in vitro* and *in vivo,* in lung cancer, that was accompanied by upregulation of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) (Massi et al.; 2008).

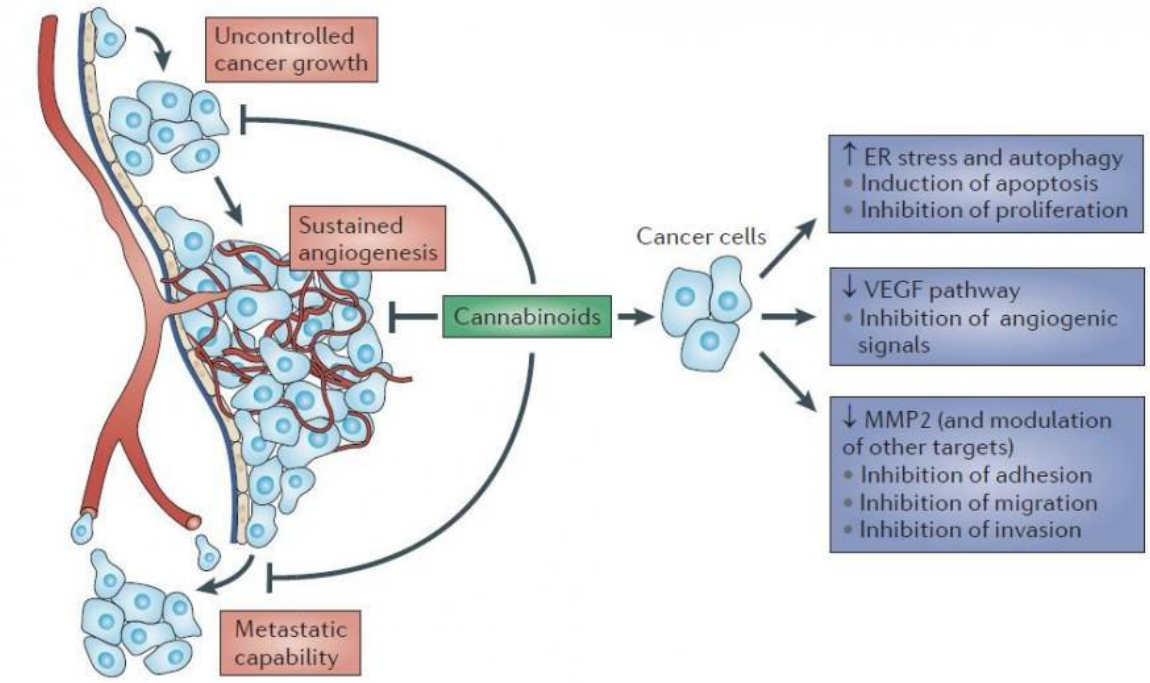


Fig. 13: Inhibition mechanism of angiogenis by cannabinoids

In addition to the anti-angiogenic effects of CBs in tumor, several evidences suggest that CBs are able to control not only the angiogenic process associated to tumoral conditions. In fact, cannabinoids may prevent, at least in part, granuloma-associated angiogenesis, controlling mast cells function (De Filippis et al., 2008). Chronic inflammatory conditions, such as granulomas, are associated with angiogenesis; selective cannabinoid receptor agonists in a model of angiogenesis-dependent granuloma formation induced by λ-carrageenin in rats, exhibited antiangiogenic properties, in fact it was demonstrated that these cannabnomimetic compound were able to decrease neovascularization in the granulomas (De Filippis et al., 2008).

3. Material and methods

3.1 Animal models

3.1.1 Animals

Female mice (6-8 weeks) C57BL/6 were obtained from Charles River (Wilmington, MA,USA) and used for the study. We, also, used female mice (6-8 weeks) CB_2 KO obtained from Charles River. Animals were provided with food and water *ad libitum*. The light cycle was automatically controlled (on 07 h 00 min; off 19 h 00 min) and the room temperature thermostatically regulated to 22±1 °C with 60±5% humidity. Prior to the experiments, animals were housed in these conditions for 3–4 days to become acclimatized.

Fig.14: C57BL/6 mouse

3.1.2 Experimental protocol

The tumour model we have used involves the induction of lung carcinoma in mice; briefly, Lewis Lung Carcinoma cells (LLC1; $2.5X10^5$), that have a high tropism for the lung, were intravenously injected in C57BL/6 mice (Sorrentino et al., 2010). Animal model of lung carcinoma will performed in collaboration with the group of Prof. Aldo Pinto, University of Salerno, that possess a demonstrated expertise in the field.

JWH 133 (0.5, 1, 5 μg/mouse), a CB_2 full agonist, and JZL184, a MAGL inhibitor (5, 10, 20 μg/mouse), from 13^{th} to 17^{th} day after cell implant, were intraperitoneally administered daily. At the 17^{th} day the mice were sacrificed and the left lung lobes were fixed in OCT medium and the 7-μm cryosections cutted were used for immunoistological analysis to measure the tumour burden, while the right lung lobes were homogenized for biochemical analysis to evaluated angiogenesis.

3.2 Drug

The compounds that we used are:

(6aR,10aR)-3-(1,1-Dimethylbutyl)-6a, 7, 10, 10 a-tetrahydro-6, 6, 9- trimethyl-6H-dibenzo[b,d]pyran (JWH133 in Tocrisolve™ 100) (TOCRIS, Ellisville MO): a potent CB_2 selective agonist (K_i = 3.4 nM), dissolved in water-soluble emulsion Tocrisolve^{TM}100 composed of a 1:4 ratio of soya oil/water that is emulsified with the block co-polymer Pluronic F68. The cannabinoid is incorporated with the emulsion as part of a specialized innovative production process. The soya oil forms a coating around the cannabinoid molecule, and the block co-polymer is added to stabilize the emulsion, preventing the lipid droplets from coalescing (merging) in the surrounding water. JWH133 is, approximately, 200-fold selective over CB_1 receptors.

4- nitrophenyl-4-(dibenzo [d] [1,3] dioxol–5–yl (hydroxy) methyl) piperidine-1- carboxylate (JZL184, TOCRIS, Ellisville MO): a potent and selective monoacylglycerol lipase (MAGL) inhibitor. Blocks hydrolysis of the endocannabinoid 2-arachidonyl glycerol (2-AG). JZL184 was dissolved in DMSO, obtained a stock solution and then, by serial dilutions, we used the appropriated concentration.

3.3 Cell culture

3.3.1 Lewis Lung carcinoma cells

Lewis Lung carcinoma cells (LLC1) (ATCC® CRL-1642™) were cultured in DMEM containing 10% FBS, L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 µg/ml) in an atmosphere of 5% CO_2 at 37 °C. Cells seeded in 96 multiwell plates ($1.5X10^5$/well) were treated with JWH133 (dissolved in ethanol) 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M. After 24 hours incubation time, cells were subjected to different assay to evaluate viability and proliferation.

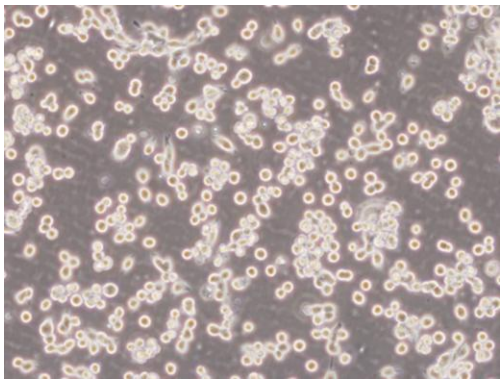


Fig.15: Lewis lung carcinoma cells

3.3.2 Cell Functionality Assay

To determinate cells functionality, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-Formazan Assay (MTT assay) was performed. Cells were plated at the density of ($1.5X10^5$/well), left to adhere at 37 °C, and then treated as described previously. After 24-h incubation time, 25µl of 5g/L 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium solution were added to the cells. Cells were incubated for an additional 3 h at 37 °C before being lysed, and then dark blue crystals were solubilized with 100 µl of a 50% N,Ndimethyl formamide/20% sodium dodecyl sulfate solution with an adjusted pH of 4.5. Optical density

(OD) was measured with a spectrophotometer (Titertek Multiskan MCC/340; Titertek Instruments, Huntsville, AL) equipped with a 620-nm filter.

3.3.3 Cell Proliferation Assay

To determine the effect of treatment of JWH133 on LLC1 cellular proliferation, Cell Proliferation ELISA BrdU assay (Roche Applied Science, Monza, Italy) was used. The assay is based on the measurement of BrdU incorporation during DNA synthesis. Cells were seeded ($1.5X10^5$/well) in 96 wells plates and cultured for 24 hours. JWH133, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M, were added for 24 hours. One control was performed:

blank: has to be performed in each experimental setup. The blank provides information about the unspecific binding of BrdU and anti-BrdU- POD conjugate to the plate. The absorbance value obtained in this control has to be subtracted from all other values.

During this labelling period, the pyrimidine analogue BrdU was incorporated in place of thymidine into the DNA of proliferating cells. After removing the culture *medium* the cells were fixed and the DNA was denaturated by adding nuclease for 30 minutes at room temperature. This step was essential to improve the accessibility of the incorporated BrdU for detection by the antibody. The anti-BrdU-POD was added to cells for 90 minutes at room temperature. It bound to the BrdU incorporated in newly synthesized endothelial cellular DNA. The immune complexes were detected by the subsequent substrate (TMB) reaction. The reaction product was quantified by measuring the absorbance at the respective wavelength using Microplate Autoreader at 405nm, reference wavelength 490nm.

3.4 Biochemical Evaluation

3.4.1 Western blot Analysis

Western Blot analysis have been performed on samples of homogenised lung. Briefly, frozen samples of lung were de-frozen and than tissue lysed in 150 µl of ice-cold hypotonic lysis buffer and incubated on ice for additional 45 min. The total protein extract was obtained by centrifugation at 14,000 rpm for 10 min at 4°C.

Samples (50 µg/mL) were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred onto nitrocellulose membrane and incubated with one of the following antibodies: rabbit anti-CB_1 (1:500 v/v; Cayman Chemical, Michigan, USA), rabbit anti-CB_2 (1:200 v/v; Cayman Chemical, Michigan, USA), rabbit anti-FAAH (1:200 v/v; Cayman Chemical, Michigan, USA), rabbit anti-MAGL (1:250 v/v; Cayman Chemical, Michigan, USA). Appropriate peroxidase-conjugated secondary antibodies (1:2000 v/v; PerkinElmer Massachusset, USA) were used, and proteins were visualized using an enhanced chemiluminescence kit (PerkinElmer Massachusset, USA). Protein expression was quantified by densitometric analysis of the acquired images by ImageQuant 400 (GE Healthcare) and a computer program (Quantity One, Bio-Rad, Hercules, CA).

3.4.2 ELISA assay

For the quantitative determination of mouse Vascular Endothelial Growth Factor (VEGF) concentrations in Quantikine® mouse VEGF Immunoassay (R&D Systems, Minneapolis, MN) was used. VEGF levels were determined in tumor extracts, obtained by homogenization, by solid phase ELISA using an immunoassay for mouse samples. This assay employs the quantitative sandwich enzyme immunoassay technique. Briefly, samples were stored at lower -20°C temperature until use. After adding 50µl of Assay Diluent RD1N, 50µl of standards (composed of dilution of mouse VEGF standard from 500pg/ml to 7.8 pg/ml; minimum

detectable dose ranged from 3.9-25 pg/ml according to manufacture data sheet), control (given with kit) and samples were added to the pre-coated plate.

The plate was covered and incubated two hours at room temperature on a horizontal microplate shaker. Five washings were performed with 400µl Wash Buffer to ensure the removal of any unbound substances. 100µl of Mouse VEGF Conjugate were added to each well and the plate was incubated for one hour at room temperature on the shaker. Five washings were performed and 100µl of Substrate Solution were added to each well. After 30 minutes of incubation at room temperature on the bench top protected from light, 100µl of Stop solution were added to each well, gently tapping the plate to ensure thorough mixing. The optical density of each well was determine within 30 minutes, using the Microplate Autoreader ELISA (BioRad) set to 450 nm, reference wavelength 570nm. Standard curve and quantitative analysis were obtained using the specific Microplate Autoreader Software.

3.4.3 Mouse Angiogenesis Array Kit/Proteome Profiler™

To analyze the expression profiles of tumor-related proteins we used the Proteome Profiler™ Mouse Antibody Array Kit (R&D Systems, Abingdon, UK), according to the Manufacturer's instructions. This kit uses an array of 53 specific antibodies directed at proteins involved in tumor angiogenesis, spotted onto a nitrocellulose membrane. Tissue lysates were centrifuged and mixed with 1.5 ml of biotinylated detection antibody for 1 h at room temperature. Then, the membranes were incubated with the sample/antibody mixtures overnight at 4°C on a rocking platform. Following a washing step to remove unbound material, streptavidin–horseradish and chemiluminescent detection reagents were added sequentially. Data on developed X-ray film were quantified by densitometric analysis with a computer program (Quantity One, Bio-Rad, Hercules, CA). An averaged signal from the positive controls of each membrane was subtract from each protein spot.

3.5 Histological Investigations

3.5.1 Hematoxylin and Eosin (H&E) staining

Left lung lobes were fixed in OCT (optimum cutted temperature) medium (Pella, Milan, Italy), and 7-μm cryosections were cut. H&E staining was performed and used to measure the tumor burden. Tumor lesions were analyzed by using serial lung cryosections and expressed as tumor lesions/lung (area mm^2), as determined by the ratio of the tumor lesions area compared with the total lung area (Image J Software, National Institutes of Health, Bethesda, MD).

3.5.2 Blood vessels Evaluation

Lung was, previously, perfused by intra-tracheal injection of formaldehyde 4%. Paraffin sections (7 μm) from lung lobes were deparaffinised and endogenous peroxidase activity was blocked by incubating with 0.3% H_2O_2 following antigenic recovery. The sections were incubated with the primary antibody against CD31 (1:100; Novus Biologicals, Cambrige, UK), diluted in PBS. CD31/ PECAM-1 is an angiogenic marker; it plays a role in adhesive interactions between adjacent endothelial cells, as well as between leukocytes and endothelial cells. The binding of CD31 to the surfaces of leukocytes results in the increase of functional leukocyte integrins and in the diapedesis across the endothelium. CD31 is expressed in all continuous endothelia, including those of the arteries, arterioles, venules, veins and not sinusoidal capillaries, but is not expressed on the discontinuous endothelium, for example in the splenic red pulp. In addition, CD31 is expressed diffusely on the surfaces of megakaryocytes, platelets, myeloid cells, natural killer cells and certain subsets of T cells, as well as B cells precursors (Muller et al., 1997).

Sections, incubated with isotype matched antibodies, were used as negative controls. Subsequently, sections were incubated with biotinylated anti-rabbit secondary antibody

(1:200, Dako, Denmark), washed and incubated for 5 min with streptavidin–HRP (1:200; Sigma-Aldrich, Milan, Italy). Finally, exposed to diaminobenzidine chromogen with haematoxylin counterstain. The slides were then dehydrated and mounted in Entellans medium. Images were acquired with Leica DFC320 video camera (Leica, Italy) connected to the microscope (Leica, DMRB) using the Leica Application Suite software V2.4.0.

3.6 Statistical Analysis

Results are expressed as the means ± S.E.M. of *n* experiments. Statistical significance was calculated by one-way analysis of variance (ANOVA) and Turkey-corrected P-value for multiple comparison tests. The level of statistically significant difference was defined as $P<0.05$. Linear associations between variables were assessed by the use of standard-least-square linear regression. Correlation coefficient (r) was presented as measure of linear association for regression relationship.

4. Results

4.1 Deregulation of ECS during tumor growth

4.1.1 Evaluation of ECS expression during tumor progression

In lung tumoral tissue, from 3th to 13th after LLC1 implantation, analysis of Western blot exhibited significant increase about the expression of FAAH levels, in tumoral tissues. In parallel with the FAAH expression, it was observed, also, a significant increase of levels of MAGL expression, in tumoral tissues.

Our experiments showed, from 13th days, an increase of FAAH and MAGL expression in the second tumoral phase, a phase of tumoral consolidation/progression.

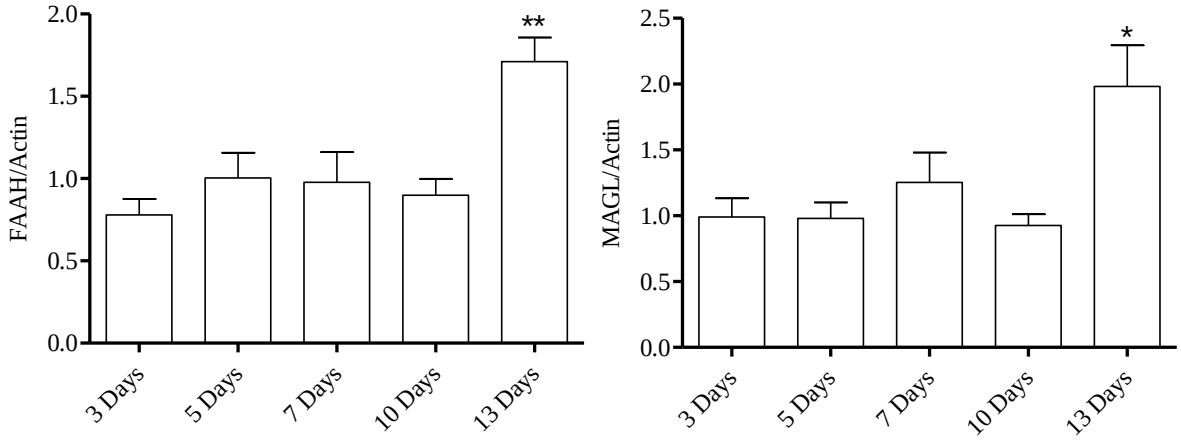


Fig. 16: FAAH and MAGL expression in lung cancer: Western blot analysis show that the expression of FAAH and MAGL, was significantly increased in lung of mice injected with LLC cells (13 days) mice. Results are expressed as mean value ± S.E.M. **P*<0.05; ***P*<0.01 *vs* 3 days.

Moreover, we, also, investigated the expression profile of the main cannabinoids receptor: CB_1 and CB_2.

Western blot analysis, showed a selected increase in CB_2 expression in tumoral tissues, 15^{th} days after tumor induction; while CB_1 receptor expression held steady in this advanced phase.

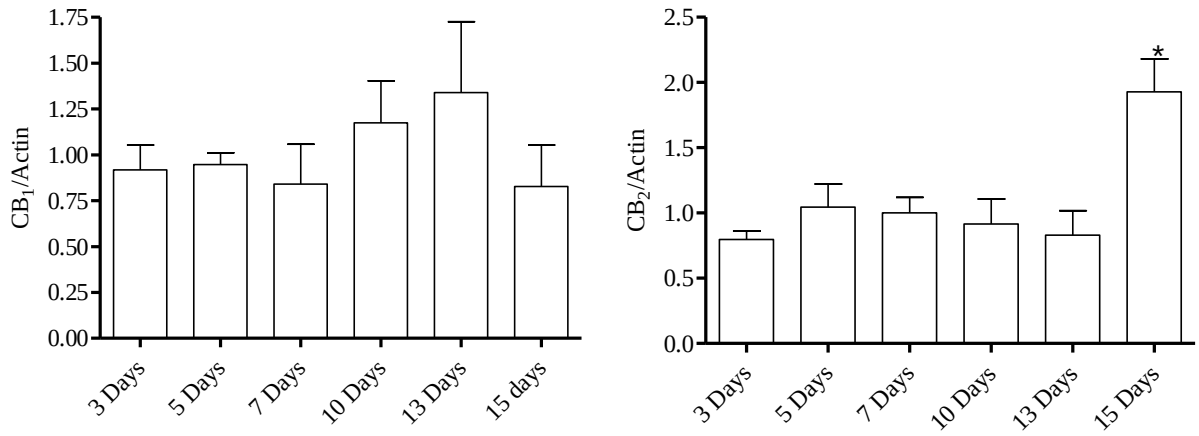


Fig. 17: CB_1 and CB_2 receptor expression in lung cancer: Western blot analysis show that the expression of CB_2, but not CB_1 receptor, was significantly increased in lung of mice injected with LLC cells (15 days. Results are expressed as mean value ± S.E.M. **P*<0.05 *vs* 3 days.

4.2 Effect of CB_2 agonist on angiogenesis during cancer process

4.2.1 Effect of JWH133 on tumor burden

Tumor burden was evaluated on tissue small slices (7 µm) stained with H&E coloration. Tumor burden was normalized in relation with tumoral mass area (mm^2).

JWH133 (0.5, 1, 5 µg/mouse i.p.) was administered, daily, in mice, from 13^{th} to 17^{th} days after cells implant. It was observed that the treatment with JWH133, significantly reduced the tumoural area in lung carcinoma mice as compared to PBS treated mice.

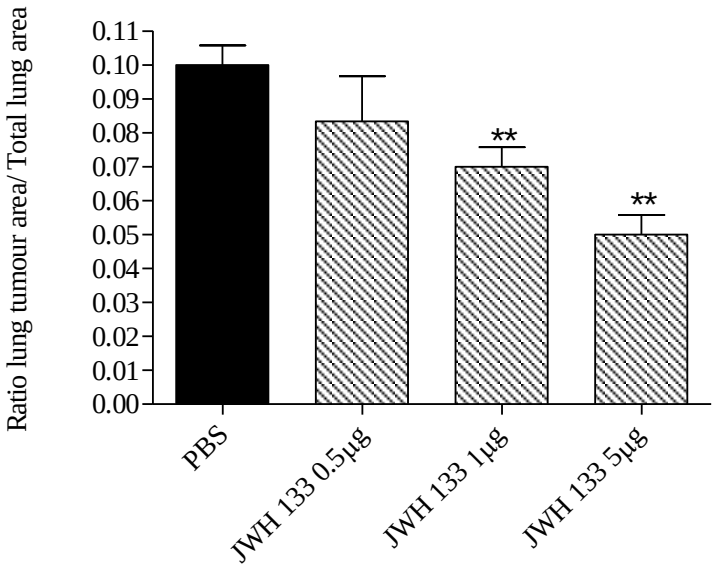


Fig.18: Effect of JWH 133 on tumor-burden. Treatment with JWH133 significantly reduced the tumor area in LLC-implanted mice compared to PBS, 17 days after cell implantation. Results are expressed as mean value ± S.E.M. $^{**}P<0.01$ *vs* PBS.

4.2.2 Effect of JWH133 on VEGF expression in lung

To evaluate if the reduction of tumoural mass was paralleled with a decrease of angiogenic process, we measured VEGF levels, since it has a key role in the process of new blood vessels formation. VEGF levels expression was evaluated by ELISA assay.

ELISA assay revealed that the administration of JWH 133 (0.5-5 µg/mouse), significantly, decreased VEGF expression in lung carcinoma, in confront to PBS treated mice.

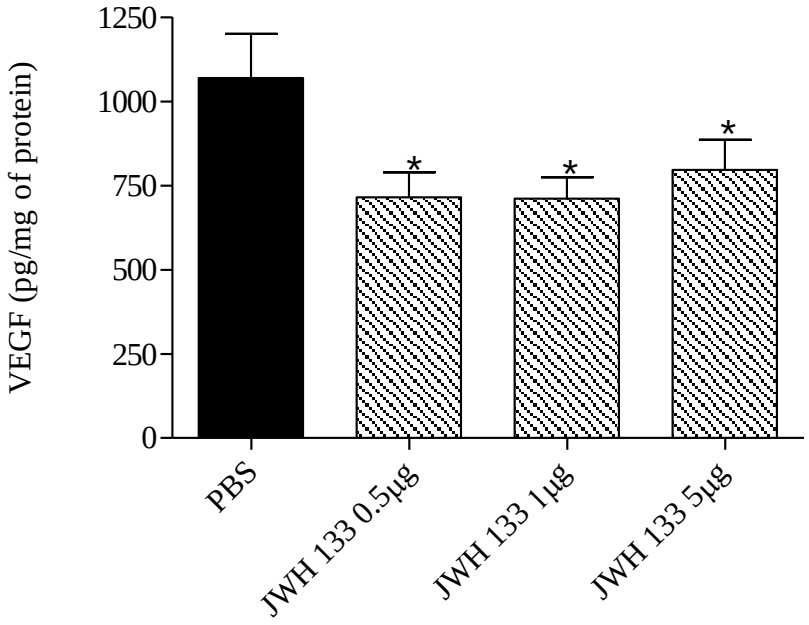


Fig.19: Effect of JWH 133 on VEGF expression in lung cancer. ELISA assay revealed that the treatment with JWH 133 (0.5-5 µg/mouse) significantly reduced VEGF level in the lung of tumor-bearing mice compared to PBS, 17 days after cell implantation. Results are expressed as mean value ± S.E.M. *P<0.05 *vs* PBS

4.2.3 Effect of JWH133 on 53 angiogenic markers expression in lung

We decided to performe a Proteome Profiler™ array; a multiarray useful to evaluated, contemporarily, the expression of 53 proteins involved in angiogenic process. In lung, analysis of these proteins revealed a decrease of numerous pro-angiogenic mediators; in particular, treatment with JWH133 (1 µg/mouse) evidenced a decrease of CXCL16, MMP-9, MMP-3, IGFBP-2, IGFBP-3 in confront to PBS treated mice (data not show). Moreover, another experiment, with JWH133 at the dose of 5 µg/mouse, evidenced the reduction of expression of a greater number of these mediators (Angiopoietins, CXCL16, MMP-9, MMP-3 SDF-1, Endoglin, HGF, IGFBP-2, IGFBP-3, Osteopontin) in treated mice compared to un-treated mice.

(A)

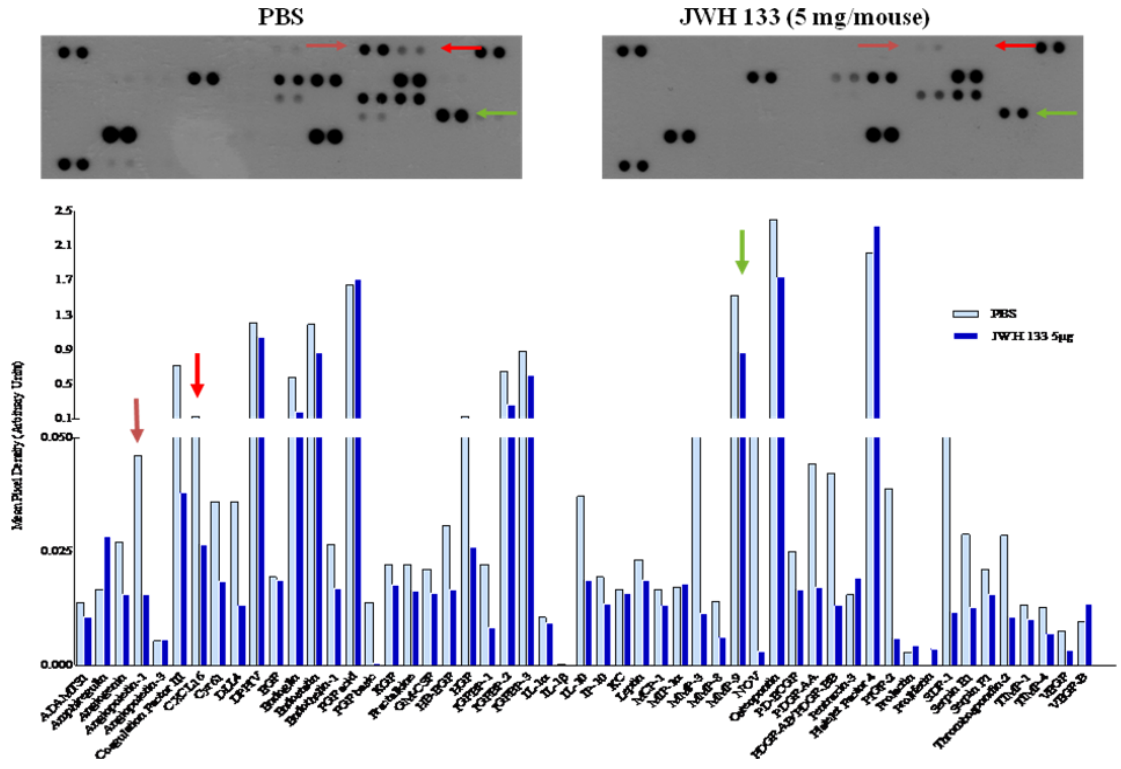


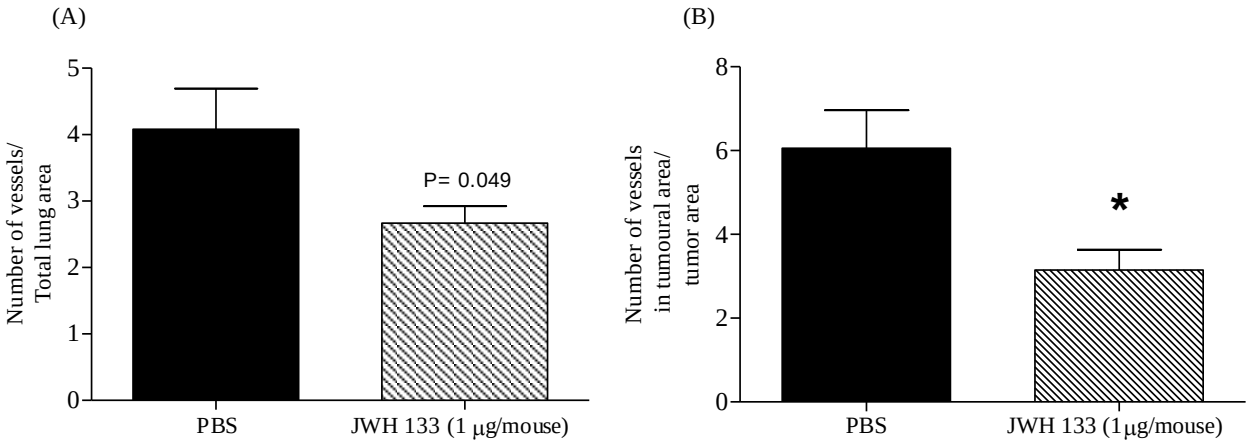
Fig.20: Effect of JWH 133 on angiogenic markers expression.Proteome Profiler™ array. (A) Representative images of two minutes exposed X-Ray film; (B) Densitometric analysis of each angiogenesis related protein. Data are expressed as average of pairs of spots.

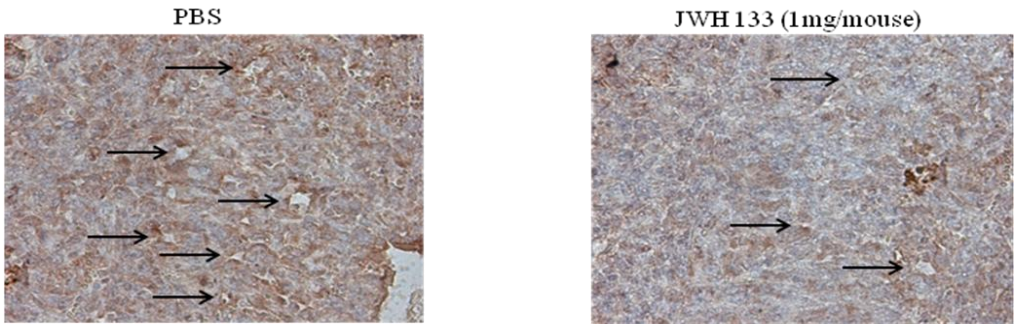
4.2.4 Effect of JWH133 on vessels number in lung carcinoma

An additional proof about the angiogenic effect of JWH133, was achieved by the count of the numbers of vessel in total lung area and in tumoral lung area. Starting to the assumption that, after release of VEGF, angiogenic process prosecutes with its binding to the endothelial cell receptor, evaluation of the expression of CD31, as a marker of endothelial cells and therefore of blood vessels, were performed.

Immunochemistry analysis evidenced that the treatment with JWH133 (1 μg/mouse), does not reduce the number of blood vessels number in total lung, compared to un-treated mice. On the other hand, it was evaluated tumoral angiogenesis counting the number of vessels only in tumoural mass. In line with previously experiments, JWH133, at the dose of 1μg/mouse, reduced, significantly, the number of tumoral vessels/mm^2 in confront to mice that received only PBS.

Finally, were counted the number of micro-vessels in tumoral mass; our data suggested that treatment with JWH133 (1μg/mouse) reduced neo-angiogenesis, as revealed by a reduction of micro-vessels.





(C)

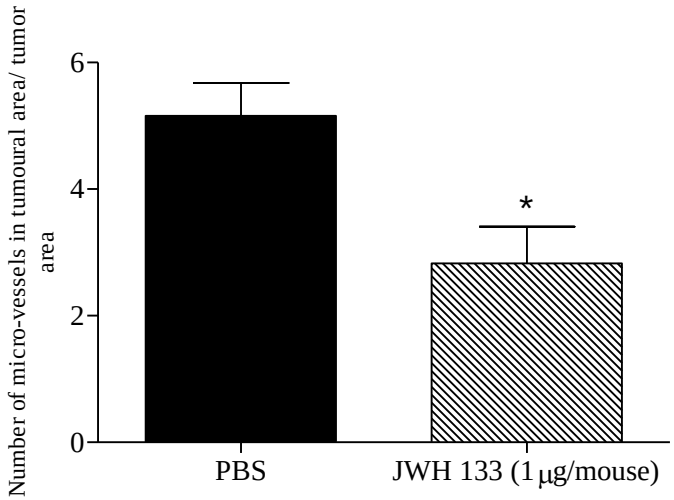


Fig.21: Effect of JWH133 on vessels number in lung carcinoma. Immunochemistry analysis evidence that treatment with JWH133 significantly reduced number of blood vessel in (A) total lung area and in (B) tumoral lung area. Moreover, JWH133, also, reduce the (C) number of micro-vessel in tumoral lung area. Results are expressed as mean value ± S.E.M. **P*<0.05 *vs* PBS.

4.3 Effect of JWH133 on LLC1 viability and proliferation *in vitro*

In order to clarify JWH133 action, distinguishing between a direct action on tumoral cells viability or on surrounding cells, *in vitro* experiments were performed.

First of all, to evaluate JWH133 effect, on LLC1 viability, MTT assay was conducted. Confluent LLC1 cells were treated with JWH133 (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M) and MTT assay was performed after 24 hours of incubation. MTT assay showed any significant variation in cells viability in LLC1 treated compared to un-treated cells.

In parallel, LLC1 stimulated with JWH133 (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M) for 24 hours, revealed any variation in their proliferation, in confront to un-treated cells as revealed by BrdU assay.

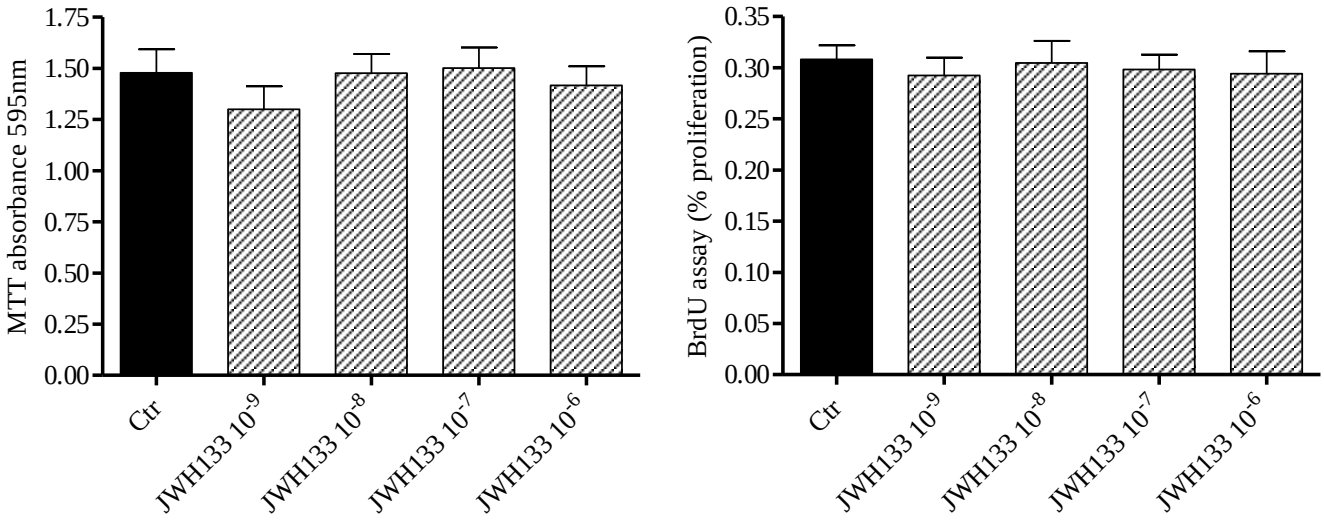


Fig.22: **Effect of JWH133 on LLC1 viability and proliferation.** MTT and BrdU assay showed any significant variation in cells viability and proliferatin.

4.4 Effect of JWH133 on tumor burden and on VEGF expression in CB_2 KO mice

To confirm the role of CB_2 receptors in the modulation of lung cancer, CB_2 knockout mice have been used.

The administration of JWH133 in CB_2 KO mice, revealed any significant variation on tumor burden of treated mice compared to PBS treated mice.

Tumor burden of treated mice is comparable to tumor burden of un-treated mice. Moreover, also the expression of VEGF levels resulted unvaried; in fact in lung of mice treated with JWH133 (5 µg/mouse) there was no change in expression of VEGF levels in confront to mice treated with PBS.

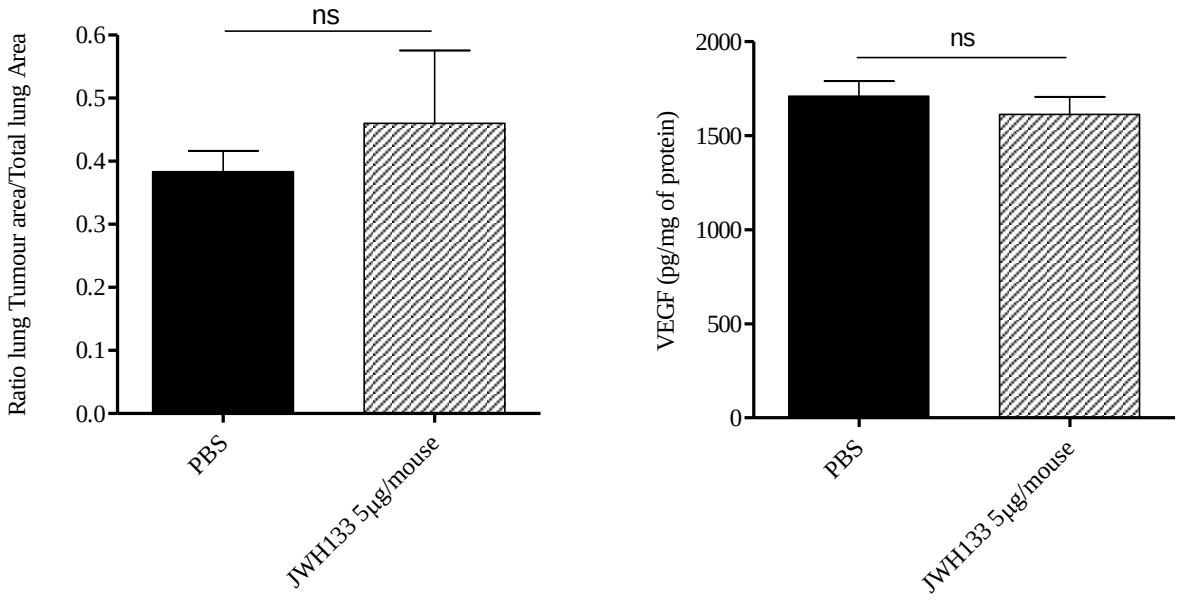


Fig.23: Effect of JWH133 on tumor burden and on VEGF expression in CB_2 KO mice. JWH133 treatment have no effect on these parameters in CB_2 KO mice.

4.5 Effect of MAGL inhibitor on angiogenesis

4.5.1 Effect of JZL184 on tumor burden

To validate the pharmacological activity of cannabinoids on the control of tumoral condition, we used a different strategy with MAGL enzyme inhibitor, JZL184..

In line with the experiments performed with CB_2 agonist, mice treated with JZL184 (5, 10, 20 μg/mouse), daily from13th to 17th, showed a, significant and dose-dependent , reduction of tumor burden in confront to PBS mice.

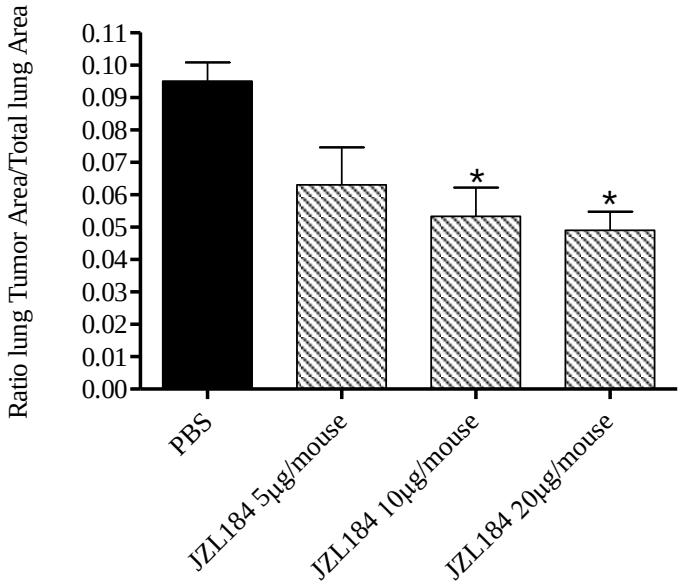


Fig.25: Effect of JZL184 on tumor-burden. Treatment with JZL184 significantly reduced the tumor area in LLC-implanted mice compared to PBS, 17 days after cell implantation. Results are expressed as mean value ± S.E.M. **P*<0.05 *vs* PBS.

4.5.2 Effect of JZL184 on VEGF expression in lung

In order to evaluated if JZL184 action on tumor burden was linked to its activity in modulating angiogenic process, ELISA assay for VEGF have been performed.

Our data reported a significant decrease of VEGF content in lung of mice treated with JZL184 20µg/mouse, compared to mice treated with PBS.

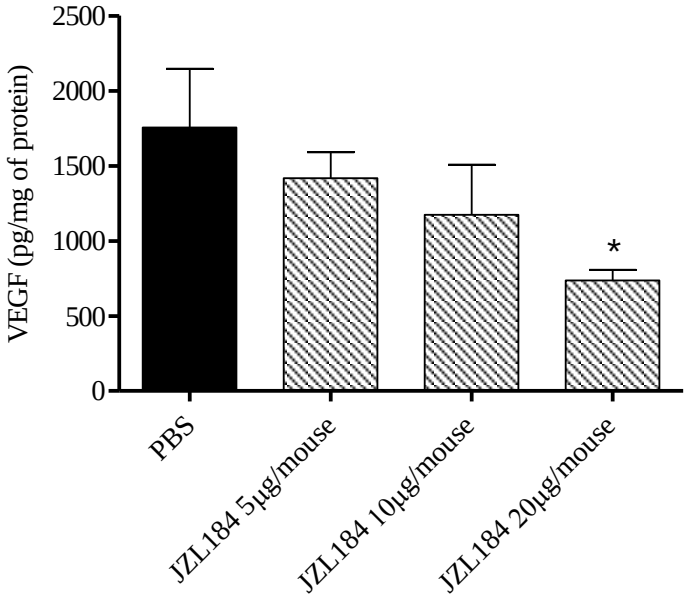


Fig.26: Effect of jzl184 on VEGF expression in lung cancer. ELISA assay revealed that the treatment with JZL1884 (0.5-5 µg/mouse) significantly reduced VEGF level in the lung of tumor-bearing mice compared to PBS, 17 days after cell implantation. Results are expressed as mean value ± S.E.M. **P*<0.05 *vs* PBS

4.5.3 Effect of JZL184 on 53 angiogenic *markers* expression in lung

Last, in order to strengthen anti-angiogenic activity of JZL184, a Proteome Profiler^{TM} array has been executed. This test showed a reduction of several pro-angiogenic factors, in mice treated with JZL184 (20µg/mouse) compared to untreated mice; these data suggested that the decreased tumor mass by JZL184 treatment may be caused by a decrease of expression of several angiogenesis-related proteins.

(A)

(B)

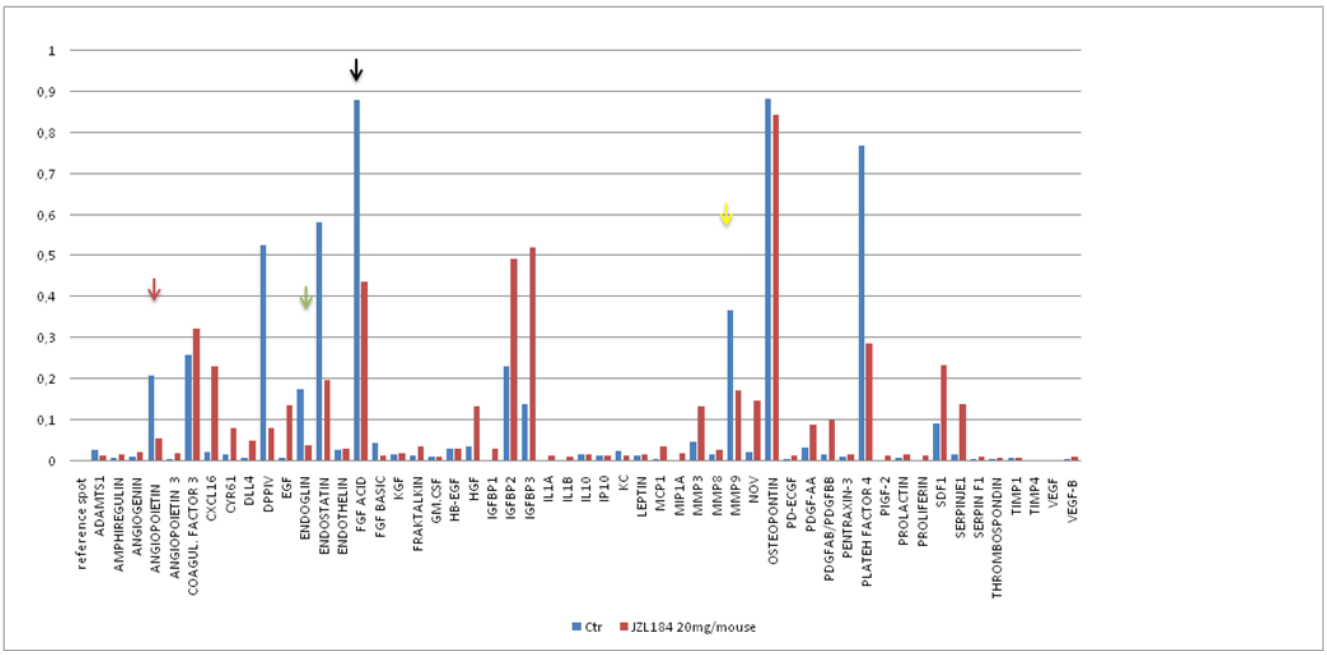


Fig.26: Effect of ZL184 on angiogenic markers expression.Proteome Profiler^{TM} array. (A) Representative images of two minutes exposed X-Ray film; (B) Densitometric analysis of each angiogenesis related protein. Data are expressed as average of pairs of spots.

5. Discussion

In 1976 for the first time, Cannabis derivates have been proposed as a potential pharmacological strategy for treating lung carcinoma (White et al., 1976). Since then, several studies have reinforced this hypothesis, although a possible in the mechanism of action of Cannabis derivates was not completely understood yet. Lung carcinoma represents, nowadays, the main cause of cancer-related death (Torre et al., 2015). The mechanisms promoting transformation and tumor growth are different but recent guidelines identify, in tumor angiogenesis, one of the main responsible. Particularly in lung, a well vascularized tissue, the inhibition of angiogenic process represents an useful target to control tumor growth (Kim and Murren, 2002).

Based on these evidences, aim of this thesis, was to investigate the role of endocannabinoid system (ECS) and its effect in a model of lung carcinoma in mice, focusing the attention on its possible role on angiogenic process.

In order to study the ECS deregulation, we evaluated the expression of the principal enzymes involved in endocannabinoids (eCBs) metabolism (FAAH, MAGL) and the expression of the main eCB receptors (CB_1, CB_2). We found a strong deregulation of ECS in the second phase of tumor growth; in particular, we found an increase of eCBs degradation enzymes and a selective increase of CB_2 receptor expression in tumor mass.

In order to clarify the role of CB_2 over-expression, we tried a pharmacological approach with JWH133, a CB_2 full agonist.

Several evidences reported in preclinical studies show that, JWH133 had anti-tumoral effects; in fact this molecule caused inhibition of AKT-Regulate COX-2/PGE_2 signaling pathway

promoting apoptosis in breast cancer (Caffarel et al., 2010); moreover, JWH133 induced G1 cell cycle arrest on melanoma cells, through the inhibition of p-Akt (Blazquez et al., 2006).

In our experiments, the treatment with JWH133, during the progression phase of tumor, significantly reduced tumor burden. Similarly, we found that the treatment with JWH133 was able to decrease the content of VEGF, the key molecule in the process of new blood vessels formation. The importance of VEGF in tumor growth is confirmed by the recent approval, in cancer therapy, of Bevacizumab (Avastin, Roche), an humanize monoclonal antibody direct on VEGF (Presta et al., 1997). The treatment with this antibody, in association with the classical chemotherapy based on platinum, showed a significant amelioration of lung carcinoma affected patients survival (Sandler et al., 2006).

In our experiments the effect of JWH133 was not limited to VEGF content reduction, but it involved other pro-angiogenic mediators as revealed by the oligoarray of 53 mouse angiogenesis related proteins, many of which (i.e angiopoietin1, chemokines as CXCL16 e SDF-1, endothelial markers of proliferation as, endoglin and metallo-proteases, MMP-3, MMP-9) resulted decreased. The restore of balance between pro- and anti-angiogenic factors by JWH133 treatment was, also, confirmed by the reduction in vessel number in the tumoral area and, particularly, by a decrease of micro-vessels number, which are typical of tumoral neo-angiogenesis. These promising results, identifying the activation of CB_2 to slow down tumor progression based on angiogenesis, are perfectly in line with several studies demonstrating antiangiogenic effects of CB_2 agonists in several types of solid tumors both *in vitro* and *in vivo* studies.

Guzman and its colleagues, in 2004, demonstrated that incubation of C6 glioma cells with both natural and synthetic cannabinoids, inhibited VEGF release into the medium in a time dependent manner (Guzman et al., 2004). They also showed that Cannabinoid-induced

attenuation of VEGF production was evident in another glioma cell line (the human astrocytoma U373 MG) (Blazquez et al., 2004).

In accordance to this, we wished to discriminate between a direct action on cancer cells or on the cells surrounding the tumor, to distinguish between JWH133 anti-proliferative or anti-angiogenic effect. First of all, we assessed the effects of JWH133 on Lewis Lung Carcinoma cells (LLC1) *in vitro*. Treatment with JWH133 had no effect on either the viability or proliferation of LLC1, suggesting that the reduction of the tumor mass, previously observed *in vivo*, could not be a direct effect on cancer cell, but rather on the micro-environment, i.e. all the events that lead to the consolidation and tumor progression, including angiogenesis (Bocci et al., 2013). This result opened up the possibility for a treatment with less side effects compared to traditional anti-tumoral drugs.

In order to understand JWH133 effect, CB_2 knockout mice have been used too. When JWH133 were administrated in CB_2 knockout mice underwent to lung cancer development, it lost its ability to reduce tumor burden and VEGF content, confirming the importance of CB_2 and this effect.

Despite the evidence of a therapeutic effect of CBs, their effective introduction into clinical use is still controversial and strongly limited by central effects shown by many of them (psychotropic effect). An alternative approach may derive from the use of indirect cannabinoid agonists, i.e. from those drugs that potentiate eCB tone inhibiting their degradation. This strategy may have lower side effects because it would allow the up-regulation of the eCB tone only in the area where the eCB machinery is activated and deregulated, i.e. in lung tumor, as it happens in our experimental model.

In our study we decided to potentiate the 2-AG pathway since this eCB is the preferential ligand on CB_2 receptor; for our aim, we used an inhibitor of Monoacylglycerol lipase, the JZL184 compound. It has been reported that JZL184 dramatically elevates brain levels of 2-

AG, in different animal model (Blankman et al., 2007; Long et al., 2009; Nomura et al., 2011).

In our experiments, the administration of JZL184, significantly and a in a dose dependent manner, reduced lung tumor mass in parallel with a decrease in VEGF levels in the lungs of treated animals. Moreover, in the same experimental conditions, we demonstrated that a large number of pro-angiogenic mediators, MMP-9, FGF, acid and basic, Endoglin and Angiopoietina1, were reduced by treatment with JZL184.

Our data corroborate the abundant evidences showing that eCB and cannabinoids, natural and synthetic, possess anti-angiogenic effects and thus can counteract the growth and progression of many cancers types (Pisanti et al., 2013).

In conclusion, the deregulation of ECS that, for the first time, we evidenced in a mice model of lung cancer, can be the target of therapeutic interventions directed to control the angiogenesis, to promote tumor shrinkage. Moreover, the results obtained in this thesis identify ECS related molecules, as CB_2 selective agonists or inhibitors of degradation of eCBs, as possible agents co-adjuvants of conventional anti-cancer therapies. In fact, these drugs, in parallel to reducing tumor growth, offer the opportunity to resolve the high costs and side effects of the classical anti-angiogenic drugs actually used in therapy.

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