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"Endocannabinoids as emerging suppressors of angiogenesis and breast tumor growth and metastatic spreading"

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“Endocannabinoids as emerging suppressors of angiogenesis and breast tumor growth and metastatic spreading”

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LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

1. Sarnataro D, Grimaldi C, **Pisanti S**, Gazzerro P, Laezza C, Zurzolo C, Bifulco M. Plasmamembrane and lysosomal localization of CB1 cannabinoid receptor are dependent on lipid rafts and regulated by anandamide in a human breast cancer cell line. FEBS Lett 2005;579:6343-9.

2. **Pisanti S**, Grimaldi C, Laezza C, Malfitano AM, Santoro A, Vitale M, Caruso MG, Notarnicola M, Iacuzzo I, Portella G, Di Marzo V, Bifulco M. Anandamide inhibits adhesion and migration of breast cancer cells. Exp Cell Res 2006;312:363-73.

3. Bifulco M, Laezza C, **Pisanti S**, Gazzerro P. Cannabinoids and cancer: pros and cons of an antitumour strategy. Br J Pharm 2006;148:123-35.

4. **Pisanti S**, Sarnataro D, Santoro A, Gazzerro P, Malfitano AM, Laezza C, Bifulco M. The Cannabinoid CB1 Receptor Antagonist Rimonabant (SR141716) Inhibits Human Breast Cancer Cell Proliferation Through A Lipid Rafts Mediated Mechanism. Mol Pharmacol 2006;70:1298-306.

5. Laezza C, **Pisanti S**, Crescenzi E, Bifulco M. Anandamide inhibits Cdk2 and activates Chk1 leading to cell cycle arrest in human breast cancer cells. FEBS Lett. 2006;580:6076-82.

6. **Pisanti S**, Borselli C, Oliviero O, Laezza C, Gazzerro P, Bifulco M. Antiangiogenic activity of the endocannabinoid anandamide. Correlation to its tumour-suppressor efficacy. J Cell Physiol 2007;211:495-503.

7. Bifulco M, Grimaldi C, Gazzerro P, **Pisanti S**, Santoro A. Rimonabant: Just an Anti-obesity Drug? Current Evidence on Its Pleiotropic Effects. Mol Pharmacol 2007;71:1445-56.

8. Bifulco M, Malfitano AM, **Pisanti S**, Laezza C. Endocannabinoids in endocrine and related tumours. Endocr Rel Cancer 2008. *in press*

9. **Pisanti S**, Laezza C, McGuire PG, Morbidelli L, Malfitano AM, Borselli C, Gazzerro P, Das A, Bifulco M. Anti-angiogenic activity of rimonabant: therapeutic potential in ocular diseases. *submitted for publication*

10. Laezza C, **Pisanti S**, Malfitano AM, Bifulco M. Anandamide controls human breast cancer cell migration via RhoA/Rho-kinase signaling pathway. *submitted for publication*

ABSTRACT

The endogenous cannabinoid system, comprising the cannabinoid receptors, their endogenous ligands (e.g. anandamide) and the enzymes for their metabolism, is an almost ubiquitous signaling system involved in the control of several physio-pathological conditions. Modulating the endocannabinoid system turned out to hold therapeutic promise in a wide range of diseases and pathological processes. In this frame, given the ubiquity of the endocannabinoids and their receptors and their modulating activity on proteins involved in cell fate control, there has been increasing evidence for a role of the endocannabinoid system in neoplastic transformation and a remarkable interest to exploit this role for therapeutic gain. However, despite the collected pharmacological evidence on cannabinoids' properties, very little is known concerning the endogenous function of the endocannabinoid system and in particular of CB1 signaling in the processes of tumor growth, metastatic spreading and angiogenesis, all steps of tumor progression. In particular we focused our attention on breast cancer, starting from the hypothesis that targeting CB1 receptor signaling could induce a non-invasive phenotype also through a direct interference on the angiogenic process. To address these issues, suitable *in vitro* and *in vivo* models of breast cancer and angiogenesis were employed. The CB1 antagonist/inverse agonist rimonabant and a stable analogue of anandamide were utilized, as critically important tools to explore, respectively, the basal functions of endocannabinoid system and CB1 signaling and their therapeutic exploitation. We found that breast tumor growth and crucial events of metastatic process are inhibited following CB1 activation. Important molecular players were identified (Rho/FAK/Src; Chk1/Cdc25A; $p21^{waf}$/cdk2). An outstanding finding was the induction of DNA checkpoint by anandamide, able to constrain tumor progression in its early phases, which further confirms a putative tumor-suppressor action of the endocannabinoid system. Moreover, looking at CB1 receptor we reported its association with lipid rafts/caveolae in breast cancer and obtained relevant evidence about rafts function as a useful platform to regulate the differential signaling to CB1 agonists and antagonists. We also suggested that compartmentalization of CB1 into lipid rafts, induced by rimonabant, could be responsible for the anti-proliferative effect observed, through the sequestration of key effectors signaling proteins downstream auto-activated tyrosine kinase receptors in breast cancer. Finally, we found that modulating the endocannabinoid system turned out to affect all the steps of angiogenesis, from the migration of endothelial cells and proliferation, to the formation of three-dimensional tube structures and neovessel stabilization. In conclusion our findings provide direct evidence for a regulatory role of endocannabinoids and of CB1 receptor signaling in tumor progression and angiogenesis, opening the way for new therapeutic opportunities linked to their pharmacological modulation.

1. BACKGROUND

1.1 From *Cannabis* ...

The first steps in the discovery of the endocannabinoid system date back almost 4000 years ago, when the therapeutic and psychotropic actions of the plant *Cannabis sativa* were first documented in India (Peters and Nahas 1999). The use of cannabis as a psychoactive substance reached Europe and the Americas through the Arab world in the 19th century. During the same period, cannabis extracts had gained widespread use for medicinal purposes until 1937, when concern about the dangers of abuse led to the banning of marijuana for further medicinal use in the United States. Over the last 40 years, the isolation and characterization of the psychoactive component of *C. sativa*, Δ^9-tetrahydrocannabinol (Δ^9-THC) represented a challenging research task, awakening a renewed interest for pharmacotherapy (Gaoni and Mechoulam 1964). To date, about 60 different plant terpeno-phenols more or less related to THC have been isolated and defined cannabinoids. The majority of these lack psychoactivity. They include cannabidiol, cannabinol, cannabigerol, and cannabichromene (Table 1). The discovery of these principles stimulated the generation of a whole range of synthetic analogs that concerned not only compounds structurally similar to phytocannabinoids, but also analogs with different chemical structures, including classic and non-classic cannabinoids and aminoalkylindoles (Howlett et al. 2002), as well as the subsequently discovered endogenous arachidonic acid derivatives or endocannabinoids.

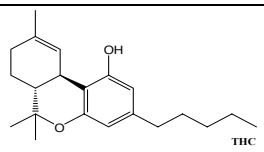
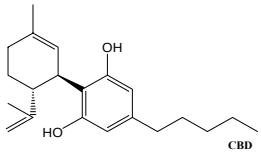
Phytocannabinoids		
Δ^9-tetrahydrocannabinol (Δ^9-THC)		CB1≈CB2 agonist
Cannabidiol (CBD)		no activity at CB1 or CB2 Inhibition of AEA uptake and metabolism

Table 1

1.2 ... to the endocannabinoid system

The endogenous cannabinoid system, comprising the cannabinoid CB1 and CB2 receptors, their endogenous ligands (endocannabinoids) – e.g. *N* – arachidonoylethanolamine (anandamide) and 2-arachidonoyl glycerol (2-AG) -

and the proteins that regulate endocannabinoid biosynthesis and degradation, is an almost ubiquitous signaling system involved in the control of several patho-physiological conditions (Di Marzo et al. 2004).

So far, two subtypes of G-protein-coupled receptors for Δ^9-THC and endocannabinoids, have been cloned: cannabinoid receptor 1 (CB1), originally named 'central' receptor (Matsuda et al. 1990) and CB2, also incorrectly known as 'peripheral' receptor (Munro et al. 1993). An increasing number of reports and pharmacological evidence suggest that endocannabinoids might also exert their biological effects through non-CB1/CB2 receptors, which, however, have not yet been cloned except for the transient receptor potential vanilloid type 1 ion channel (TRPV1), that is activated by various lipids including anandamide (Begg et al. 2005). Of particular interest are the abnormal cannabidiol-sensitive endothelial receptor, involved in vasodilatation and endothelial cell migration and the orphan receptors GPR-119, for oleoylethanolamide, and GPR-55, which recognizes a variety of cannabinoid ligands (Brown 2007).

CB1 and CB2 receptors share only 44% sequence homology and 68% within the transmembrane domains, which are thought to contain the binding sites for cannabinoids.

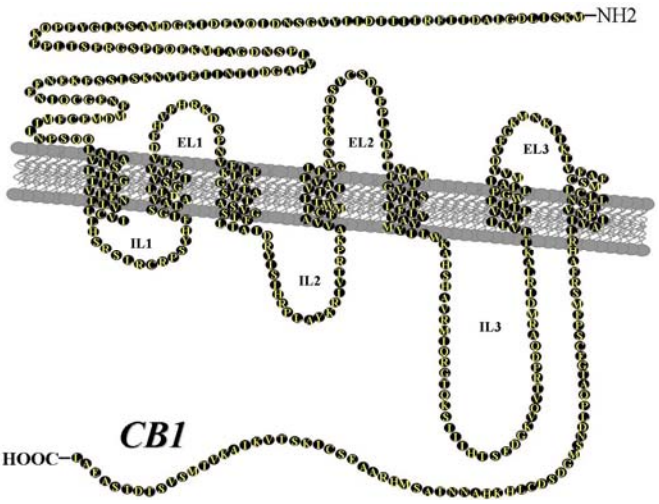


Figure 1: CB1 receptor.

Both the CB1 and CB2 genes encode a seven-transmembrane-domain protein belonging to the $G_{i/o}$ protein-coupled receptor family (Fig. 1) (Munro et al. 1993). The signal transduction pathway downstream cannabinoid receptors includes inhibition of adenylate cyclase (Howlett et al. 1986), stimulation of mitogen-activated protein kinase (MAPK) (Bouaboula et al. 1995) and phosphatidylinositol-3-kinase pathway (Gomez Del Pulgar et al. 2002a) and, in the case of CB1, ion channels (Mackie and Hille 1992). Different structural classes of cannabinoid receptor agonists have the unique ability to activate different signaling cascades which, in turn, influences agonist efficacy. CB1

receptors were found to efficiently couple and activate both G_i and G_o, whereas CB2 only G_o, showing also an agonist-selective G protein signaling (Glass and Northup 1999).
The central and most of the peripheral effects of cannabinoids rely on CB1 activation. This receptor is preferentially expressed in the central nervous system (Matsuda et al. 1990) and is detectable in several brain areas, at very high levels in the basal ganglia, hippocampus, cerebellum and cortex, where it mediates cannabinoid psychoactive effects; its expression during brain development is significantly different from the one observed in the adult stage (Berrendero et al. 1999). CB1 receptors are also present in peripheral nerve terminals, as well as in extra-neural tissues such as testis, uterus, vascular endothelium, eye, spleen, ileum, adipocytes and in several tumors (Matsuda et al. 1990; Munro et al. 1993; Felder and Glass 1998; Straiker et al. 1999; Liu et al. 2000; Cota et al. 2003; Bifulco et al. 2006). The CB2 receptor is the predominant form expressed in immune cells and it is unrelated to cannabinoid psychoactive effects (Felder and Glass 1998). The CB2 is normally expressed in areas enriched of B lymphocytes, such as the spleen marginal zone, the lymph node cortex, the nodular corona of Peyer patches and the mantle zones of secondary follicles in tonsils, suggesting a role for this receptor in the immune response (Munro et al. 1993; Galiegue et al. 1995). Recently it was found to be present also in the brain (Van Sickle et al. 2005), in non-parenchymal cells of the cirrhotic liver (Julien et al. 2005), in the endocrine pancreas (Juan-Pico et al. 2005), and in the bone (Ofek et al. 2006). A recent study (Jorda et al. 2004) showed that CB2 was over-expressed in several human myeloid leukemia cell lines; interestingly, in retrovirus-induced myeloid leukemia models, the Cb2 gene was located in a common virus integration site, EVI1, suggesting that Cb2 could be a proto-oncogene involved in transformation (Valk et al. 1997). CB2 receptors were also found in microglia cells (Nunez et al. 2004), in glioma and other tumors such as in skin, breast and pancreatic tumors (Casanova et al. 2003; Di Marzo et al. 2004; Caffarel et al. 2006; Carracedo et al. 2006).
The discovery of cannabinoid receptors suggested the existence of endogenous ligands capable of activating them, the so-called ‘endocannabinoids’.
Endocannabinoids are lipid molecules containing long-chain polyunsaturated fatty acids, amides, esters and ethers, with different selectivity for the two receptor types (Mcallister and Glass 2002). The best studied are anandamide (AEA), isolated from porcine brain, and 2-arachidonoylglycerol (2-AG) (Devane et al. 1992; Mechoulam et al. 1995; Sugiura et al. 1995).
During the last few years, several other bioactive lipid mediators have been described: 2-arachidonoyl-glyceryl-ether (noladin ether), o-arachidonoyl-ethanolamine (virodhamine), N- arachidonoyl-dopamine, and possibly oleamide (Hanus et al. 2001; Porter et al. 2002; Leggett et al. 2004) (Table 2). All these molecules derive from the non-oxidative metabolism of arachidonic acid, an essential ω6-polyunsaturated fatty acid. Moreover, N-palmitoylethanolamine (PEA), N-oleoylethanolamine (OEA) and N-

stearoylethanolamine (SEA) compounds called 'endocannabinoid-like' are present in human, rat and mouse brain (Di Marzo 1998).

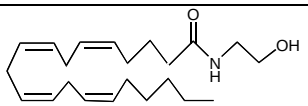
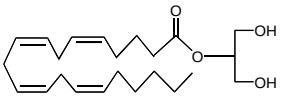
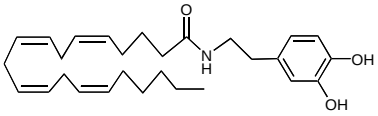
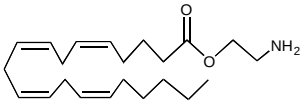
Endocannabinoids		
Anandamide (AEA)		CB1>>CB2 agonist TRPV1 agonist
2-Arachidonoylglycerol (2-AG)		CB1≈CB2 agonist
N-Arachidonoyl dopamine		CB1>>CB2 agonist TRPV1 agonist
Virodhamine		CB2 full agonist CB1 antagonist
Noladin-ether		Selective CB1 agonist

Table 2

Anandamide is a partial or full agonist of CB1 receptors, depending on the tissue and biological response measured. Although it also binds CB2 receptors, it has very low efficacy as CB2 agonist (Devane et al. 1992). 2-AG activates both CB1 and CB2 receptors (Mechoulam et al. 1995).

Endocannabinoids are very lipophilic and thus cannot be stored in vesicles like other neurotransmitters in resting cells, but like prostanoids are produced 'on demand', following physiological or pathological stimuli. Consequently, the regulation of endocannabinoid signaling is tightly controlled by their synthesis, release, uptake and degradation. Several different stimuli, including membrane depolarization and increased intracellular Ca^{2+} and/or metabotropic receptor stimulation, can active complex enzymatic machineries, which lead to the cleavage of membrane phospholipids and eventually to the synthesis of endocannabinoids (Bisogno et al. 2005). A putative membrane endocannabinoid transporter involved in the cellular uptake of endocannabinoids may also be involved in their release.

The *in vivo* biosynthesis of anandamide (and its congeners N-acylethanolamines), is believed to occur through the enzymatic hydrolysis catalyzed by a phospholipase D (PLD) of a membrane lipid precursor, *N*-arachidonoylphosphatidylethanolamide (NAPE), which itself is generated by the enzymatic transfer of arachidonic acid in the *sn*-1 position in phosphatidylcholine to the amide group of phosphatidylethanolamine (PE) (Di Marzo et al. 1994). Although a specific trans-acylase for the latter reaction has not yet been identified, a NAPE-specific PLD has recently been cloned (Okamoto et al. 2004). A secretory phospholipase A2 (PLA2) that can catalyze the hydrolysis of *N*-acyl-PE to *N*-acyl-lysoPE, which is then acted on by a

lysoPLD to generate *N*-acyl-ethanolamides, including anandamide, was recently identified in the stomach (Sun et al. 2004). An alternative pathway has been identified, which involves the hydrolysis of NAPE to phosphor-anandamide by a phospholipase C (PLC), followed by dephosphorylation through a phosphatase (Liu et al. 2006).
2-AG is generated from diacylglycerol (DAG) by a DAG lipase selective for the *sn*-1 position (Bisogno et al. 2005) (Fig. 2).

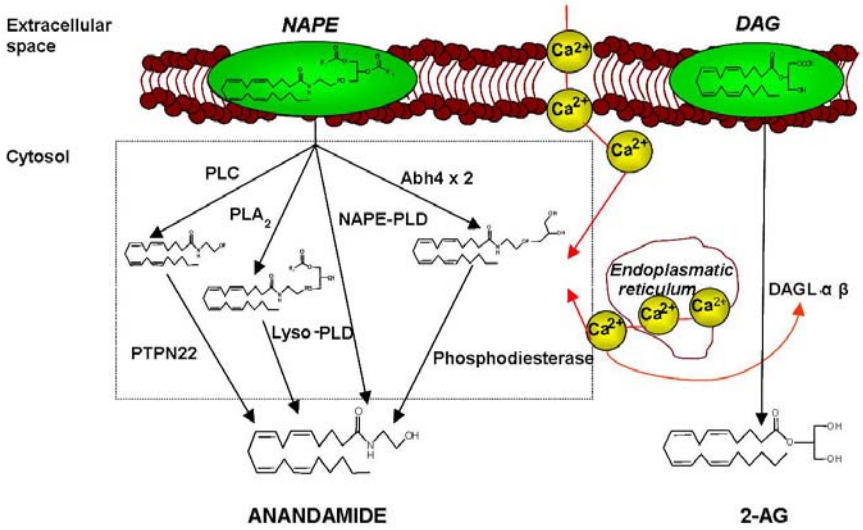


Figure 2: Proteins and pathways for the biosynthesis of endocannabinoids. Abh4, alpha/beta-hydrolase 4; lyso-PLD, lysophospholipase D; PTPN22, protein tyrosine phosphatase N22.

After synthesis, endocannabinoids can activate cannabinoid receptors, either after previous release into the extracellular space or directly moving within the cell membrane. Endocannabinoid signaling is limited by very efficient degradation processes, involving facilitated uptake from the extracellular space into the cell and enzymatic catabolism mediated by specific intracellular enzymes. The facilitated diffusion process is driven by transmembrane concentration gradient, is saturable and temperature dependent, and does not require ATP or sodium ions. The molecular nature of the carrier protein(s) involved in endocannabinoid uptake (EMT, Endocannabinoid Mediate Transport) has not yet been elucidated. However, the enzymes able to degrade endocannabinoids are quite well characterized. They are fatty acid amide hydrolase (FAAH) for anandamide and related compounds (Giang and Cravatt 1997) and monoacylglyceride lipase (MAGL) for 2-AG, although other enzymes might be partially involved in the degradation of this latter (Dinh et al. 2002) (Fig. 3). The unique role of FAAH in terminating signaling by anandamide was indicated by the phenotype of FAAH knockout mice, which displayed 10 to 15 times elevated levels of anandamide across the brain,

supersensitivity to the actions of exogenous anandamide, and the appearance of tonic signaling by endogenous anandamide resulting, for example, in CB1 receptor-mediated hypoalgesia (Cravatt et al. 2001).

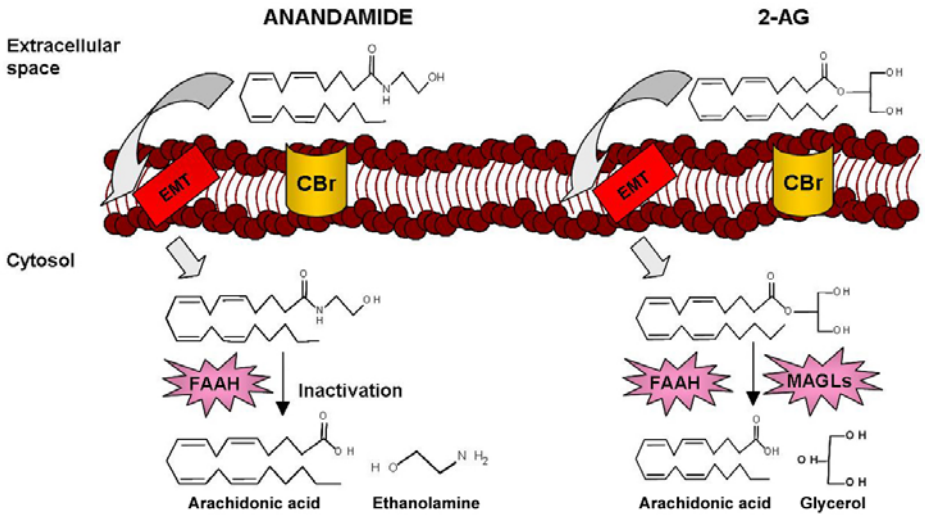


Figure 3: Proteins and pathways for the degradation of endocannabinoids.

A recent study investigated whether endocytic processes could be involved in the uptake of endocannabinoids and found that about half of the AEA uptake occurs via a caveolae/lipid raft-related process (McFarland et al. 2004).
Ethanolamine, arachidonic acid, and glycerol, the hydrolysis products of AEA and 2-AG, are recycled into membrane phospholipids in order to be used again (Bisogno et al. 2005). Furthermore, because of the presence of arachidonate moiety, the possibility that endocannabinoids can also be susceptible to oxidative mechanisms catalyzed by lipoxygenases, cyclooxygenases and cytochrome *P*450 oxidases has been investigated (Kozak and Marnett 2002).

1.3 The endocannabinoid system as therapeutic target in physiopathological conditions

During the past 15 years a remarkable amount of studies have been performed in order to understand the biological role of the endocannabinoid system and its regulatory functions in health and disease. Such studies have been greatly facilitated by the introduction of selective cannabinoid receptor antagonists and inhibitors of endocannabinoid metabolism and transport, as well as mice deficient in cannabinoid receptors or the endocannabinoid-degrading enzyme FAAH, whereas synthesis inhibitors are not yet available. The endocannabinoid system has been implicated in a growing number of

physiological functions, both in the central and peripheral nervous systems and in peripheral organs. More importantly, modulating the activity of the endocannabinoid system turned out to hold therapeutic promise in a wide range of disparate diseases and pathological conditions, ranging from mood and anxiety disorders, movement disorders such as Parkinson's and Huntington's disease, neuropathic pain, multiple sclerosis and spinal cord injury, to cancer, atherosclerosis, myocardial infarction, stroke, hypertension, glaucoma, obesity/metabolic syndrome and osteoporosis, to name just a few (Pacher et al. 2006).
In the central nervous system endocannabinoids act as neuromodulators or retrograde messengers. Retrograde endocannabinoid signaling is crucial for certain forms of short-term and long-term synaptic plasticity at excitatory or inhibitory synapses in many brain regions, and thereby contributes to various aspects of brain function including cognitive functions -learning and memory-, emotions and the reinforcement of substances of abuse in the mesolimbic system (Wilson and Nicoll 2002; Gerdeman et al. 2003). CB1 receptors and endocannabinoids are very abundant in the basal ganglia and cerebellum, where they control movement and posture, for example by influencing dopaminergic signaling (Van der Stelt and Di Marzo 2003). The neuromodulatory actions of endocannabinoids in the sensory and autonomic nervous systems result, mostly via CB1 receptors, in the regulation of pain perception (Iversen and Chapman 2002) and of cardiovascular (Randall et al. 2002) and gastrointestinal functions (Di Carlo and Izzo 2003). Their cross-talks with steroid hormones and with hypothalamic hormones and peptides, help modulate food intake, the pituitary-hypothalamus-adrenal axis, and reproduction (Wenger and Maccarrone 2005). CB2 receptors, instead, are involved in cellular and particularly humoral immune response, with possible implications for (neuro)inflammation and chronic pain (Klein 2005).

1.4 Current thoughts about the endocannabinoid system in cancer

The ubiquity of the endocannabinoids in both vertebrate and invertebrate tissues, and their modulating activity on proteins and nuclear factors involved in cell proliferation, differentiation and apoptosis, suggests that the endocannabinoid signaling system could be involved, among other effects, in the control of cell survival, death and neoplastic transformation (Bifulco and Di Marzo 2002; Bifulco et al. 2006).
Numerous recent studies have suggested that (endo)cannabinoids might directly inhibit tumor growth *in vitro* and *in vivo*. Various cannabinoids, including Δ^9-THC and cannabidiol, endocannabinoids like anandamide, its congeners and 2-AG, and endocannabinoid-transport or -degradation inhibitors (VDM-11 and AA-5-HT), have been shown to inhibit tumor growth and progression of numerous types of cancers including glioma, glioblastoma

multiforme, breast, prostate and thyroid cancer, colon carcinoma, leukemia and lymphoid tumors, to name just a few. The proposed mechanisms are complex and may involve induction of apoptosis in tumor cells, anti-proliferative action, anti-metastatic effect through inhibition of neo-angiogenesis and tumor cell migration (Bifulco et al. 2006). Moreover the effect, depending on the type of agonist and target tissue, is CB1, CB2 or TRPV1 receptor-dependent or independent (e.g., cyclooxygenase).

As mentioned above, (endo)cannabinoids inhibit the proliferation of various tumor cells, possibly through inhibition of proliferative pathways like: adenylyl cyclase and cAMP/protein kinase A pathway (Melck et al. 1999), cell cycle blockade with induction of the cyclin-dependent kinase inhibitor $p27^{kip1}$ (Portella et al. 2003), decrease in epidermal growth factor receptor (EGF-R) expression and/or attenuation of EGF-R tyrosine kinase activity (Casanova et al. 2003; Mimeault et al. 2003), decrease in the activity and/or expression of nerve growth factor, prolactin or vascular endothelial growth factor tyrosine kinase receptors (De Petrocellis et al. 1998; Melck et al. 2000; Portella et al. 2003). In 1998, De Petrocellis et al. investigated the possible anti-mitogenic effects of anandamide on epithelial human breast cancer cell lines EFM-19 and MCF-7. In these models, treatment with sub-micromolar concentration of anandamide (as well as of 2-AG or HU-210) significantly inhibited the G1–S transition of cell cycle. Moreover, anandamide inhibited the expression of prolactin receptors, induced down-regulation of the brca1 gene product (De Petrocellis et al. 1998), and of trk proteins, the high-affinity neurotrophin receptors (Melck et al. 2000). The block of the G1–S transition was ascribed to the inhibition of adenylyl cyclase and, consequently of cAMP-protein kinase A pathway and to the activation of MAPK (Melck et al. 1999). Several intraepithelial or invasive prostatic cancers showed increased expression of EGF-R, EGF and transforming growth factor α (TGFα). Mimeault and colleagues (2003) showed that a micromolar concentration of anandamide inhibited EGF-induced proliferation of DU145 and PC3 prostate cancer cells, as well as of androgen-stimulated LNCaP cells, via G1 arrest, and downregulated EGF-R levels. Both phenomena were CB1-mediated. Similar growth arrest and receptor modulation were also reported for prolactin- and nerve growth factor-stimulated DU145 (De Petrocellis et al. 1998; Melck et al. 2000; Bifulco et al. *in press*).

The pro-apoptotic effect of cannabinoids in tumor cells is complex and may involve increased synthesis of the pro-apoptotic sphingolipid ceramide (Galve-Roperh et al. 2000; Gomez del Pulgar et al. 2002a,b), ceramide-dependent up-regulation of the stress protein p8 and several downstream stress-related genes expressed in the endoplasmic reticulum (ATF-4, CHOP, and TRB3) (Carracedo et al. 2006), prolonged activation of the Raf-1/extracellular signal-regulated kinase cascade (Galve-Roperh et al. 2000), and inhibition of Akt (Gomez del Pulgar et al. 2000; Ellert-Miklaszewska et al. 2005), c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase (Galve-Roperh et al. 2000; Sarker et al. 2003; Hinz et al. 2004; Powles et al. 2005). The anti-

tumoral action of cannabinoids on glioma may be exerted either via the CB1 or the CB2 receptor. THC induced apoptosis of C6 glioma cells by a pathway involving CB1 receptor, sustained generation of the pro-apoptotic lipid ceramide and prolonged activation of Raf1/MEK/ERK cascade (Galve-Roperh et al. 2000). A role for BCL-2 family members, such as Bad, has also been hypothesized (Ellert-Miklaszewska et al. 2005). Pro-apoptotic effect may rely also on a CB1 receptor-independent stimulation of sphingomyelin breakdown (Sanchez et al. 1998). Furthermore, the mechanism through which anandamide induces apoptosis in cells expressing both functional cannabinoid and vanilloid receptors is still controversial and might depend on the experimental conditions used. It is important to remark that prolonged anandamide incubation times (5–6 days) in DU145 and PC3 cells were able to induce massive apoptosis. This effect was mediated by CB1/2 via cellular ceramide accumulation, and was absent in LNCaP cells (Mimeault et al. 2003). However cannabinoid receptors could have a protective role against programmed cell death, as reported in human neuroblastoma and C6 cells, where anandamide induced apoptosis, via vanilloid receptors, increasing intracellular calcium concentration, activating COX, releasing cytochrome *c* and activating caspase 3 (Maccarrone et al. 2000) (see also Table 3).

Table 3. Effect of (endo)cannabinoids in cancer treatment: pro and cons evidence.

Tumor (cell type)	*Cannabinoid (concentration or dose)*	*Effect*	*Mechanism of action*	*References*
Human breast cancer cell lines (MCF7; EFM-19; T47D)	AEA (2-10μM) 2-AG (2-10μM) HU210(≥4μM)	−	Inhibition of the mitogen-induced stimulation of the G0/G1-S phase	De Petrocellis et al. 1998
	AEA (≥2μM) 2-AG, HU210 (≥1μM)	−	Inhibition of NGF-induced proliferation Inhibition adenylyl ciclase; down-regulation PRLr TRK	Melck et al. 2000 Melck et al. 1999
Human breast cancer cell line (MDA-MB-231)	AEA (10 μM)	−	S phase arrest; induction Chk1 intra-S phase checkpoint	Laezza et al. 2006
Human breast cancer cell line (MDA-MB-231) Mouse breast cancer cell line (TSA-E1)	AEA (10 μM and 0.5mg/kg/dose)	−	Inhibition of adhesion and migration *In vivo*, reduction of number and dimension of metastatic nodes	Grimaldi et al. 2006
Human breast cancer cell lines (MCF7; MDA-MB-231) Mouse mammary carcinoma (4T1)	THC (≤5μM)	+	Increased tumor growth and metastasis; *in vivo*, decreased anti-tumor immune response	McKallip et al. 2005
Human breast cancer cell lines (MCF7; T47D; MDA-MB-231; MDA-MB-468)	THC (≥12μM)	−	G2/M phase transition blockade through Cdc2 and apoptosis induction	Caffarel et al. 2006
Human breast cancer cell lines (MCF-7; MDA-MB-231)	Cannabidiol (8-12μM)	−	Inhibition of proliferation; apoptosis induction	Ligresti et al. 2006
Human breast cancer cell lines (MCF-7; MDA-MB-231; T47D)	Rimonabant (0.1 μM and 0.7mg/kg/dose)	−	Inhibition of proliferation; G1 arrest *In vivo*, growth inhibition of breast xenografts tumors	Sarnataro et al. 2006

Tumor (cell type)	*Cannabinoid (concentration or dose)*	*Effect*	*Mechanism of action*	*References*
Androgen-independent prostate cancer cells (PC3, DU145)	AEA, R-(+)-MET (≥2μM)	−	Inhibition of mitogen-induced proliferation, G1 arrest	Mimeault et al. 2003
	THC (1μM)	−	Apoptosis	Melck et al. 2000 Ruiz et al.. 1999
Androgen-dependent prostate cancer cells (LNCaP)	AEA, R-(+)-MET (≥2μM)	−	Inhibition of mitogen-induced proliferation, G1 arrest	Mimeault et al. 2003
Androgen-dependent prostate cancer cells (LNCaP)	WIN-55,212-2 (≥2.5μM)	−	Dose- and time-dependent induction of apoptosis; decreased expression of AR and PSA	Sarfaraz et al. 2005
Androgen-dependent prostate cancer cells (LNCaP)	R-(+)-MET (0.1-0.2μM)	+	Increased proliferation and AR expression	Sanchez et al. 2003
Rat glioma cell line (C6)	THC (1μM)	−	Apoptosis *via* ceramide *de novo* synthesis *In vivo*, regression of C6-derived glioma	Galve-Roperh et al. 2000
	JWH133, WIN-55,212-2 (0.1μM)	−	Apoptosis *via* ceramide *de novo* synthesis	Sanchez et al. 2001
	WIN-55,212-2 (15μM)	−	Apoptosis *via* activation of caspase cascade	Ellert-Miklaszewska et al. 2005
Human astrocitoma (grade IV)	JWH-133 (50μg/die)	−	*In vivo*, inhibited growth of tumors induced in deficient mice	Sanchez et al. 2001
Human glioblastoma multiforme cell line (GBM)	THC (1μM) WIN-55,212-2	−	Decreased proliferation and increased cell death	McAllister et al. 2005
Human neuroglioma cells	R-(+)-MET (1-10μM)	−	Apoptosis induction	Hinz et al. 2004
K-ras-transformed FRTL-5 thyroid cells (KiMol)	Met-F-AEA (0.5mg/kg/ dose)	−	*In vivo*, inhibited growth of tumors induced in nude mice	Bifulco et al. 2001
Thyroid tumor xenografts Experimental lung metastases	Met-F-AEA (0.5mg/kg/ dose)	−	*In vivo*, inhibited growth of thyroid tumor xenografts induced in athymic mice *In vivo*, inhibited the development of lung metastases	Portella et al. 2003
Thyroid tumor xenografts	Met-F-AEA (0.5mg/kg/d); VDM-11, AA-5HT(5mg/kg/d); Rimonabant (0.7mg/kg/dose)	−	*In vivo*, inhibited growth of thyroid tumor xenografts induced in athymic mice	Bifulco et al. 2004
Mouse skin carcinoma cells (PDV-C57)	JWH-133, WIN-55,212-2 (1.58 μg)	−	*In vivo*, inhibited growth of tumors induced in nude mice	Casanova et al. 2003
Mantle cell lymphoma cell lines	AEA, WIN-55,212-2, Rimonabant (1-10μM)	−	Growth inhibition; apoptosis induction	Flygare et al. 2005
Lung carcinoma	THC 100mg/kg	−	Growth inhibition	Munson et al. 1975
Lung cancer cells (NCI-H292) Glioblastoma cell line (U373-MG)	THC (0.1-0.3μM)	+	Increased proliferation	Hart et al. 2004
Pancreatic tumor cells (Panc1; MiaPaCa2)	THC (2μM and 15mg/kg/die)	−	Apoptosis induction through ceramide *in vivo*, inhibited growth of xenografts and intrapancreatic tumors	Carracedo et al. 2006

− inhibitory effect; + stimulatory effect

More importantly, systemic or local treatment with cannabinoids inhibited the growth of various types of tumors or tumor cell xenografts *in vivo*, including lung carcinoma (Munson et al. 1975), glioma (Galve-Roperh et al. 2000; Sanchez et al. 2001; Massi et al. 2004), thyroid epithelioma (Bifulco et al. 2001), lymphoma (McKallip et al. 2002), and skin carcinoma (Casanova et al. 2003) in mice. Based on the *in vivo* efficacy, it has been suggested that anti-tumor effect of cannabinoid-related drugs could be partially ascribed to the inhibition of tumor metastatic spreading and neoangiogenesis.

However, in sharp contrast to the above, Hart et al. (2004) have demonstrated that treatment of lung cancer (NCIH292), squamous cell carcinoma (SCC-9), bladder carcinoma (5637), glioblastoma (U373-MG), astrocytoma (1321N1), and kidney cancer (A498) cells with nanomolar concentrations of cannabinoids such as THC, anandamide, HU-210, and WIN 55,212-2 leads to rapid EGFR- and metalloprotease-dependent cancer cell proliferation. However, the same study also documented that at micromolar concentrations cannabinoids induced cancer cell apoptosis, in agreement with previous reports (Hart et al. 2004) (see also Table 3). These results highlight a likely bimodal action of (endo)cannabinoids on cancer cell growth, with low concentrations being pro-proliferative and high concentrations having anti-proliferative effects. The variability of the effects of (endo)cannabinoids in different tumor models may be also related to the differential grade of expression of CB1 and CB2 receptors.

It has been documented that the expression of CB1 receptor was regulated in an opposite way in normal or transformed cells. In FRTL-5 normal cells or in K-*ras*-transformed FRTL-5 cells (KiMol), as well as in the tumors derived from the latter, the expression of CB1 receptors was regulated in opposite ways upon treatment with anandamide, by being down-regulated in normal cells and up-regulated in transformed cells. (Bifulco et al. 2001). Thus, CB1 receptor expression determines the extent of the responsiveness of normal or transformed FRTL-5 cells to endocannabinoids. A similar situation occurs in human astrocytomas, where the extent of CB2 receptor expression is directly related with tumor malignancy (Sanchez et al. 2001). Apparently, an opposite regulation of CB1 expression in transformed *versus* normal cells was a common mechanism: THC induced apoptosis in several human cancer cell lines but showed less efficacy in non-transformed cell counterparts, that might even be protected from cell death (Sanchez et al. 1998; Ruiz et al. 1999; Galve-Roperh et al. 2000; Casanova et al. 2003; McAllister et al. 2005; Caffarel et al. 2006). An increasingly accepted notion is that the endocannabinoid system very often induces opposing effects in normal and neoplastic cells, on important physiological processes, such as proliferation and migration (Guzman et al. 2001). For instance, CB1 agonists stimulate migration in normal cells, whereas inhibit migration in tumor cells (Song and Zhong 2000). The molecular basis of this "*ying-yang*" action is yet unknown: it could depend on the coupling of the CB receptors to different types of G-protein or on a differential capacity of cells to synthesize ceramide (Howlett 2004). Enhanced

endocannabinoid signaling was found in some cancer *vs*. non-cancer human tissues, or in human tumor cells with an increasingly higher degree of invasiveness (Petersen et al. 2005; Ligresti et al. 2003). This could be an endogenous anti-proliferative mechanism acting through selective cannabinoid receptor activation.
Noteworthy, cannabinoids' immunosuppressive effects may compromise anti-tumor immune responses. Thus, cannabinoids may be effective in killing tumors that abundantly express cannabinoid receptors, such as gliomas, but may increase the growth and metastases of other types of tumors, with no or low expression of cannabinoid receptors, due to the prevalent suppression of the anti-tumor immune response (McKallip et al. 2005).

1.5 Lipid rafts in the organization of the endocannabinoid system

Lipid rafts are membrane domains enriched in sphingolipids and cholesterol and also in plasmenylethanolamines, particularly those containing arachidonic acid. They are biochemically defined by the insolubility of their components in cold non-ionic detergents (like Triton X-100) (Simons and Ikonen 1997). Because of their ability of forming more liquid ordered domains within the membranes, lipid rafts segregate particular proteins and regulate their intracellular trafficking, folding and signal transduction functions within (Simons and Toomre 2000).
One subset of organized raft domains is represented by caveolae, flask-shaped invaginations of the membrane formed from lipid rafts by polymerization of caveolins, a family of integral membrane proteins that tightly bind cholesterol.
The general function of caveolae is not clear. They have been implicated in endocytosis ad trancytosis of various proteins across the endothelial monolayer and during signal transduction. Lipid rafts and caveolae have also been shown to play an important role in the regulation of various cellular functions including organization of cell signaling machinery such as receptor tyrosine kinases and GPCRs, cholesterol transport, endocytosis, cell polarization and migration (Simons and Ikonen 1997).
Interestingly, it has been reported that lipid rafts/caveolae play a key role in breast tumor cell invasion (Bourgignon et al. 2004) and that caveolin 1 is a potent suppressor of mammary tumor growth and metastasis (Williams et al. 2004; Sloan et al. 2004).
Interestingly, some observations on the caveolae/lipid raft-mediated uptake and recycling of anandamide (McFarland et al. 2004) and the intracellular trafficking and regulation of CB1-mediated signal transduction (Bari et al. 2005) suggest that there are connections between the endocannabinoid system and cholesterol-enriched lipid rafts. It has been recently reported that detergent-resistant membranes play a role in the cellular accumulation of anandamide by mediating an endocytic process responsible for anandamide

internalization. The enzyme primarily responsible for anandamide metabolism, FAAH, is excluded from lipid rafts. However, the metabolites of anandamide accumulate in these detergent-resistant membrane microdomains. There is some preliminary evidence that makes it reasonable to propose that anandamide metabolites enriched in lipid rafts may act as precursors to anandamide synthesis. Overall, experimental evidence is mounting that lipid rafts may play a role in the cellular regulation of anandamide inactivation and production (McFarland et al. 2006). Moreover CB1 co-localizes almost entirely with caveolin-1 in neuronal cells (Bari et al. 2007).
Finally, some evidence suggests a possible involvement of lipid rafts in anandamide-induced cell death (Sarker and Maruyama 2003) and that anandamide induces apoptosis, at least in hepatoma cell line (HepG2), interacting with cholesterol present in the cell membrane (Biswas et al. 2003).

1.6 Glancing at angiogenesis

As proposed in 1971 by Folkman, tumor growth and metastasis are angiogenesis-dependent processes and hence, blocking angiogenesis is turned out to be a fundamental therapeutic strategy to arrest tumor growth (Ferrara and Kerbel 2005).
The *de novo* development of blood vessels takes place via one of two processes: vasculogenesis, in which progenitor cells give rise to new endothelial cells that form tubes and provide blood supply to tissues, and angiogenesis, in which new blood vessels arise from pre-existing mature ones (Risau 1997; Dimmeler 2005). The process of vasculogenesis only occurs during embryogenesis, whereas angiogenesis occurs regularly in adult tissues in a variety of physiological and pathological conditions, including wound healing, inflammation, rheumatoid arthritis, endometriosis, diabetic retinopathy, macular degeneration, and tumor progression (Carmeliet 2003). Several complex steps are involved in angiogenesis, the first one represented by localized enzymatic degradation of the basement membrane of the existing vessels, followed by the detachment of endothelial cells from adhesive proteins in the extracellular matrix and migration into the perivascular space, where endothelial cells proliferate rapidly. Then, the new endothelial cells morphologically differentiate into tube-like structures that eventually join to form new capillaries (Bussolino et al. 1997) (Fig. 4).
The entire process, at different stages, is controlled by a finely tuned balance between positive and negative effectors, such as growth factors, integrins, angiopoietins, chemokines, oxygen sensors, endogenous inhibitors and many others (Carmeliet 2003). Among these factors, vascular endothelial growth factor (VEGF), a soluble angiogenic factor produced by both normal and tumor cells, plays a key role in regulating normal and abnormal angiogenesis (Ferrara and Kerbel 2005).

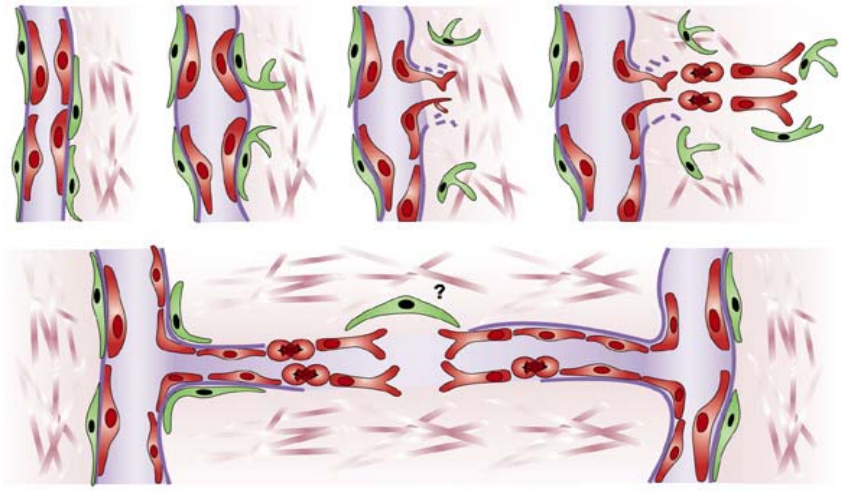


Figure 4: New blood-vessel formation. Pericytes (green) detach and blood vessels dilate before the basement membrane and extracellular matrix is degraded. Endothelial cells (red) migrate into the perivascular space towards angiogenic stimuli and proliferate. Behind the migration columns, endothelial cells adhere to each other and create a lumen, which is accompanied by basement-membrane formation and pericyte attachment. Finally, blood-vessel sprouts will fuse with other sprouts to build new circulatory systems (Bergers and Benjamin 2003).

Both stimulating and inhibiting angiogenesis are fundamental strategies to human health. Recently, anti-angiogenesis therapies have been introduced successfully in the clinic, representing a turning point in tumor and macular degeneration therapies and heralding a new era for the treatment of several commonly occurring angiogenesis-related diseases (Carmeliet 2005; Ferrara and Kerbel 2005).

1.7 Endocannabinoids and angiogenesis: a challenging research task

Recent studies on the endocannabinoid system have provided strong evidence for a key role of the endocannabinoids in the control of cell-signaling pathways involved in cancer cell growth, invasion and metastasis processes, in a way dependent on CB receptor activation. In particular it has been suggested that anti-tumor effect of cannabinoid-related drugs could be partially ascribed to the inhibition of tumor neo-angiogenesis *in vivo* (Portella et al. 2003). Indeed, endocannabinoids inhibited angiogenesis in thyroid tumors *via* inhibition of VEGF and its receptor Flt-1 expression, interfered with endothelial cell migration also inducing endothelial cell apoptosis, thereby counteracting thyroid, skin and glioma cancer growth *in vivo* (Portella et al. 2003; Casanova et al. 2003; Blazquez et al. 2003). Cannabinoid treatment of gliomas, inhibited

the expression of VEGF, angiopoietin-2, matrix metalloproteinase-2 (MMP-2), hypoxia-inducible factor 1-α (HIF-1α). Moreover, intra-tumoral administration of THC to two patients affected by glioblastoma multiforme, was reported to decrease both VEGF and VEGFR-2 activation in the tumors (Blazquez et al. 2004). Neverthless, the mechanism and the extent of endocannabinoids anti-angiogenic effects have not been fully explored to date.

1.8 Role of CB1-endocannabinoid axis in physiopathology: focus on CB1 antagonist/inverse agonist rimonabant (SR141716)

The fundamental importance of the CB1-endocannabinoid axis in physio-pathology is reflected in the ongoing development of high-affinity CB1 antagonists and inverse agonists as therapeutic drugs (see enclosed Bifulco et al. 2007, Table 3). Among the increasing number of compounds sharing CB1 receptor antagonistic properties, known so far are diarylpyrazoles, aminoalkylindoles and triazole derivatives. The first highly selective CB1 receptor antagonist was discovered by Sanofi-Aventis and was the diarylpyrazole [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride], so-called rimonabant (SR141716, Acomplia^{TM}), which showed a number of biological effects *in vitro* and *in vivo* in several pathological situations. Binding studies have demonstrated that rimonabant is a potent (pK_i = 8.4) and selective ligand for CB1 receptors, showing a high affinity (K_i = 5.6 nM) for the CB1, and low affinity (K_i >1000 nM) for the CB2 receptor (Rinaldi-Carmona et al. 1994). Moreover, it displays a weak affinity to Galanin$_2$, MC$_5$, opioid$_k$, and pA_2 receptors (Compton et al. 1996; Shire et al. 1996). Functional studies confirmed its potent (pA_2:7.98–8.85) and selective CB1 receptor antagonistic activity. This compound readily displaced the cannabinoid synthetic agonist [^{3}H]CP 55,940 from specific binding sites (K_i= 1.98 nM) and has been shown to prevent cannabinoids from producing several of their typical effects, both *in vitro* and *in vivo*. Rimonabant potency as an antagonist has been shown by comparing its ability to attenuate WIN 55,212-2-induced inhibition of electrically evoked contractions of the mouse isolated vas deferens (K_d= 2.4 nM) with that of WIN 56,098, bromopravadoline, and iodopravadoline. It was also an effective antagonist *in vivo* by suppressing the hypothermia elicited by WIN 55,212-2 and psychomotor effects in mice and rats (Perio et al. 1996). Saturation binding experiments with membranes prepared from rat cerebellum have shown that radiolabeled rimonabant

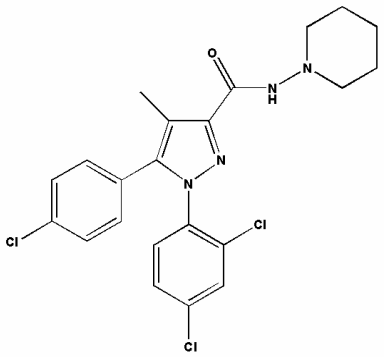


Figure 5. Chemical structure of the CB1 antagonist rimonabant.

undergoes specific, rapid, saturable, high-affinity binding to a single class of sites. This specific binding is readily attenuated by the cannabinoids CP 55,940, WIN 55,212-2, Δ^9-THC, hydroxy-Δ^9-THC and anandamide. Interactions with rimonabant are competitive in nature for CP 55,940, WIN 55,212-2, and Δ^9-THC, but non-competitive for anandamide, because the latter compound decreases both the affinity constant and the B_{max} of radiolabeled rimonabant (Rinaldi-Carmona et al. 1994). Fourth and fifth transmembrane domains of the CB1 receptor are essential for high-affinity binding of rimonabant, whereas the extracellular loop between these two domains is unimportant (Shire et al. 1996). In some experiments, rimonabant has been found to produce effects that are opposite in direction from those produced by cannabinoid receptor agonists. Increasing evidence suggests that rimonabant behaves also as an inverse agonist in some membrane preparations. Indeed, Bouaboula et al. (1997) found that Chinese hamster ovary cells, transfected with the CB1 receptor, displayed high constitutive activity of both MAPK and adenylate cyclase and this increase was inhibited by rimonabant. They also observed that guanosine 5'-*O*-(3-[^{35}S]thio)triphosphate enhanced the binding of rimonabant, a feature usually described for inverse agonists (Pertwee 2005).
The involvement of the endocannabinoid system in the physiological control of appetite and energy metabolism has prompted out to develop an anti-obesity strategy through CB1 receptor antagonism. The assessment of the clinical efficacy of rimonabant as an antiobesity drug was carried out in several multinational, randomized and placebo-controlled trials on patients who were overweight (body mass index higher than 27 kg/m^2) or obese (body mass index $\geq$30 kg/m^2). A cumulative weight loss and a significant change of waist circumference from the baseline were observed in patients receiving 20 mg/day of rimonabant. It is noteworthy that rimonabant also caused an increase of high-density lipoprotein (HDL) cholesterol levels and a reduction of triglycerides, fasting insulin, and insulin resistance, positively affecting risk factors for a number of obesity related co-morbidities through weight loss-dependent and -independent pathways. Although the precise mechanism of action of rimonabant have to be further dissected, it is emerging from both preclinical and clinical research, that rimonabant can have a plethora of pharmacological effects, besides weight reduction, affecting a broad range of diseases from obesity-related comorbidities (type II diabetes, metabolic syndrome) to drug dependence (nicotine, alcohol, psycho stimulants abuse), fertility and cancer (for a review see enclosed Bifulco et al. 2007).

1.9 Targeting peripheral CB1 for an anti-proliferative strategy

It is well known that, upon stimulation, both CB1 and CB2 receptors induce the activation of the MAPK pathway (Galve-Roperh et al. 2002), whereas CB1 antagonist rimonabant inhibits MAPK activation by pertussin toxin-sensitive

receptor-tyrosine kinase, therefore displaying negative intrinsic activity that is referred to inverse agonism (Bouaboula et al. 1997). Starting from the hypothesis that the rimonabant potent anti-obesity effects in rodent models might result also from its metabolic peripheral action on adipose tissue, and since hyperplasia of adipose tissue is a critical event for the development of obesity, Gary-Bobo and colleagues (2006) investigated the effect of rimonabant on mouse pre-adipocytes proliferation *in vitro*, showing inhibition of proliferation and induction of adipocyte late maturation without lipid droplets accumulation. Among the possible molecular mechanisms involved in such effect, they proposed inhibition of basal and serum-induced p42/44 MAPK pathway. The regulation by rimonabant of MAPK activity could be mediated by the CB1 receptor coupled to PTX-sensitive G_i protein, in agreement with its inverse agonist properties. A recent evidence for a rimonabant control of cell proliferation arises from a study by Teixeira-Clerc et al. (2006) on the possibility to use CB1 antagonism as an innovative strategy for the treatment of liver fibrosis. This study starts from the observation that the endocannabinoids and their receptors are implicated in the pathogenesis of chronic liver diseases associated with hepatic fibrosis (Mallat and Lotersztajn, 2006). CB1 receptor antagonism by rimonabant administration to mice, counteracts the wound-healing response to three types of acute liver injury, by decreasing the accumulation of hepatic myofibroblasts and the levels of the profibrogenic cytokine TGF-β1. The proposed mechanism of anti-fibrotic properties rely on the inhibited proliferation of the hepatic myofibroblasts in response to platelet-derived growth factor (PDGF)-BB both in mice and humans. The anti-proliferative effect depends on CB1 receptor signaling, as revealed by the absence of effect in the $Cnr1^{-/-}$ (CB1 knock-out) hepatic myofibroblasts. As previously observed in other cell systems, the molecular pathways mediating CB1 antagonism effects on hepatic myofibroblasts involve decreased phosphorylation of ERK MAPK and Akt, both in $Cnr1^{-/-}$ and in rimonabant wild-type treated cells.

1.10 Therapeutic exploitation of CB1 antagonism/inverse agonism in cancer

Several plant-derived cannabinoids (e.g. THC and cannabidiol), synthetic cannabinoid receptor agonists (e.g. HU-210), endocannabinoids (e.g. anandamide, 2-AG), inhibitors of endocannabinoid degradation (e.g. VDM-11, AA-5-HT) are now known to exert anti-tumor activity *in vitro* in a plethora of tumor cells including breast, brain, skin, thyroid, prostate and colorectal, and in animal models of these tumors (for a review see Bifulco et al. 2006). The involvement of CB1 and/or CB2 receptors signaling in the anti-tumor effects observed has been revealed also by a pharmacological approach using cannabinoid receptors selective antagonists. In fact, several studies reported

that rimonabant counteracts the anti-tumor effects of anandamide related compounds or other cannabinoid agonists in thyroid, breast and prostate cancers (Grimaldi et al. 2006; Portella et al. 2003; Bifulco et al. 2001; Sarfaraz et al. 2005) where the effects depend on CB1 receptor activation. In other tumor types, such as glioma, rimonabant fails to revert the anti-proliferative action of cannabinoid agonists, whereas the selective CB2 antagonist SR144528 (Sanchez et al. 2001) or a combination of the CB1/CB2 antagonists can partially prevent this effect (Jacobsson et al. 2001). However, it has also been reported that pre-incubation with these antagonists potentiates the anandamide-mediated cell death of glioma cells (Maccarrone et al. 2000). Considering the anti-tumor properties of the cannabinoid receptor agonists, it could be expected that cannabinoid receptor antagonists, such as rimonabant, if used alone, would enhance proliferation of malignant and normal cells leading to cancer. Collected data excluded this possibility, reporting rather that not only agonists to cannabinoid receptors but also antagonists, when used alone, are able to inhibit tumor growth (Bifulco et al. 2004) or induce apoptosis in cancer cells (Powles et al. 2005; Derocq et al. 1998). The first observation of a rimonabant potential anti-tumor action was provided in rat thyroid cancer cells (KiMol) *in vitro* and in thyroid tumor xenografts induced by KiMol injection in athymic mice. In this model, rimonabant was able to partially prevent the anti-tumor effect of VDM-11 and AA-5-HT, inhibitors of endocannabinoid degradation. However, rimonabant, when used alone, in the same model and at the same dose shown previously to counteract anandamide effect (0.7 mg/kg intra-tumoral, twice a week for two weeks), did not enhance tumor growth, instead exerting a small but significant anti-tumor effect on thyroid tumors, both *in vitro* and *in vivo* (Bifulco et al. 2004). It is noteworthy that micromolar concentrations of rimonabant decreased viability of primary mantle lymphoma cells isolated from tumor biopsies of two patients (Flygare et al. 2005). Moreover rimonabant showed an additive negative effect on the viability of the mantle cell lymphoma cell line Rec-1 when combined with equipotent doses (5 μM) of anandamide. Long-term growth of Rec-1 cells was also inhibited by rimonabant, but at a dose ten fold higher (10 μM) in comparison of the more efficacious CB1/CB2 agonist WIN-55,212-2. However it has not yet provided a molecular mechanism for the anti-tumor action of rimonabant. It has been proposed that the observed effects could be ascribed to: 1) a tonic anti-proliferative action mediated by the local endocannabinoids through mechanisms independent from CB1 receptor, particularly when CB1 receptors are blocked by the antagonist rimonabant; and 2) the inverse agonist properties of rimonabant on the receptor. It may also act by CB1 receptor independent mechanisms. These possibilities could explain the paradox whereby both CB1 agonists and antagonists display anti-tumor activity.

2. AIMS OF THE STUDY

Modulating the endocannabinoid system turned out to hold therapeutic promise in a wide range of diseases and pathological processes. In this frame, given the ubiquity of the endocannabinoids and their receptors and their modulating activity on proteins and nuclear factors involved in cell fate control, there has been increasing evidence for a role of the endocannabinoid system in neoplastic transformation and a remarkable interest to exploit this role for therapeutic gain. Based on the *in vivo* efficacy, it has been suggested that anti-tumor effect of (endo)cannabinoids beyond tumor growth arrest and apoptosis induction, could be partially ascribed to the inhibition of metastatic spreading and neoangiogenesis. However, despite the collected pharmacological evidence on cannabinoids' properties, very little is known concerning the endogenous function of the endocannabinoid system and in particular of CB1 signaling in the processes of tumor growth, metastatic spreading and angiogenesis, all steps of tumor progression. In particular we focused our attention on breast-cancer, the number-one cause of cancer in women, starting from the hypothesis that targeting CB1 receptor signaling could induce a non-invasive phenotype in breast cancer also through a direct interference on the angiogenic process.
Therefore, the main aims of this doctorate thesis were:
1) to provide key information on the involvement of the endocannabinoid system in breast cancer proliferation, cell cycle regulation, invasiveness and metastatic spreading;
2) to characterize the specific role of CB1 receptor in the above processes, looking also at its association with lipid rafts/caveolae and at their role in the observed CB1-mediated effects;
3) to study the endogenous role of the endocannabinoids and CB1 receptor signaling in angiogenesis, with particular emphasis to tumor-induced angiogenesis, providing also new potential targets for the anti-angiogenic therapeutic strategy.
To address these issues, suitable *in vitro* and *in vivo* models of breast cancer and angiogenesis were employed. The CB1 antagonist/inverse agonist rimonabant and a stable analogue of the endocannabinoid anandamide were utilized as critically important tools to explore, respectively, the basal functions of endocannabinoid system and CB1 signaling and its therapeutic exploitation.

3. MATERIALS AND METHODS

Cell lines

The human breast cancer cell lines used in this study were: MDA-MB-231, MCF-7, T47D. All cell lines were from Interlab cell line collection (IST, Genova) and were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. Human umbilical endothelial cells (HUVECs) were isolated from freshly delivered umbilical cords, as described (Hisano et al. 1999) and grown in M199 medium supplemented with 10% foetal bovine serum, aFGF, bFGF, EGF, hydrocortisone and heparin. Pig aortic endothelial (PAE) cells were kindly donated by Prof. F. Bussolino (IRCC, University of Turin, Italy). Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Drugs

The selective CB1 antagonist rimonabant, SR141716, was kindly provided by Sanofi-Aventis Research (Montpellier, France). Met-F-AEA (2-methyl-2'-F-anandamide), a metabolically stable analogue of anandamide, was purchased from Sigma-Aldrich. Met-F-AEA is indicated in the text as anandamide.

Proliferation assay

Cell proliferation was evaluated, *in vitro*, by measuring [^{3}H]thymidine incorporation. In brief, $5x10^3$ cells/ml were seeded into 96-well plates, immediately treated with the drugs, incubated for 24 hr at 37°C (5% CO_2), then pulsed with 0.5 μCi/well of [^{3}H]-thymidine (Amersham Biosciences Europe, Italy) and harvested 4 h later. Radioactivity was measured in a scintillation counter (Wallac, Turku, Finland).

Flow cytometry

For assessment of apoptosis the cells, subjected to various treatments, were collected at the indicated time points, washed with PBS and resuspended at $1x10^6$ cells/ml in annexin V binding buffer (0.01 M HEPES, pH 7.4; 0.14 M NaCl; 2,5 mM $CaCl_2$). Apoptotic cell death was identified by double supra-vital staining with recombinant FITC-conjugated annexin-V-antibody (Dakocytomation) and propidium iodide. Flow cytometric analysis was performed immediately after staining. Data acquisition and analysis were performed in a Becton Dickinson FACSCalibur flow cytometer using CellQuest software. Each sample was analysed using 10,000 events.

For cell cycle analysis cells were collected, fixed in 300 μl of PBS plus 700 μl of ethanol 70% and kept at -20ºC o.n. Propidium iodide (10 μg/ml) in PBS containing 100 U/ml DNase-free RNase A was added to the cells; after 15 min at room temperature cells were subjected to flow cytometric analysis using ModFit LT v3.0 program from Verity Software House, Inc. (Topsham, ME).

Each sample was analysed using 10,000 events corrected for debris and aggregate populations.

Immunoprecipitation and Western blot analysis

For the immunoprecipitation assay cells were lysed at 4°C in RIPA buffer (500 µl) and centrifuged at 13,000 rpm. The samples (500 µg of total cell extract) were incubated for 12 h at 4°C with 3–4 µg of the desired monoclonal antibodies, then added with 50 µl of protein A/G–agarose (Sigma, St Louis, Mo) and incubated overnight at 4°C. About 50 µg of proteins were loaded on 12% SDS-polyacrylamide gels under reducing conditions. After SDS–PAGE, proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% non fat dry milk (Bio-Rad Laboratories Inc., Richmond, CA) and incubated with the specific antibody. After three washes, filters were incubated for 1hr at room temperature with horseradish peroxidase-conjugated secondary antibody (Bio-Rad). The membranes were then stained using a chemiluminescence system (ECL-Amersham Biosciences) and then exposed to X-ray film (Kodak). Immunoreactive bands were quantified using Quantity One 1-D analysis software (Bio-Rad).

Reverse-Transcriptase Polymerase Chain Reaction.

Total RNA was extracted from cell lines by guanidinium thiocyanate-isopropanol method. Reverse transcription (RT) was performed using Moloney murine leukemia virus reverse transcriptase and random oligonucleotide primer. The first-strand cDNA was then amplified using two different sets of primers. The sense primer CB1-F (5'-GATGTCTTTGGGAAGATGAACAAGC-3') and the antisense primer CB1-R (5'-GACGTGTCTGTGGACACAGACATGG-3') were used to amplify the CB1R; the primers for amplification of alpha actin were A1F (5'-ATGATCTGGACCATCATCCT-3') and A1R (5'-CTRATGTGGAAGTTRTGCATG-3'). Polymerase chain reactions (PCR) were performed 30 s at 93°C, 1 min at 59°C, and 1 min at 69°C for 25 to 28 cycles. Amplified DNA was extracted with chloroform and electrophoresed in a 2% agarose gel in 0.5x Tris-borate/EDTA.

Human angiogenesis array

Human angiogenesis antibody array on MDA-MB-231 tumor-conditioned medium was performed following manufacturer's instructions (RayBiotech, Inc.). The array membranes were incubated with conditioned medium, stained using a chemiluminescence system (ECL-Amersham Biosciences) and then exposed to X-ray film (Kodak). Immunoreactive spots were quantified using Quantity One 1-D analysis software (Bio-Rad).

Endothelial cell migration assay

Chemotactic motility of breast cancer and endothelial cells was assayed using Transwell (Corning Costar, Cambridge, MA) with 6.5-mm diameter

polycarbonate filters (8-μm pore size). Briefly, the lower surface of the filter was coated with 50 μg/ml of collagen IV or matrigel. The fresh medium containing the chemoattractant (10% FBS or bFGF) was placed in the lower compartment. Cells were harvested and suspended at a final concentration of $20x10^4$ cells/ml in medium containing 1% FBS. Cells were subjected to the treatments before seeding. The chamber was incubated at 37°C for 4 h. Migrated cells were fixed and stained with Hoechst. Non-migrating cells on the upper surface of the filter were removed by wiping with a cotton swab. The number of migrated cells were counted by a light microscope at 20X magnification: ten randomly chosen microscopic fields were counted per well and the mean number was determined. Background levels of cells migrated in the absence of chemotactic stimuli (chemokinesis) were subtracted from all the experimental points.

Capillary-like tube formation assay

A 48-well plate was coated with 120 μl/well Matrigel for 30 minutes at 37°C. Serum-starved HUVEC cells ($4x10^4$ cells/ml) were seeded in 250 μl of M199 medium in the presence of anandamide or rimonabant with or without bFGF (10 ng/ml). Complete medium plus bFGF and not supplemented basal medium were used as positive and negative controls, respectively. After 6 h of incubation, capillary-like tube formation was examined under an inverted phase microscope. Cells were fixed with PBS containing 0.2% glutaraldehyde, 1% paraformaldehyde, photographed and analysed by Scion Image software.

Gelatin zymography

Quiescent HUVECs were serum-starved, pre-treated with anandamide or rimonabant and then stimulated with bFGF (10 ng/ml) for 24 h. The conditioned media were collected and clarified by centrifugation. Conditioned media containing equal amounts of secreted proteins were mixed with non reducing Laemmli sample buffer (2X) and loaded on 7.5% SDS-polyacrylamide gels containing 1 mg/ml gelatine (Sigma). The gels were then incubated for 30 min at room temperature twice in 2.5% (v/v) Triton X-100 and rinsed 5 times in distilled water. The gels were incubated at 37°C for a further 18 h in developing buffer (200 mM NaCl, 5 mM $CaCl_2$, 50 mM Tris/HCl pH 7.6), then stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad), followed by destaining in 10% (v/v) acetic acid/30% (v/v) methanol. Gelatinolytic activity of MMP-2 was detected as unstained bands on a blue background. HUVEC stimulated with PMA (phorbol myristate acetate) (0.1 μM, 24 h) were used as positive control for MMP-2 activity.

Chick embryo chorioallantoic membrane assay

Fertilized chicken eggs were incubated under conditions of constant humidity at 37 °C. On the forth day of incubation, a window was opened in the eggs-shell after removal of 3-4 mL of albumen so that the developing chorioallantoic membrane (CAM) was kept detached from the shell. The window was sealed

with a piece of adhesive tape and the eggs were incubated at 37 °C. On the ninth day rimonabant, pre-encapsulated in 1.5% alginate bead at the final concentration of 0.1 μM, was applied onto CAM of the eggs. The allantoic vessels around the cells' pellets were counted under a stereomicroscope and photographed with a digital camera at the time of cell implants and 72 h later.

Rabbit cornea assay

Angiogenesis was studied in the cornea of albino rabbits since this is an avascular and transparent tissue where inflammatory reactions and growing capillaries can be easily monitored and changes quantified by stereomicroscopic examination. Experiments have been performed in accordance with the guidelines of the European Economic Community for animal care and welfare (EEC Law No. 86/609).

Slow-release pellets were prepared in sterile conditions, incorporating the test substances into a casting solution of an ethynil-vinyl copolymer (Elvax-40, DuPont-De Nemours, Wilmington, DE). In the lower half of New Zealand white rabbit eye (Charles River, Lecco, Italy), anaesthetized by sodium pentothal (30 mg/kg), two adjacent micro-pockets were surgically produced using a pliable iris spatula. The angiogenic inducer bFGF (200 ng/pellet) was implanted in one pocket, the test compound or the control (Elvax-40 alone) in the other one. Daily observations of the implants were made with a slit lamp stereomicroscope by two independent operators in a blinded manner. An angiogenic response was considered positive when budding of vessels from the limbal plexus occurred after 3-4 days and capillaries progressed to reach the implanted pellets. Angiogenic activity was expressed as the number of implants exhibiting neovascularization over the total implants studied. Potency was scored by the number of newly formed vessels and by their growth rate. Data are expressed as angiogenic score calculated as [number of vessels x distance from the limbus] (Morbidelli et al. 2003).

Oxygen Induced Retinopathy

Briefly, litters of 7-day-old C57Bl/6J mice were placed with their nursing mothers into an oxygen chamber maintained at 75% oxygen until postnatal day 12. Mice were removed from the chamber at day 12 and were maintained in room air until day 17. By day 17, retinal neovascularization was present in 100% of the experimental animals. Newborn mice exposed only to room air served as controls.

Quantization of neovascular response in retinal whole mounts

New vessel tufts on the surface of the retina were stained using Isolectin GS-IB4 conjugated to Alexa fluor 488 (Invitrogen). Eyes from day 17 control and experimental animals were removed and fixed with 2% paraformaldehyde in PBS for 2 h at 4°C. The cornea was cut approximately 2 mm anterior to the limbus and removed. The lens was removed from the eye and any remaining hyaloid vessels were teased from the retina. The resulting eye cups were

incubated for 30 minutes at room temperature with 5 μg/ml of lectin in PBS. The eyes were washed extensively with PBS and the retina was removed, cut into eights and mounted on glass slides. Images were obtained of the retinal segments and the percentage of the total retinal area occupied by new vessels was quantified using the MetaMorph image analysis program.

Statistical analysis

All data were presented as means ± SD. Statistical analysis was performed using one-way ANOVA. In the case of a significant result in the ANOVA, Student T test was used for dose-response curve and Bonferroni's test for post hoc analysis for all other experiments. A *P* value less to 0.05 was considered statistically significant.

4. RESULTS AND DISCUSSION

4.1 Anandamide as emerging suppressor of breast tumor growth: molecular aspects

In the present work we aimed to demonstrate whether stimulation of CB1 receptors could interfere not only with breast cancer growth but also with metastatic process. Our starting hypothesis was that CB1 activation could induce a non-invasive phenotype in breast cancer cells. To this end we investigated the effects of anandamide on the growth, invasiveness and metastatic capabilities *in vivo* of a highly invasive and metastatic human breast cancer cell line, MDA-MB-231, which harbors constitutive ras activation and impaired p53 and ER expression. MDA-MB-231 expressed high levels of CB1 receptor, analyzed by RT-PCR and Western blot. CB1 was localized on the plasma membrane, mainly concentrated in large spots as observed by confocal microscopy (Fig. 6A and B) (see enclosed Sarnataro et al. 2005).

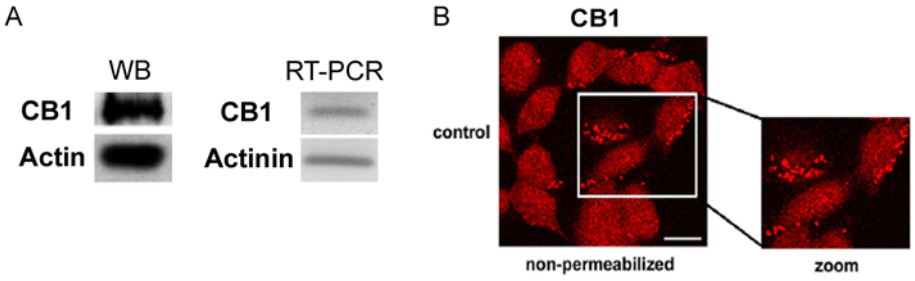


Figure 6: Expression of CB1 in MDA-MB-231 cells. (A) Cell extracts were analyzed by Western blot and RT-PCR. (B) Surface distribution of CB1 (red spots) in non-permeable conditions was determined by confocal microscopy.

Anandamide strongly inhibited the proliferation of MDA-MB-231 in a dose-dependent manner, evaluated by [^{3}H]-thymidine incorporation into DNA. Interestingly, the inhibitory effect was higher than the one observed in T47D, a poorly invasive, non-metastatic breast cancer cell line, which also express CB1 receptor (Fig. 7A). Noteworthy, anandamide has been previously reported to inhibit the growth of a metastasis-derived thyroid cancer cell line more potently than a primary cancer cell line (Bifulco et al. 2001). The lowest effective dose, 10 μM, was used in all following experiments. Anandamide did not induce apoptosis or necrosis, as revealed by a flow cytometric assay with annexinV/propidium iodide double staining (see enclosed paper Grimaldi et al. 2006). To further investigate the nature of growth inhibition, the cell cycle phase distribution was assessed by flow cytometry. Exponentially growing

MDA-MB-231 cells, incubated for 24 h with 10 μM anandamide, accumulated in the S phase of the cell cycle (Fig. 7B).

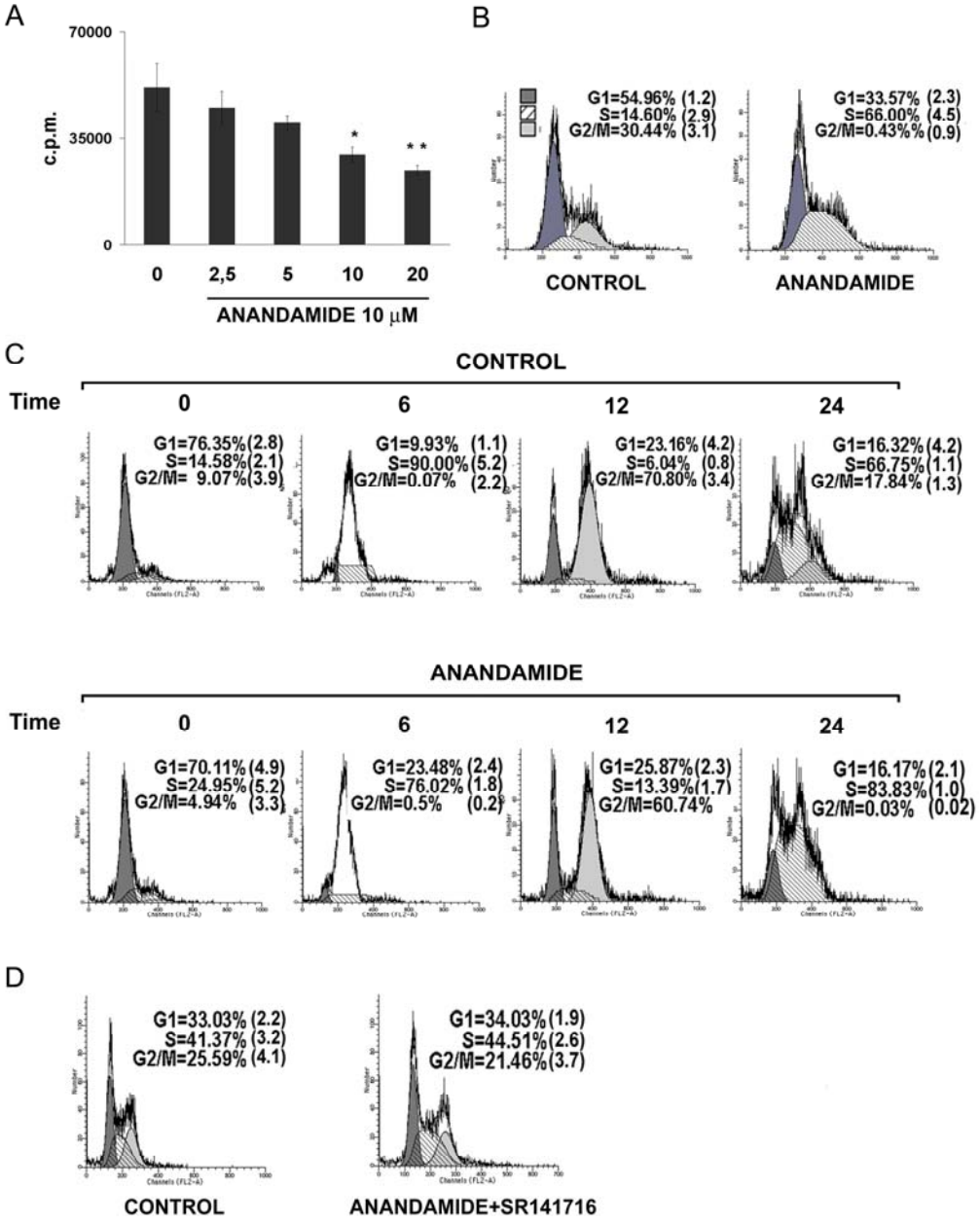


Figure 7: Effect of anandamide on cell proliferation and cell cycle distribution of MDA-MB-231. (A) The graph reports the [^{3}H]-thymidine incorporation levels (counts per minute) expressed as mean ± SD values of three experiments in triplicates. ANOVA, **P*<0.05, ***P*<0.01. (B-D) The graphs report the percentage (mean of three experiments in triplicate) of cells in each cell cycle phase, with SD in parentheses. SR141716, rimonabant.

To analyze the effect of anandamide on cell cycle progression in more detail, cells were synchronized in late G1-S phase by hydroxyurea (HU, 2 mM) (for further experimental details see enclosed paper Laezza et al. 2006). The cell cycle distribution was analyzed when cells were released from this blockade with complete medium in the presence or absence of anandamide. Treatment with anandamide determined a delay in S phase progression. By 24 h approximately 84% of the treated cells were arrested in S phase (Fig. 7C). Removal of anandamide from the culture medium led to reversibility of the cytostatic effect (data not shown). Moreover pre-treatment with the CB1 receptor selective antagonist rimonabant (SR141716) (30 min, 0.1 μM) before anandamide exposure (24 h, 10 μM) prevented the S phase blockade, suggesting a CB1 receptor-dependent signaling pathway (Fig. 7D) (see Laezza et al. 2006). As expected, cell cycle machinery was deeply altered by anandamide: cyclins A and E protein levels were decreased, whereas Cdk inhibitors $p27^{kip1}$ and $p21^{waf}$ were increased compared to control (see Figure 2 in Laezza et al. 2006). Since Cdk2, activated by the interaction with cyclin E in late G1 phase and cyclin A in S phase, determines the ability of cells to enter into, and progress through S phase, we assayed anandamide-treated cell lysates for Cdk2 kinase activity. Cdk2 protein complexes were immunoprecipitated from total cell lysates and assayed *in vitro* for kinase activity, monitored by the ability to phosphorylate histone H1. We observed a reduction in Cdk2 activity, whereas protein levels were not affected by anandamide treatment. Moreover, cyclin E was decreased in immunocomplexes with Cdk2, whereas $p27^{kip1}$ and $p21^{waf}$ were augmented (Fig. 8A).

Thus, it is highly likely that the S phase arrest occurs as a consequence of the specific loss in Cdk2 activity because of the up-regulation of $p21^{waf}$ and the reduced formation of the active complex cyclin E/Cdk2. Furthermore, we speculated that the arrest of cell cycle progression in S phase induced by anandamide might be related to the activation of an intra S-phase checkpoint. Recently, it has been demonstrated that the checkpoint kinase Chk1 mediates both intra-S and G2 phase checkpoints by targeting the Cdc25A phosphatase to proteolysis following DNA damage (Zhou and Bartek 2004). The Cdc25A positively regulates the cell cycle by activating Cdks. Chk1 is required for the majority of Cdc25A phosphorylations *in vivo* during interphase; these events target Cdc25A for rapid degradation. After anandamide treatment, we observed a time-dependent increase of the ATR-phosphorylated Chk1 species (Chk1 S345P) phosphorylation, without variation of its protein levels. To investigate whether Chk1 phosphorylation induced by anandamide treatment results in Chk1 activation, we analyzed the stability of Cdc25A protein, which was completely degraded at 24 h. In presence of staurosporine (8 nM, 24 h), a well-known Chk1 inhibitor, the Cdc25A degradation induced by anandamide was reverted (Fig. 8B).

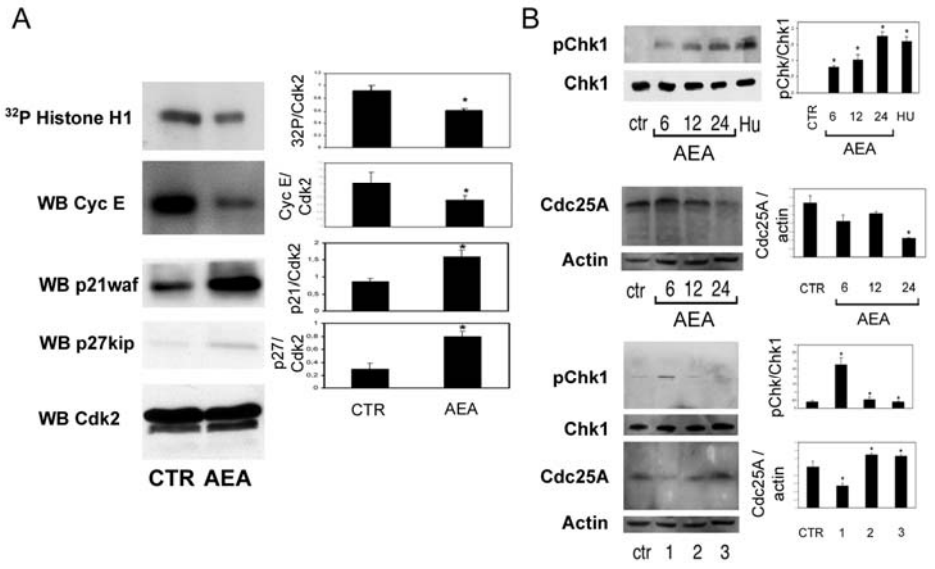


Figure 8: Effect of anandamide on cell cycle machinery. (A) The kinase activity of Cdk2 in immunoprecipitated immunocomplexes was measured with histone H1 as substrate. The amounts of Cdk2 and Cyclin E into immunocomplexes were analyzed by Western blotting. The diagram shows quantification of the intensity of bands, calibrated to the intensity of the Cdk2 bands, expressed as mean ± SD. **P* < 0.05 for AEA vs CTR. (B) Evaluation of Chk1 activity. Treatments: 1, anandamide; 2, staurosporine; 3 anandamide + staurosporine. The diagrams show quantification of the intensity of bands, calibrated to the intensity of the Chk1 or actin bands. **P* < 0.05.

For the first time we observed that anandamide activates Chk1 by arresting cells in S phase through Cdc25A proteolysis, preventing Cdk2 activation by dephosphorylation on critical inhibitory residues (Thr14/Tyr15), and thus inhibiting cyclin E-Cdk2 and Cyclin A-Cdk2 complexes. In the breast cancer cell line MCF-7, Cdc25A activity is necessary for both the activation of Cdk2 and subsequent induction of S phase entry suggesting that this potential oncogene could represent a therapeutic target in breast cancer (Cangi et al. 2000). Moreover, MDA-MB-231 cells present prerequisites (over-expression of the proto-oncogenes cyclin E, Cdc25A and mutated p53) able to activate the ATM/ATR-regulated DNA damage network. Actually, we are investigating through what kind of DNA damage anandamide may activate the S phase checkpoint. We could speculate that it may be a consequence of a replication stress rather than of a genotoxic action of anandamide. Then we propose that both the transient cell cycle checkpoint response (Cdc25A degradation) and the delayed one ($p21^{waf}$ cascade) are induced by anandamide and converge on suppression of Cdk2 activity leading to cell cycle arrest in human breast cancer cells (Fig. 9).

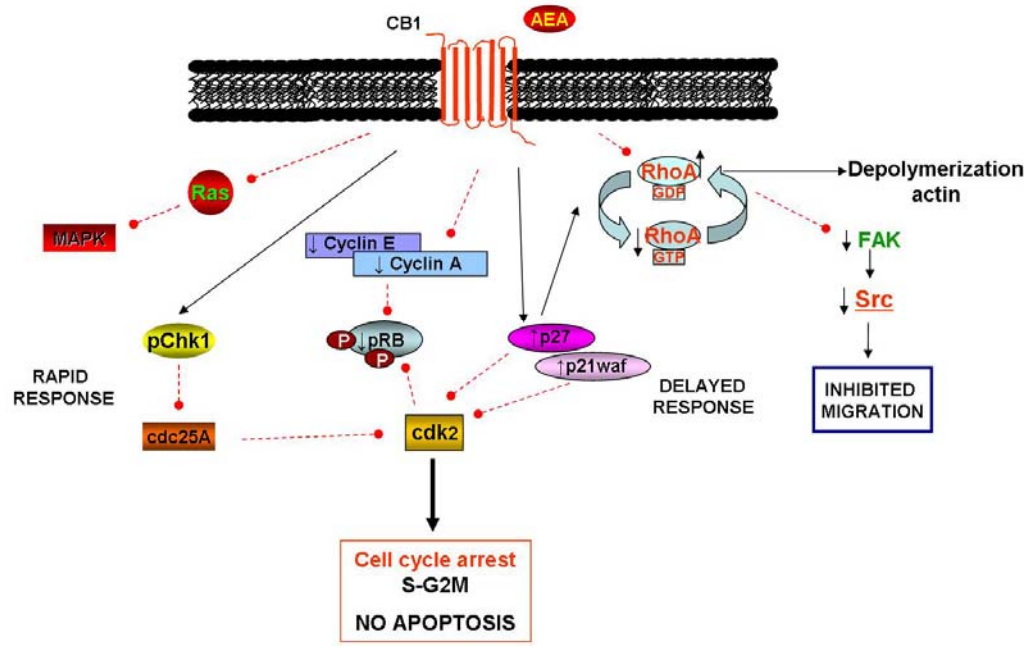


Figure 9: Schematic model depicting anandamide effect on cell cycle machinery and relevant signal transduction pathways.

This mechanism of action, already reported for chemotherapeutic drugs such as gemcitabine, a potent radio-sensitizer provides strong evidence for a direct role of anandamide related compounds in the activation of cell cycle checkpoints. This could be of great medical interest since recent findings suggest that DNA damage checkpoints might become activated in the early stages of human tumorigenesis leading to cell-cycle blockade or apoptosis and could act as a barrier against cancer and genetic instability, thereby constraining tumor progression (Bartkova et al. 2005). This notion matches with the proposed tumor-suppressor action of an endogenous sustained tone of endocannabinoids and with therapeutic exploitation of CB1 agonists and compounds (like FAAH inhibitors) aimed to augment the levels of endocannabinoids.

4.2 Anandamide induces a non-invasive phenotype in breast cancer: *in vitro* and *in vivo* evidence

Many studies have described the potential anti-tumor properties of (endo)cannabinoids (Bifulco et al. 2006). However, the possibility that stimulation of CB1 receptor interferes with metastatic processes has not been fully explored. Therefore we decided to evaluate anandamide effect on MDA-MB-231 cell migration. We performed migration assays using Boyden chambers coated with type IV collagen, the most prevalent component of the

basement membrane. We observed that anandamide inhibited for at least 60% cell migration toward a chemotactic stimulus and this effect was antagonized by pre-treatment with the CB1 antagonist (Fig. 10A). We also performed wound-healing assay founding that in control cells the wound was closed as early as 6 h, due to the influx of highly migratory cells, wherease in anandamide-treated cells only a little percentage was able to migrate (Fig. 10B).

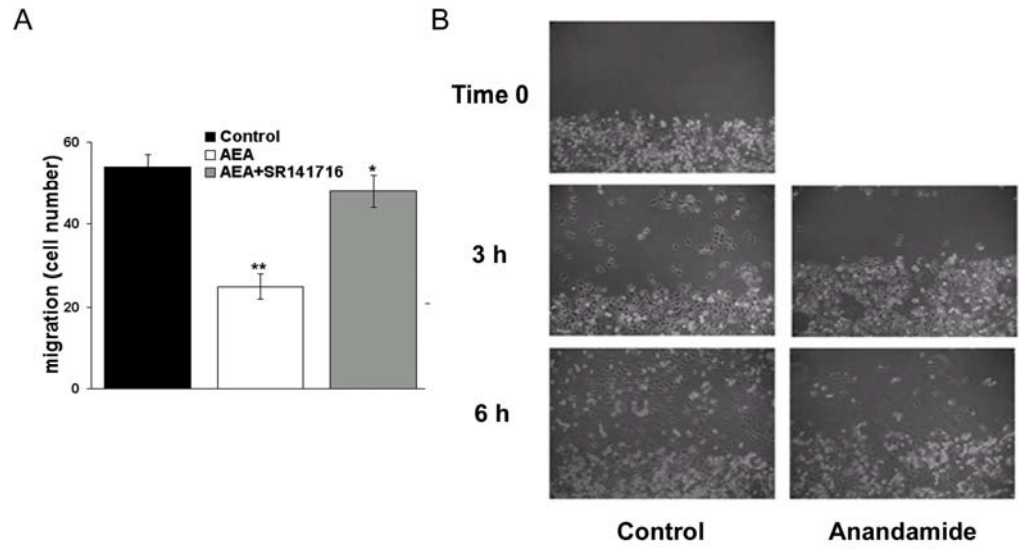


Figure 10: Anandamide inhibits breast cancer cell migration. (A) Boyden chambers transwell migration assay. Shown is the mean of migrated cells number ± SD values of triplicates from four experiments. ANOVA, with post hoc Bonferroni test, **P*<0.05, ***P*<0.001. The background represented by the number of migrated cells in absence of the chemoattractant factor was subtracted from each experimental point. (B) Wound healing assay. Plates were photographed at different time points. AEA, anandamide; SR141716, rimonabant.

The hypothesis that CB1 receptor stimulation could interfere with metastatic processes was also verified in a model of metastatic infiltration *in vivo*. To this end the murine breast cancer TSA-E1 cells were used. This cell line responded to anandamide treatment as well as MDA-MB-231, through inhibition of proliferation and migration (see Grimaldi et al. 2006). TSA-E1 cells were injected in syngenic C57BL/6N mice to induce lung metastases. Animals were divided into three groups and anandamide plus vehicle (0.5 mg/kg/ dose), anandamide plus rimonabant (0.7 mg/kg/dose), or the vehicle alone, were injected i.p. every 72 h. Anandamide significantly reduced the number and dimension of metastatic nodes, evaluated 21 days after, this effect being reduced by CB1 antagonist (Fig. 11).

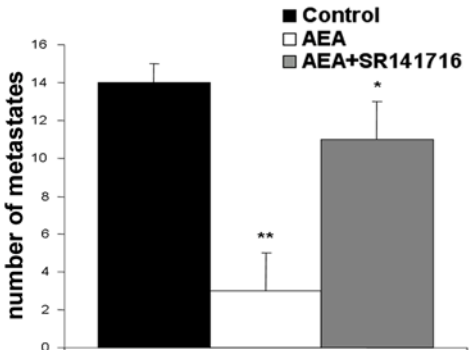


Figure 11: Anandamide reduces the number of lung metastatic nodes. Metastatic nodes, fixed in Bouin's fluid, were scored with a stereoscopic microscope. Shown is the mean and SD values of each group. ANOVA, with posthoc Bonferroni test, $^{**}P<0.001$, $^{*}P<0.05$. AEA, anandamide; SR141716, rimonabant.

Several molecules have been associated with the development of a more invasive and metastatic phenotype, such as integrins, focal adhesion kinase (FAK), and CD44. We hypothesized that CB1 receptor stimulation might induce a non-invasive phenotype in metastatic breast cancer cells through the interference on one of these targets. Our data indicate that, although integrin expression on cell membrane surface was not significantly changed by anandamide, cancer cell affinity for collagen IV and migration were strongly reduced, thus suggesting a change of the activation state of certain integrins (see enclosed paper Grimaldi et al. 2006). Since up-stream events in the signal pathways generated by integrin clustering are FAK and Src phosphorylation, in order to clarify the role of the endocannabinoid system in tumor progression and to gain further insights into the molecular mechanisms involved, we therefore analyzed the status of FAK and Src in anandamide-treated cells. FAK and Src, two tyrosine kinases both located at adhesion plaques, are involved in cell motility, adhesion, and invasion as well as in cell proliferation and survival (Hsia et al. 2003). After integrin clustering, FAK is autophosphorylated and recruits kinases of the Src family to focal adhesions to mediate cell motility. We found that total FAK and Src levels were not modified by anandamide treatment, whereas a remarkable decrease of FAK and Src phosphorylation was induced, in a way antagonized by rimonabant (Fig. 12A and B). We then hypothesized that anandamide could alter endogenous Rho signaling in these cells and that this alteration might decrease their metastatic potential *in vitro*. Indeed the Rho family of small GTPases (RhoA, Rac1, Cdc42) may play an essential role in the dissemination of carcinomas, transducing extracellular signals into intracellular events that lead to the remodeling of the actin cytoskeleton, ultimately controlling the cell morphology and a variety of functions such as cell motility, aggregation, polarity, and contraction (Bar-Sagi and Hall 2000). Anandamide (10 μM, 24 h) inhibited RhoA activity and blocked its translocation from cytosol to cell membrane, an indispensable event for RhoA activation (Fig. 12C) (see enclosed manuscript Laezza et al. *submitted*).

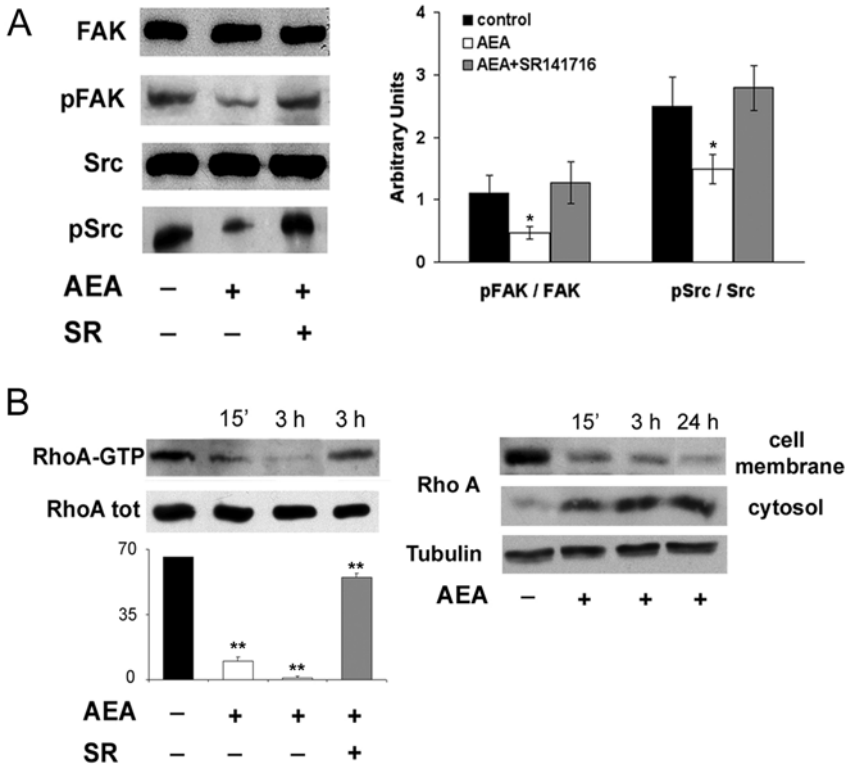


Figure 12: Anandamide decreases FAK and Src phosphorylation and inhibits RhoA activity and membrane translocation. (A) Immunoprecipitates with anti-FAK and –Src were analyzed by Western blot with anti-phosphorylated tyrosine. The graph reports mean ± SD values of optical density of three independent experiments. ANOVA, with posthoc Bonferroni test, **P*<0.05. (B) RhoA activity is represented as the relative ratio of the density of GTP-RhoA against that of total RhoA. The relative ratio of control is 100%. All data are presented as the mean ± SD of three experiments. ANOVA, ***P*<0.001. AEA, anandamide; SR, rimonabant.

Our data clearly demonstrate that crucial events of the metastatic process are targeted following CB1 receptor activation (Fig. 9). This leads to inhibition of metastases formation in mice models. We suggest that CB1 receptors might be a target for therapeutic strategies aimed at retarding not only the growth but also the progression of breast carcinomas.

4.3 Anandamide inhibits tumor-induced angiogenesis

Although we have clearly demonstrated that anandamide inhibits cell migration and related molecular pathways, it is not possible to exclude that other crucial molecular steps are concomitantly inhibited by anandamide *in vivo*, to determine the inhibition of tumor progression. One of these steps, could be the inhibition of tumor-induced angiogenesis, which providing nutrients to

proliferating cancer cells, is a critical event involved in the progression of solid tumors (Ferrara and Kerbel 2005). Increasing evidence suggests that anti-tumor activity of cannabinoid-related drugs could be at least in part ascribed to the inhibition of tumor-driven neo-angiogenesis in animal models. Anandamide was previously reported to inhibit the growth of thyroid tumors, down-regulating the expression of VEGF and its receptor Flt1 (Portella et al. 2003). In order to evaluate the effect of anandamide on breast cancer induced angiogenesis we performed a simple *in vitro* angiogenesis assay using as angiogenic stimulus the tumor-conditioned medium (TCM) derived from MDA-MB-231 cell cultures. Briefly, MDA-MB-231 cells were treated with anandamide (10 μM, 24 h) and then the TCM was tested on human umbilical vein endothelial cells (HUVECs) proliferation by [^{3}H]-thymidine incorporation into DNA. TCM significantly induced endothelial basal proliferation. We found that TCM from anandamide-treated MDA-MB-231 cells strongly reduced the proliferation of endothelial cells (Fig. 13).

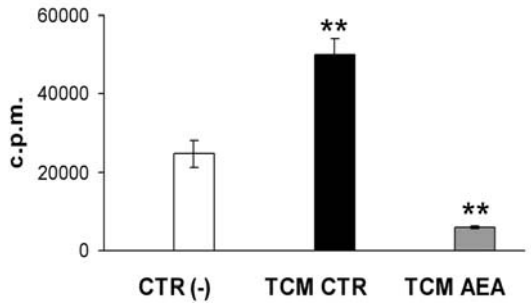


Figure 13: Tumor conditioned medium from anandamide-treated MDA-MB-231 inhibits angiogenesis. The graph reports the [^{3}H]-thymidine incorporation levels (counts per minute) expressed as mean ± SD values of three experiments in triplicates. ANOVA, $^{**}P<0.001$. CTR, control; AEA, anandamide.

This could be the result of an anandamide-switched balance between pro-angiogenic and anti-angiogenic factors produced by breast cancer cells in behalf of angiogenesis inhibitors. The next step was to investigate the nature and levels of pro- and anti-angiogenic molecules present in breast cancer TCM and their modulation after anandamide treatment. To this end we used an angiogenesis protein array (RayBiotech, Inc). We found that all tested pro-angiogenic factors were significantly reduced by anandamide treatment (Table 4). For instance VEGF was deeply down-regulated in comparison to the control (47%). Noteworthy, most growth factors and chemokines inhibited were those under the regulation of PPARγ pathway. PPARγ ligands can inhibit tumor-associated angiogenesis by blocking the production of ELR+CXC chemokines (Keshamouni et al. 2005). It has been reported that anandamide induces transcriptional activation of PPARγ, binding directly to PPAR-ligand binding domain (Bouaboula et al. 2005). We are now investigating whether PPARγ activation could be directly involved in the anti-tumor effect of anandamide.

Table 4

Angiogenic factors	TCM	TCM+ Anandamide	% inhibition (*vs* TCM)
Angiogenin	20 ± 0.8	19.3 ± 2.3	5%
ENA-78	3.8 ± 1.4	2.7 ± 1.5	29%
bFGF	10.2 ± 2	9.8 ± 3.3	4%
GRO	12.5 ± 0.1	10.7 ± 1.5	15%
IFNγ	1.4 ± 0.07	1.1 ± 0.08	21%
IL6	18 ± 0.5	14.9 ± 4.6	17%
Leptin	2.2 ± 0.2	0.9 ± 0.07	58%
MCP-1	57.1 ± 6.0	49.4 ± 9.5	13%
PDGF-BB	9.7 ± 1.9	6.8 ± 1.1	29%
PlGF	10.5 ± 1.8	8.3 ± 1.2	21%
RANTES	8.6 ± 0.1	7.9 ± 0.5	7%
TGFβ1	35.2 ± 3.7	26.7 ± 1.8	23%
TIMP1	37.1 ± 2.6	30.2 ± 1.8	18%
TIMP2	75.7 ± 10.7	50 ± 2.8	33%
Thrombopoietin	10.5 ± 1.3	6.8 ± 0.6	35%
VEGF	42.6 ± 7.6	22.4 ± 3.2	47%
VEGF-D	10.6 ± 2.3	6.8 ± 1.9	36%

Data are presented as mean ± SD of two experiments in triplicates.

4.4 Direct anti-angiogenic activity of anandamide: correlation to its tumor-suppressor efficacy

Despite some collected evidence about inhibition of angiogenesis in mice models of thyroid, skin and glioma tumors treated with cannabinoid-related drugs (Portella et al. 2003; Casanova et al. 2003; Blazquez et al. 2003), the mechanism and the extent of (endo)cannabinoids anti-angiogenic effects have not been fully explored. Therefore, keeping on our studies on anandamide activity, we aimed to investigate its potential direct anti-angiogenic effect using porcine aortic endothelial cells (PAE) and human umbilical vein endothelial cells (HUVECs) in two-dimensional and three-dimensional angiogenic models *in vitro* and in the chick chorioallantoic membrane assay (CAM) *in vivo*. It has already been reported that HUVECs express functional cannabinoid CB1 receptors (Liu et al. 2000). At first we confirmed, through a Western blot analysis, that also PAE cells, express CB1 receptors. Moreover we observed that incubation with anandamide (10 μM, 24 h) decreases CB1 protein expression in both endothelial cell types (see enclosed paper Pisanti et al. 2007, Figure 1A). In order to evaluate anandamide effect on endothelial cell proliferation *in vitro*, its inhibitory potency on bFGF-induced proliferation (10 ng/ml, 24 h) was assessed by [^{3}H]-thymidine incorporation. We found that anandamide inhibits endothelial cell proliferation in a dose-dependent manner, with a dose-dependency between 5 and 10 μM (Fig. 14A). The inhibitory effect was not due to cytotoxicity since anandamide had no effect on endothelial cell normal growth without bFGF stimulation (Fig. 14B). Furthermore, the anti-

proliferative effect observed was almost totally counteracted by pre-incubation with rimonabant, indicating that inhibition of proliferation depends on CB1 receptor signaling (Fig. 14A).

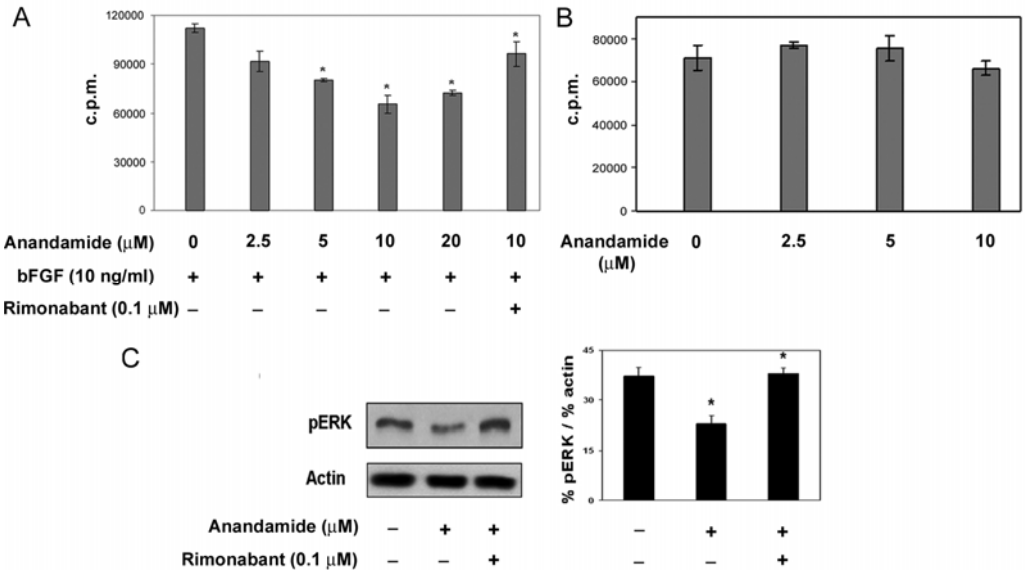


Figure 14: Anandamide inhibits bFGF-induced endothelial cell proliferation through inhibition of ERK phosphorylation. (A-B) The graphs report the [^{3}H]-thymidine incorporation levels (c.p.m.) expressed as mean ± SD values of three experiments in triplicates. ANOVA, **P*<0.05. (C) Cell lysates were blotted with anti-pERK antibody. The diagram shows quantification of the intensity of the bands, calibrated to the intensity of the actin bands, expressed as means ± SD (ANOVA, **P*<0.05).

Angiogenesis requires coordinated regulation of multiple signaling pathways. Mitogen activated protein kinases are activated by growth factors, including bFGF and VEGF, and have been implicated to play critical roles during angiogenesis (Beckner, 1999). ERK is usually strongly activated by growth factors and is important for cell proliferation. We found that ERK phosphorylation was reduced of 40% by anandamide pre-treatment before stimulation with bFGF, the inhibition being reverted by CB1 antagonist, in agreement with the observed inhibition of proliferation (Fig. 14C). ERK is known to be usually activated by cannabinoid agonists (Bouaboula et al. 1995). However, in this case, the observed inhibition of bFGF-induced ERK activation via CB1 signaling, already reported in neuronal cells for NGF-induced ERK, constitutes an interesting paradigm for the signaling link between GPCRs and RTKs (transinactivation) (Rueda et al. 2002). Cell cycle phase distribution was not altered by anandamide treatment (10 μM, 24 h), whereas we observed apoptosis induction (20% of sub-G1 positive cells compared to the control), confirmed also by a flow cytometric analysis with annexinV/propidium iodide double staining (Fig. 15A and B). It has been previously reported that anandamide induces apoptosis in HUVECs via

vanilloid receptor VR1 stimulation (Yamaji et al. 2003). Therefore we also investigated the role of CB1 receptor signaling pathway in the pro-apoptotic action of anandamide; pre-treatment with the CB1 antagonist SR141716 (0.1 μM, 30 min) partially prevented anandamide-induced apoptosis (Fig. 15B).

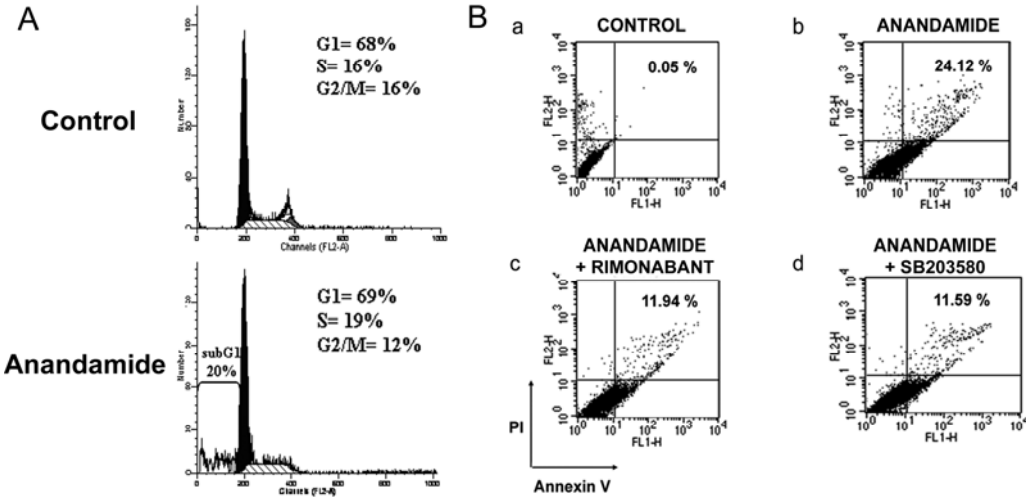


Figure 15: Effect of anandamide on apoptosis and cell cycle progression of endothelial cells. (A) Control and anandamide parts show the relative percentage of cells in each phase. Apoptosis was calculated as the percentage of sub-diploid (sub-G1) DNA peak. (B) In each part is shown the percentage sum of annexinV^+ and annexinV^+/propidium iodide$^+$ cells. The parts are representative of four independent experiments.

Among the pathways that control cell survival in endothelial cells we investigated p38 MAPK and NFkB pathways. We observed that p38 MAPK inhibition by SB203580 (10 μM, 1 h) partially prevents anandamide-apoptosis, as the CB1 antagonist, demonstrating that p38-MAPK-signaling pathway is required for CB1-mediated apoptotic signals in endothelial cells. Another important factor that regulates cell death or survival is the transcription factor NFkB, which has been proposed to function either as a pro-apoptotic or as an anti-apoptotic protein depending on cell type and apoptotic inducers (Barkett and Gilmore 1999). In our model we found that with increasing time there was a decrease of non-phosphorylated form of IkBα, targeted for degradation by phosphorylation on Ser 32, which indicated the involvement of NFkB activation following anandamide treatment in endothelial cells (Fig. 16A). NFkB activation by CB1 agonist-CP55,940 was reported in PC12 cells, where apoptosis was also observed (Erlandsson et al. 2002). A potential link between the THC-induced apoptosis and the activation of NFkB was also proposed in dendritic cells (Do et al. 2004). However, in our study we did not find a causal relation between NFkB activation and apoptosis, indeed when the activity of NFkB was inhibited with synthetic phosphorothioate oligodeoxynucleotides,

endothelial cells were not prevented from undergoing apoptosis, rather there was a little increment in the percentage of annexin V^+ cells (Fig. 16B).

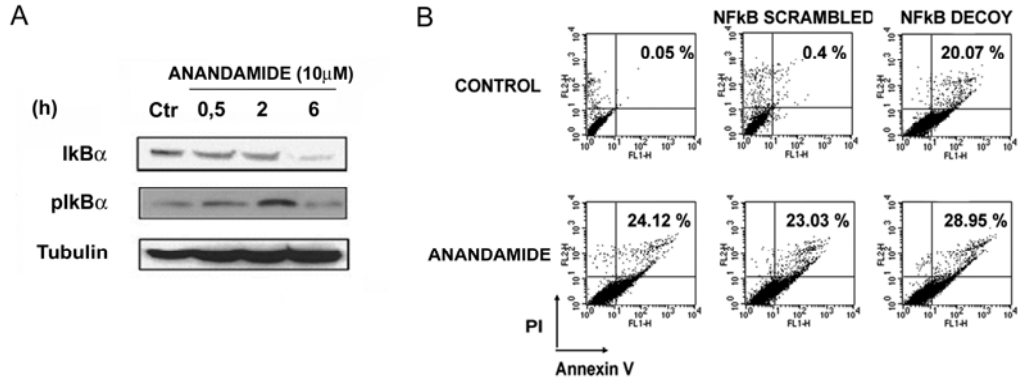


Figure 16: Effect of anandamide on NF-kB activation. (A) Cell lysates blotted with anti-IkBα, anti-pIkBα (Ser32) and tubulin antibodies. (B) HUVECs were pre-treated or not with NFkB synthetic phosphorothioate oligonucleotides (scrambled and decoy, 1 μM, 1 h) before anandamide exposure. In each part is shown the percentage sum of annexin V^+ and annexin V^+/propidium $iodide^+$ cells. The parts are representative of three independent experiments.

Maybe only one of the possible homo- or heterodimers of the five members of NFkB family (p65, Rel B, c-Rel, p50/p105, p52/p100) could be activated by anandamide and this activation could be liable for the regulation of vascular cell functions not related to apoptosis, such as the expression of vascular cell adhesion molecules (VCAM-1, E-selectin), COX-2 or i-NOS (De Martin et al. 2000). Afterwards, anandamide was tested for its inhibitory potency on the morphological differentiation of endothelial cells into tube-like structures, another endothelial cell function crucial to angiogenesis. *In vitro* two-dimensional tube formation assay was carried out plating endothelial cells onto a matrigel coat. Anandamide seemed to be effective in inhibiting bFGF-induced capillary network formation by significantly reducing the number of tube-like structures' intersections (Fig. 17A and B). Matrix metalloproteinases (MMPs), expecially the gelatinases (MMP-2 and MMP-9) secreted by endothelial cells, are hypothesized to play a key role in the processes of matrix remodeling and endothelial sprouting during angiogenesis, owing to their ability to degrade components of the basement membrane (Bussolino et al. 1997). To assess whether anandamide also interferes with MMP-2 (72 kD gelatinase A) activity, gelatin zymography was performed. The effect on MMP-9 secretion could not be assessed in HUVECs because very low to undetectable levels of MMP-9 are secreted (Pisanti et al. 2007). Anandamide treatment for 24 h led to a significant decrease of MMP-2 gelatinolitic activity, reverted by CB1 antagonist pre-treatment (Fig. 17C).

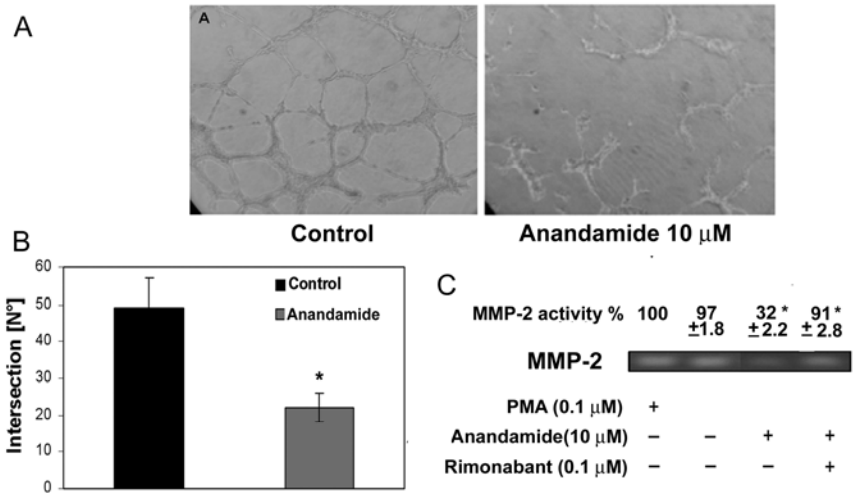


Figure 17: Anandamide inhibits capillary tube formation and MMP-2 gelatinolytic activity. (A) Capillary-like tube formation on matrigel after 6 h. Photographs, magnification 10X. (B) Histogram represents the number of intersections calculated by means of Scion Image Software, expressed as mean ± SD of three independent experiments. ANOVA, **P* <0.05. (C) Gelatin zymography of endothelial conditioned medium. Data of relative pixel density are presented as mean ± SD of four independent experiments of each sample. ANOVA, **P* <0.05.

Finally, we found that anandamide displays anti-angiogenic activity also *in vivo* as evaluated by CAM assay. 1.5% alginate slow releasing beads incorporating anandamide (10 μM) were implanted on the embryonic CAM at day 9 of incubation. After 72 h of implantation, a decrease of vascular reaction was detectable around the beads incorporating anandamide when compared with the control (see Figure 5 in Pisanti et al. 2007). In conclusion our findings document the inhibitory actions of anandamide on several fundamental angiogenic steps, including endothelial cell growth and survival and two-dimensional angiogenesis. It has been previously reported that high doses of anandamide produced by macrophages during shock condition, could play a crucial role in endothelial injury inducing apoptosis via VR1 receptor (Yamaji et al. 2003). Very interesting was the finding that anandamide inhibits bFGF-induced endothelial cell proliferation but not basal one, whereas it induces apoptosis also in absence of pro-angiogenic stimulation (data not shown). This suggests that anandamide could be involved not only in endothelial cell injury in shock conditions, but also in vascular remodeling during particular physiopathological processes.

Our starting hypothesis about a correlation between the anti-tumor activity of anandamide and angiogenesis inhibition was further tested and confirmed using a valuable three-dimensional *in vitro* model of endothelial and tumor cell spheroids co-cultures in different three-dimensional polymeric matrices. This system allows to mimic the *in vivo* tumor microenvironment and architecture. In particular, collagen (1.2 mg/ml) and collagen-hyaluronic acid (C=1.2

mg/ml; HA=5mg/ml) semi-interpenetrating networks were used in this study. The matrices composed of collagen, fibrin or hyaluronan represent the major matrix environments where angiogenic or vasculogenic events take place, acting as scaffold proteins for vessel formation (Davis et al. 2002). At first we observed that both tumor spheroids co-cultured or tumor-conditioned medium enhanced the angiogenic sprouting of endothelial cell spheroids (see Figure 6 in enclosed paper Pisanti et al. 2007). Moreover, as in 2D assays, anandamide seemed to be effective in inhibiting the process of sprouting angiogenesis inducing a strong reduction of both the sprout number and the average sprout length. In conclusion we documented the inhibitory actions of anandamide on several fundamental angiogenic steps *in vitro* and *in vivo*. It is plausible that the reported anti-angiogenic effects may play an important role in mediating its anti-tumor activity documented in animal models. So our results open the way to further investigations to assess and define the role of the endocannabinoid system in the angiogenic process and the therapeutic potential linked to its pharmacological modulation.

4.5 Going into CB1 receptor signaling in breast cancer growth: involvement of lipid rafts/caveolae

In order to deeply explore the role of the endocannabinoid system and in particular of CB1 receptor signaling in breast cancer growth, in absence of its pharmacological stimulation with agonists, we decided to use a critically important tool, that is pharmacological blockade of CB1 receptors by rimonabant. All above presented findings on anandamide activity in breast cancer and previous studies on several CB1 agonists, reported that pre-treatment with the CB1 selective antagonist rimonabant, but not the CB2 antagonist, counteracts most of the observed anti-tumor effects in thyroid, prostate and breast cancers, thus highlighting the involvement of CB1 signaling (Bifulco et al. 2001; Portella et al. 2003; Bifulco et al. 2004; Sarfaraz et al. 2005; Grimaldi et al. 2006). Considering the anti-tumor properties of the cannabinoid receptor agonists, it could be expected that antagonists, such as rimonabant, if used alone, would induce null effects or enhance proliferation of malignant and normal cells leading to cancer. Collected data excluded both this possibilities, reporting rather that not only agonists to cannabinoid receptors but also antagonists, when used alone, are able to inhibit tumor growth (Bifulco et al. 2004) or induce apoptosis in cancer cells (Powles et al. 2005; Derocq et al. 1998). Furthermore rimonabant displayed anti-proliferative properties in preadipocyte and hepatic myofibroblasts *in vitro* and *in vivo* (Gary-Bobo et al. 2006; Teixeira-Clerc et al. 2006). However the mechanism by which rimonabant exert these effects remains unknown.
In the second section of this thesis work we aimed to investigate whether pharmacological interference of CB1 signaling by rimonabant plays any role in

regulating breast cancer growth, as well as to characterize the mechanism underlying such effect. To check the effect of CB1 blockade on tumor cell proliferation *in vitro*, different breast cancer cells such as highly invasive metastatic ER−, MDA-MB-231 and less invasive ER+ T47D and MCF-7 cells, were tested for [^{3}H]-thymidine incorporation in response to increasing doses of rimonabant. We found that rimonabant inhibited cell proliferation in a dose-dependent manner. It is noteworthy that the anti-proliferative effect was completely lacking in the absence of the CB1 (CHO cells), suggesting that it was CB1-dependent and further related to the dose used and to CB1 expression levels (Fig. 18A). Indeed, the growth of highly invasive metastatic MDA-MB-231 cells was inhibited more efficaciously and at very low doses (0.1 μM) in comparison to less-invasive T47D and MCF-7 cells, which both express lower levels of CB1 receptor, assessed by RT-PCR and Western blot analysis (Fig. 18B). In confirmation of the involvement of CB1 receptor in rimonabant anti-proliferative activity, we found that after preincubation with CB1 antisense oligonucleotides, rimonabant failed to inhibit proliferation, reflecting a decline of functional CB1 caused by the inhibition of mRNA translation (Fig. 18C). Comparable results were obtained with CB1 RNA interference approach (data not shown).

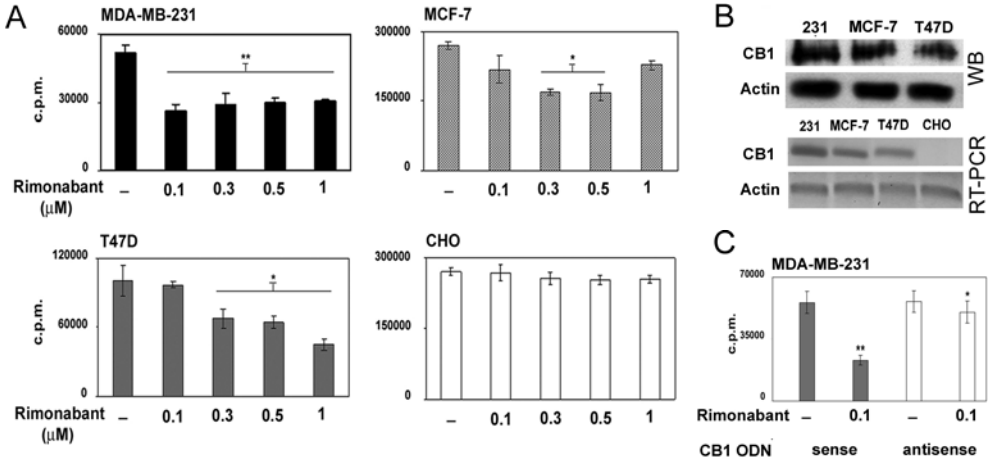


Figure 18: Antiproliferative effect of rimonabant in human breast cancer cell lines. (A) The graphs report the [^{3}H]-thymidine incorporation levels (c.p.m.) expressed as mean ± SD values of three experiments in triplicates. ANOVA, **P*<0.05, ***P*<0.01. (B) Comparison of CB1 expression levels in the different breast cancer cell lines, through Western blot and RT-PCR analyses. (C) Cell proliferation in MDA-MB-231 cells transfected with CB1 sense and antisense oligonucleotides. ANOVA, **P*<0.05, ***P*<0.01.

We chose to carry on our study on metastatic MDA-MB-231 cells, where the highest inhibition of proliferation was reached at 0.1 μM, without induction of apoptosis and/or necrosis (see Figure 2 in enclosed paper Sarnataro et al. 2006). It is noteworthy that the same concentration was able to counteract the anti-proliferative effect of pharmacological doses of anandamide in the same

cell model. Moreover, this very low concentration did not exert any cytotoxic effect in human lymphocytes (see enclosed papers Sarnataro et al. 2006; Malfitano et al. 2007). To determine how CB1 blockade influences cell growth, cell cycle phase distribution of asynchronous and synchronous serum-starved cells was assessed by flow cytometry in MDA-MB-231 cells treated with rimonabant (0.1 μM, 24 h). In both cases we found that rimonabant induced a reduction in the percentage of cells in S phase with a parallel increase of cells in G0/G1, thus blocking the G1/S transition of the cell cycle (Fig. 19), similarly to what it had been reported with endocannabinoids in MCF-7 cells (De Petrocellis et al. 1998). This effect was also confirmed by both the inhibition of cyclin E and D1 expression levels and the increase of the cyclin-dependent kinase inhibitor $p27^{kip1}$, which is known to mediate cell cycle arrest in response to various anti-proliferative signals.

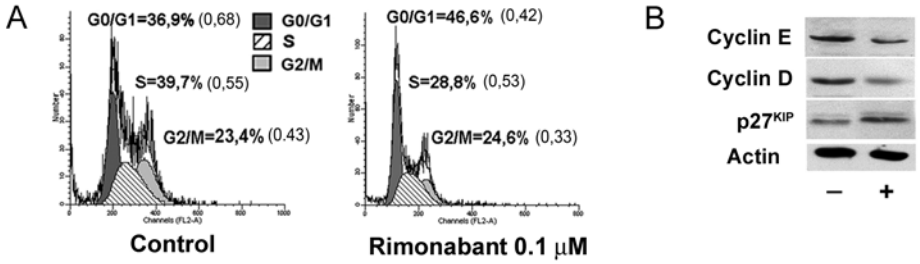


Figure 19: Rimonabant affects G1/S phase transition through modulation of cell-cycle related proteins. The panels show the relative percentage of cells in the G0/G1, S, and G2/M phases and the SD (in parenthesis) of the combined results of three independent experiments. (B) Immunoblots of the indicated proteins. Loading control: actin.

On the other hand, we also found that rimonabant (0.7 mg/kg/dose) inhibits MDA-MB-231-induced xenografts tumor growth *in vivo*, the effect being statistically significant starting from 3 weeks of treatment (Fig. 20). The overall observation suggest an anti-tumor activity of rimonabant in breast cancer.

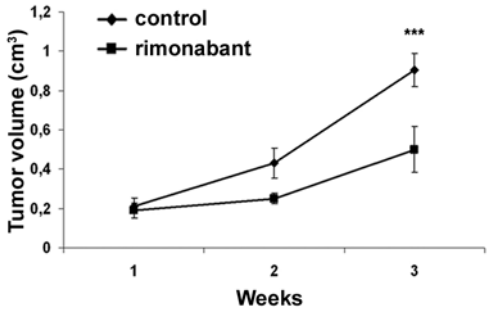


Figure 20: Rimonabant inhibits growth of xenografts tumors induced by MDA-MB-231 injection in mice. Tumor volume at different weeks from mice inoculation is shown. Data were shown as mean ± SE of n=5 animals. ANOVA followed by Bonferroni's test, ***P<0.001.

We have recently shown that the CB1 receptor is associated with lipid rafts/caveolae in MDA-MB-231 cells (Sarnataro et al. 2005). At first we decided to check whether the CB1 receptor associated with lipid rafts in the presence of its antagonist rimonabant and then to investigate the implication of raft microdomains in rimonabant effect by using a cholesterol sequestering agent, methyl-β-cyclodextrin (MCD), which is known to perturb lipid rafts composition (Sarnataro et al. 2002). In order to isolate lipid rafts we performed OptiPREP density gradient flotation assay in TX-100 detergent (see enclosed paper Sarnataro et al. 2006, for further technical details). As shown in Fig. 21A we confirmed the enrichment of CB1 in lipid rafts/caveolae (R). Next we investigated the localization of a downstream molecule relevant for CB1 signaling, ERK1/2 and its activated phosphorylated form pERK (Thr202/Tyr204). We found that in control steady-state conditions (24 h) approximately 20% of total ERK was present in the raft fractions (R). No detectable levels of active pERK were found. Interestingly, in starved MDA-MB-231 cells stimulated for 15 min with fresh serum, ERK was almost totally present in non-raft (NR) fractions, also in the active form. In this condition also CB1 was excluded from rafts, probably as a consequence of rafts perturbation by serum depletion, not yet restored. It seems that induction of MDA-MB-231 proliferation occurs outside lipid rafts through pERK activation and subsequent downstream signaling. In cells treated with rimonabant (0.1 μM, 15 min) we observed a substantial shifting of total ERK protein from non-rafts to rafts fractions and a concomitant inhibition of ERK phosphorylation. In addition more than 50% of CB1 was lipid rafts-associated (Fig. 21B). We could speculate that rimonabant treatment early shifts CB1 into lipid rafts, therefore sequestrating upstream signaling components essential for transduction of proliferative stimuli (e.g. G protein, p21ras or ERK itself), which cannot be more activated in non-rafts fractions, interrupting the mitogenic cascade. It is of strong relevance the notion that rimonabant, besides its antagonist properties also displays inverse agonist effects (Pertwee 2005), since it can block CB1 receptor high constitutive activity at both levels of MAPK and adenylyl ciclase in CB1 transfected CHO cells and also switch off MAPK activation from some tyrosine kinase receptors (RTKs) (Bouaboula et al. 1997). Indeed, MAPKs represent a point of convergence of mitogenic signals emerging from several distinct types of G protein-coupled receptors (GPCRs) and RTKs, suggesting the integration of redundant information. We might speculate from our results and available literature that the binding of rimonabant to CB1 could induce biological responses that negatively interfere with particular RTKs pathways, especially those that are autoactivated and overexpressed in cancer cells like MDA-MB-231 (Takabatake et al. 2007). In this frame, the ability of rimonabant to early compartmentalize CB1 receptors in lipid rafts and segregate effector key signaling proteins (G proteins, p21ras, ERK) physically sequestering them from RTKs, could represent the explanation for its anti-proliferative activity in breast tumor cells.

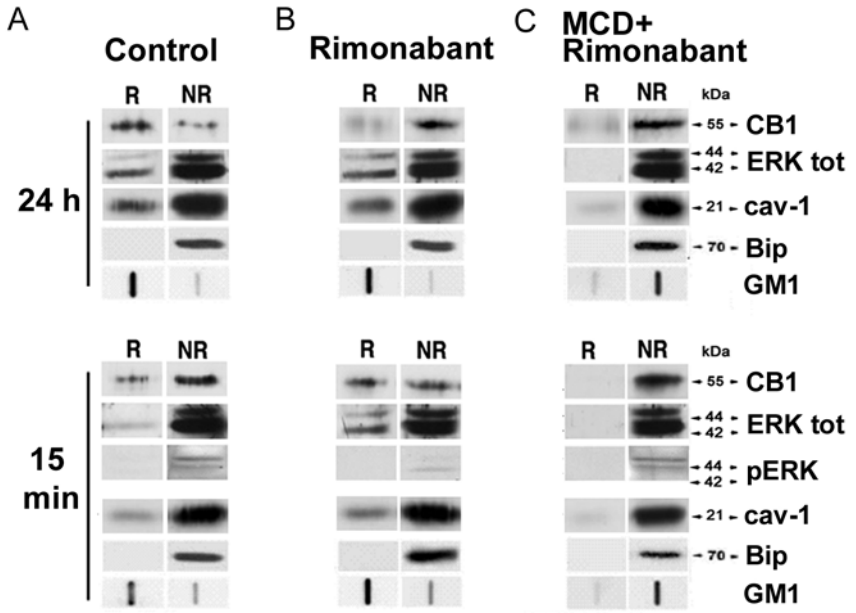


Figure 21: CB1 and ERK localization and activation in non-rafts and lipid rafts fractions. Cell lysates were subjected to OptiPREP density gradient flotation assay in TX-100 detergent. Both lipid rafts fractions (pooled fractions 4 and 5, R) and the nonraft material (pooled fractions 9–12, NR) were immunoblotted for the reported proteins at the indicated time points in control conditions (A) and after rimonabant (B) or MCD+rimonabant (C) treatments.

Interestingly, with longer incubation times (24 h) rimonabant almost completely abolished the receptor raft association, inducing CB1 to be excluded from lipid rafts. Such a phenomenon might be related to the trafficking and recycling of CB1 receptor in response to prolonged exposure to its antagonist. Ongoing studies will clarify this finding and its pharmacological relevance.

To directly evaluate the role of lipid raft integrity in the functional effects of rimonabant, we first perturbed MDA-MB-231 raft composition with MCD (10 mM, 15 min), which we have previously shown to displace CB1 from lipid rafts (Sarnataro et al. 2005), and subsequently checked for the gradient distribution of the CB1 in the OptiPREP preparation following treatment with rimonabant. After both early (15 min) and prolonged (24 h) rimonabant treatment, CB1 receptors remained in the heavy fractions of the gradient (non-rafts), as well as total ERK (Fig. 21C). Moreover ERK was activated at 15 min time point in the non-raft fractions, in a way comparable to control conditions, suggesting that rafts disruption affects rimonabant signaling. In order to check the relevance of this findings and to gain further insight into the involvement of lipid rafts and associated intracellular proteins in the anti-proliferative effect of rimonabant, MDA-MB-231 cell proliferation was tested after treatment with rimonabant (0.1 μM, 24 h) in basal conditions and after perturbation of lipid raft composition with MCD (10 mM, 15 min) (Fig. 22).

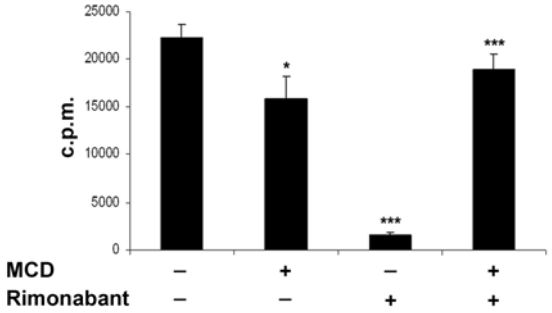


Figure 22: Lipid rafts perturbation prevents rimonabant antiproliferative effect. Cells were pre-treated with MCD (15min, 10 mM) and then with rimonabant (0.1 μM, 24 h). The graph report the [^{3}H]-thymidine incorporation levels (c.p.m.) expressed as mean ± SD values of three experiments in triplicates. ANOVA, with post hoc Bonferroni, **P*<0.01, ****P*<0.001.

Interestingly, we found that the anti-proliferative effect exhibited by rimonabant was almost completely reverted by pretreatment with MCD, indicating again that rimonabant-induced cell growth arrest requires lipid rafts integrity to occur and, high likely, CB1 compartmentalization inside them. In support of our data, there is a large body of evidence that G protein coupled receptor functions depend on the lipid raft integrity (Simons and Toomre 2000). The little anti-proliferative effect of MCD *per se* is probably associated with its affinity for cell membrane components, particularly cholesterol, which plays a major role in the structure and function of the cell membrane (Grosse et al. 1998). In conclusion, we showed that rimonabant inhibits human breast cancer growth *in vitro* and *in vivo*. Moreover we provided a novel mechanism of action for the anti-proliferative effect which requires lipid rafts/caveolae integrity to occur. This observation is particularly intriguing because lipid rafts play a critical role in breast tumor cell invasion (Bourguignon et al. 2004). Complementarily with these data, we have previously shown that CB1 is associated with lipid rafts/caveolae and suggest that they might represent a cellular device for its intracellular trafficking, as well as a favorable platform to regulate CB1 signaling (McFarland et al. 2004; Bari et al. 2005) and its link with some growth factor receptors belonging to the RTK family. We therefore sustained the evidence that functional effects of rimonabant, when used in absence of exogenous CB1 agonists, could depend on its inverse agonist properties at CB1 receptor (Pertwee 2005).

An often raised alternative interpretation of the inhibition of autoactivated receptors by inverse agonists could be the blockage of endogenous agonists present in culture medium or produced by the cells. This could be likely for some inverse cannabimimetic effects observed in diseases where there are high levels of endogenous cannabinoids (Di Marzo and Petrosino 2007). However, MDA-MB-231 breast cancer cells express high levels of fatty acid amide hydrolase, the enzyme mostly involved in anandamide degradation, and therefore low levels of endocannabinoids (Grimaldi et al. 2006). Moreover we have reported that also pharmacological doses of anandamide (10 μM) inhibit

human breast cancer cell proliferation (Grimaldi et al. 2006). In this case the antiproliferative effect observed was not dependent on lipid rafts, since their distruption by MCD pre-treatment did not revert anandamide inhibitory action on cell growth (Fig. 23).

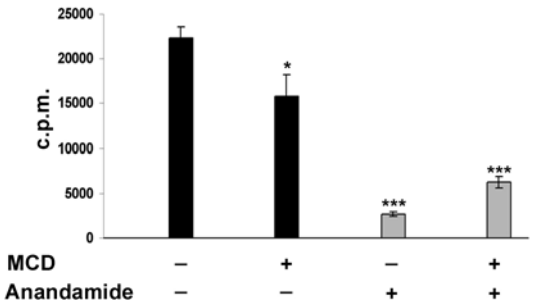


Figure 23: Lipid rafts perturbation does not prevent anandamide antiproliferative effect. Cells were pre-treated with MCD (15 min, 10 mM) and then with anandamide (10 μM, 24 h). The graph reports the [^{3}H]-thymidine incorporation levels (c.p.m.) expressed as mean ± SD values of three experiments in triplicates. ANOVA, with post hoc Bonferroni, **P*<0.01, ****P*<0.001.

Moreover we found that anandamide affects breast cancer cell growth inducing intracellular cascades which lead to S phase cell cycle blockade (Laezza et al. 2006). It appears clear that either anandamide or the antagonist/inverse agonist rimonabant targeting CB1 signaling display a comparable inhibitory efficacy on breast cancer growth, although through a different mechanism of action. This is unsurprising, since there are multiple oncogenic cascades and potential tumor-suppressing targets in breast cancer.

4.6 Evidence for a role of CB1 receptor signaling in angiogenesis: pharmacological blockade by rimonabant inhibits angiogenesis and pathological neovascularization

The CB1 antagonist/inverse agonist rimonabant has been showed to counteract the anti-tumor effects of anandamide related compounds and other cannabinoid agonists in thyroid, prostate and breast cancers (Portella et al. 2003; Sarfaraz et al. 2005; Grimaldi et al. 2006). Unexpectedly, rimonabant *per se* showed a powerful anti-proliferative action in thyroid, mantle cell lymphoma and breast tumors, as we reported above (Bifulco et al. 2004; Flygare et al. 2005; Sarnataro et al. 2006) as well as in adipocytes and hepatic myofibroblasts (Gary-Bobo et al. 2006; Teixeira-Clerc et al. 2006). Furthermore, we recently reported direct evidence for an anti-angiogenic activity of anandamide *in vitro* and *in vivo* supporting its anti-tumor efficacy and providing also new evidence for a role of the endocannabinoid system in the angiogenic process (see

enclosed paper Pisanti et al. 2007). In light of these results, to display the role of the endocannabinoid system and in particular of CB1 receptor signaling in angiogenesis, we aimed to investigate how rimonabant, either as antagonist or as inverse agonist of CB1 receptor, was able to directly modulate the angiogenic process *in vitro* and *in vivo*. We used the same experimental approach as for anandamide studies. At first, in order to determine if rimonabant affects angiogenesis, its activity on bFGF-induced endothelial proliferation was evaluated. We found that rimonabant inhibits dose-dependently endothelial proliferation, evaluated by MTT and by measuring [^{3}H]-thymidine incorporation (Fig. 24A and B). The EC_{50} was approximately 0.3 μM. The inhibitory effect was not due to cytotoxicity of rimonabant in endothelial cells since it did not change the proliferation of unstimulated endothelial cells and it did not induce cell death, as assessed by trypan blue incorporation (Fig. 24B).

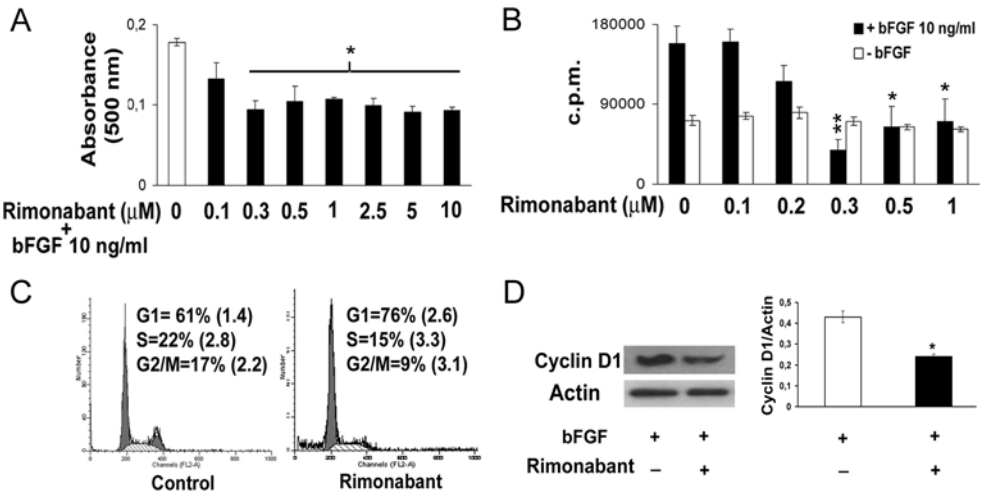


Figure 24: Rimonabant inhibits bFGF-induced endothelial cell proliferation and cell cycle progression through cyclin D1 down-regulation. The graphs report absorbance values for MTT assay (A) and [^{3}H]-thymidine incorporation (c.p.m.) (B), as mean ± SD of three independent experiments in triplicates (Student's *t* test *vs* control, ***P*<0.001, **P*<0.01). (C) Control and rimonabant panels show the mean of the relative percentage of cells in the G_1, S and G_2/M phases analyzed by ModFit software, with SD in parentheses. (D) Immunoreactive bands of cyclin D1. The diagram shows quantification of the intensity bands calibrated to the intensity of actin bands, expressed as means ± SD of three independent experiments. **P*<0.05.

Moreover, it did not induce apoptosis as assessed by flow cytometric analysis with annexinV/propidium iodide double staining (see enclosed manuscript Pisanti et al. *submitted*). Rimonabant pre-treatment (0.3 μM, 30 min) followed by bFGF (10 ng/ml, 24 h) resulted in the accumulation of endothelial cells in the G1 phase with a corresponding decrease in the number of cells in the S and G2/M phase (Fig. 24C). This effect is in agreement with the reduced [^{3}H]-thymidine incorporation, which provides an estimate of DNA synthesis. Since

the transition of cells from G1 to S phase is in part regulated by cyclin D1, we examined whether rimonabant could exert its anti-proliferative effects through the regulation of cyclin D1. Rimonabant slightly down-regulated cyclin D1 expression at 24 h (Fig. 24D). These results indicated that rimonabant affects cell cycle progression at the G1/S phase. Sprouting angiogenesis includes successive phases of microvessel formation, neovessel growth and stabilization. Steps in the neovessel growth process are the migration of endothelial cells toward an angiogenic factor, proliferation and organization into three-dimensional capillary-like structures interconnected to form a polygonal network (Vailhe et al. 2001). The effect of rimonabant on chemotactic motility of endothelial cells was determined by Transwell migration assay. Cells were pre-incubated with rimonabant (0.3 μM) and plated in the upper compartment of Boyden chambers coated with Matrigel. In the lower compartment as chemoattractant was used M199 20% serum or M199 1% BSA plus bFGF (10 ng/ml). Rimonabant alone had no significant effect on basal migration through a Matrigel coat, whereas it inhibited serum-induced and, more efficiently, bFGF-induced migration of HUVECs (Fig. 25A).

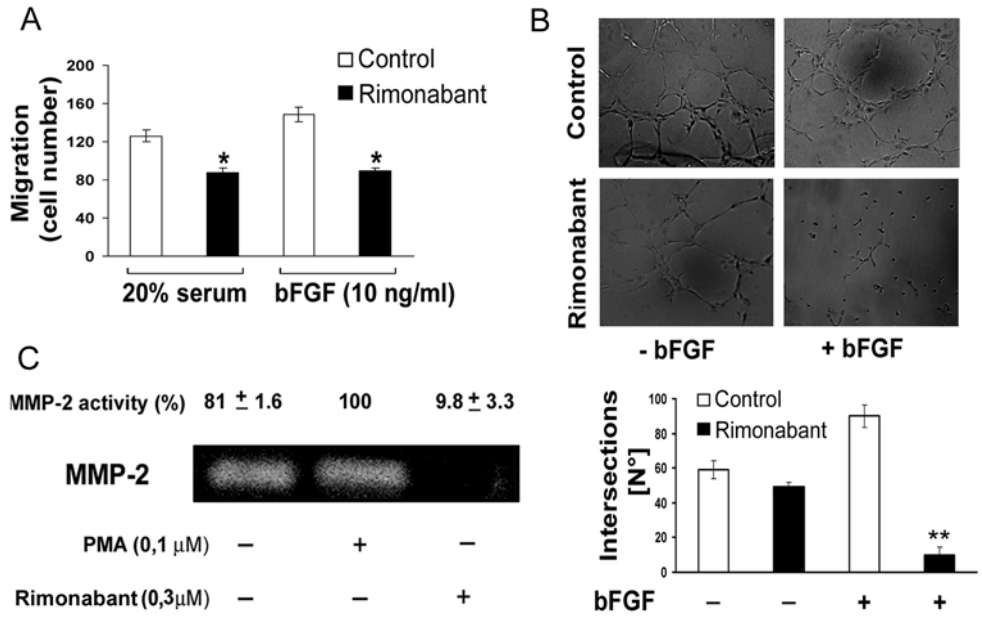


Figure 25: Rimonabant inhibits bFGF-induced migration, capillary tube formation and MMP-2 gelatinolytic activity. (A) Histogram reports the number of cells migrated expressed as mean ± SD values of triplicates from at least four independent experiments. Background levels of cells migrated in the absence of chemotactic stimuli (chemokinesis) were subtracted from all the experimental points. ANOVA, **P*<0.05. (B) Capillary-like tube formation on matrigel after 6 h. Photographs, magnification 10X. Histogram represents the number of intersections, as mean ± SD of three independent experiments. ANOVA, ***P*<0.001. (C) Gelatin zymography of endothelial conditioned medium. Data of relative pixel density are presented as mean ± SD of four independent experiments of each sample. ANOVA, **P*<0.05.

This result indicates that rimonabant is able to suppress endothelial cell migration in responding to chemotaxis agents. Next, the effect of rimonabant on morphological differentiation of endothelial cells into capillary-like tube structures was investigated using two-dimensional matrigel coat (Pisanti et al. 2007). Rimonabant seemed to be strongly effective in inhibiting two-dimensional bFGF-induced capillary network formation by significantly reducing the number of tube-like structures' intersections (Fig. 25B). As detected by gelatin zymography analysis rimonabant (0.3 μM, 24 h) also decreased MMP-2 gelatinolytic activity (Fig. 25C), a crucial event for angiogenesis, being necessary for endothelial cell migration and tube formation (Van Hinsbergh et al. 2006).

The above reported observations raised the important question of the molecular mechanism by which rimonabant induces its anti-angiogenic effect. We evaluated the modification of cell signaling induced by rimonabant on bFGF-stimulated endothelial cells. We first probed the expression and phosphorylation of ERK, that is usually strongly activated by growth factors and is an important early step for cell proliferation during angiogenesis and for endothelial cell motility promotion (Beckner 1999; Eliceiri et al. 1998). Serum starved HUVECs were pre-treated 30 min with rimonabant followed by the addiction of bFGF (10 ng/ml) for increasing time points (15 min, 30 min, 24 h). Our results showed that ERK phosphorylation (Thr202/Tyr204) is reduced by rimonabant with highest inhibition at 30 min. At 24 h ERK phosphorylation was restored to control levels, therefore long-term ERK activity was not affected by rimonabant treatment (Fig. 26A). Early activation of ERK is required for the activities of many key enzymes and transcription factors that ultimately regulate cell cycle progression (Chang et al. 2003). Therefore inhibition of ERK phosphorylation by rimonabant correlated with the observed G_1-S cell cycle transition. We also investigated the hypothesis that the inhibition of cyclin D1 and ERK activity was not sufficient to explain the anti-proliferative effect induced by rimonabant. To address this point, we were prompted to analyze the effect of rimonabant on FAK/PI3K/Akt pathway. Indeed, binding of the angiogenic growth factors VEGF and bFGF to their respective receptors, leads to receptor phosphorylation and subsequent activation of signaling proteins such as phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ (Cross and Claesson-Welsh 2001). One of the best-characterized PI3K downstream targets is the serine/threonine protein kinase B, also known as Akt. PI3K-Akt signaling axis is activated by a variety of stimuli in endothelial cells and regulates multiple critical steps in angiogenesis, including endothelial cell survival, migration, and capillary-like structure formation (Dimmeler and Zeiher 2000). Indeed, it has been recently reported that a number of endothelial cell responses involved in angiogenesis were impaired in $Akt^{-/-}$ endothelial cells (Chen et al. 2005). The activation of FAK leads to its association with PI3K, which is required for Akt stimulation. FAK become phosphorylated at six different tyrosine residues after the engagement of integrins with ECM proteins and these phosphorylations are important for

cell migration (Sieg et al. 2000). We found that rimonabant induced a decrease in the tyrosine phosphorylated FAK (Fig. 26B). Concomitantly, a decrease in the phosphorylated Akt (Ser473) was induced already at 15 min treatment and maintained at 24 h (Fig. 26A).

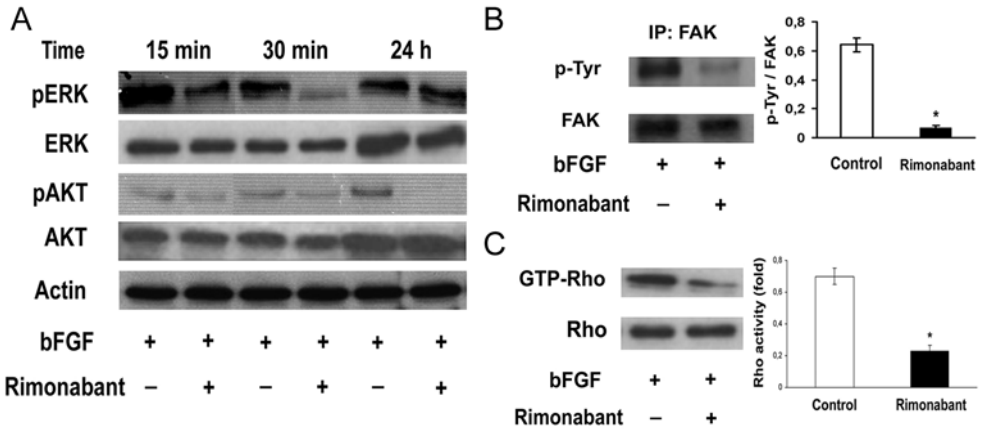


Figure 26: Rimonabant interferes with multiple signaling pathways involved in angiogenesis. (A) Total cell lysates were analyzed by western blot with anti-pERK (Thr202/Tyr204), anti-ERK, anti-pAKT (Ser473) and anti-AKT antibodies. Same filters were stripped and reprobred with anti-actin as loading control. (B) Anti-FAK immunoprecipitates (IP) were immunoblotted with anti-phosphotyrosine antibody. (C) RhoA activity is represented as the relative ratio of the density of GTP-RhoA against that of total RhoA. The relative ratio of control is 100%. The diagrams show quantification of the intensity of bands calibrated to the intensity of total protein bands, expressed as means ± SD (B and C). *P<0.05.

These reductions were clearly related to a decrease in phosphorylation because the total amount of both FAK and Akt was not modified by rimonabant treatment. Akt kinase is an important component in prosurvival but also in migratory signaling pathways. This observation, together with the result that rimonabant inhibited endothelial cell migration and morphogenesis *in vitro*, suggested that it may preferentially target cellular motility. The ability of rimonabant to prevent FAK activation also would be anticipated to affect the Rho-mediated signal pathways, as Rho signaling is facilitated by FAK (Palazzo et al. 2004). The Rho family of small GTPases is involved in the regulation of several components of cell migration, including the development of cell polarization, the assembly of focal adhesions, the formation of directional cell protrusions, and the rapid reorganization of actin filaments. Actually Rho has been demonstrated to regulate endothelial cell organization critically during angiogenesis (Van Nieuw Amerongen et al. 2004). To evaluate the effect of rimonabant on bFGF-induced RhoA activity, we used a pull-down assay with the fusion protein GST-RBD, which recognizes only RhoA-GTP, the active form of RhoA. We found that rimonabant strongly inhibits RhoA activation reducing RhoA-GTP levels. Total Rho levels in the cell lysates were not affected (Fig. 26C). Therefore rimonabant, by inhibiting RhoA activation,

strongly inhibited endothelial cell chemotaxis and MMP-2 secretion, both involved in cell invasion and capillary tube formation. Taken together these data indicate that rimonabant interferes with multiple signaling pathways involved in endothelial cell activation in response to the angiogenic factor bFGF, leading to the inhibition of several angiogenesis critical steps.
Whatever the mechanism of action is, the anti-angiogenic activity of rimonabant was directly confirmed in three well-established *in vivo* angiogenesis models. Rimonabant strongly reduced spontaneous vasculature development in the CAM assay (see enclosed paper Pisanti et al. *submitted*). Indeed, the formation of new microvessels and branching were powerfully inhibited by rimonabant treatment around the alginate beads incorporating the substance, whereas a great number of neovessels was formed in the control. Noteworthy, rimonabant treatment did not destabilize the pre-existing mature large vessels (Fig. 27).

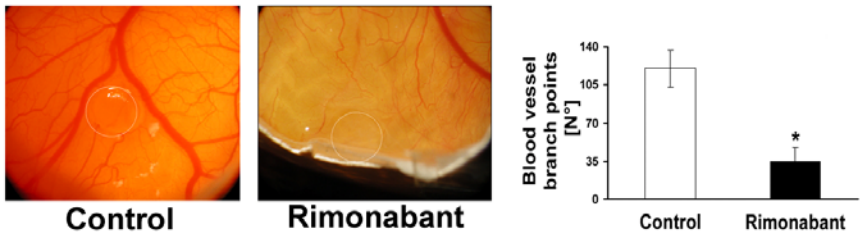


Figure 27: *In vivo* antiangiogenic effect of rimonabant on spontaneous angiogenesis in chick CAM. The alginate beads incorporated rimonabant (0.1 μM). The pictures are representative of three independent experiments conducted in duplicates. the histogram reports the mean ± SD of blood vessel branch points. ANOVA, **P*<0.05.

In rabbit cornea assay rimonabant was firstly tested on angiogenesis and inflammation using slow release pellets, bearing 1 or 10 μg of rimonabant, implanted in the cornea stroma. Rimonabant pellets were devoid of any inflammatory and angiogenic activity (Fig. 28A). Rimonabant was then tested on angiogenesis induced by bFGF (200 ng/pellet), which produced a neovascular growth progressing in the cornea stroma from the surrounding limbic vessels (Fig. 28B). Rimonabant was tested at 1 and 10 μg/dose in pellets implanted adjacently to bFGF releasing pellets (Fig. 28C-E). Rimonabant inhibited bFGF-induced angiogenesis. The effect was not dose-dependent during the early stages (first 10 days of observation), but over the longer term, while the higher concentration completely prevented bFGF-induced neovascular growth, 1 μg of rimonabant was less potent and some capillaries grew (Fig. 28).

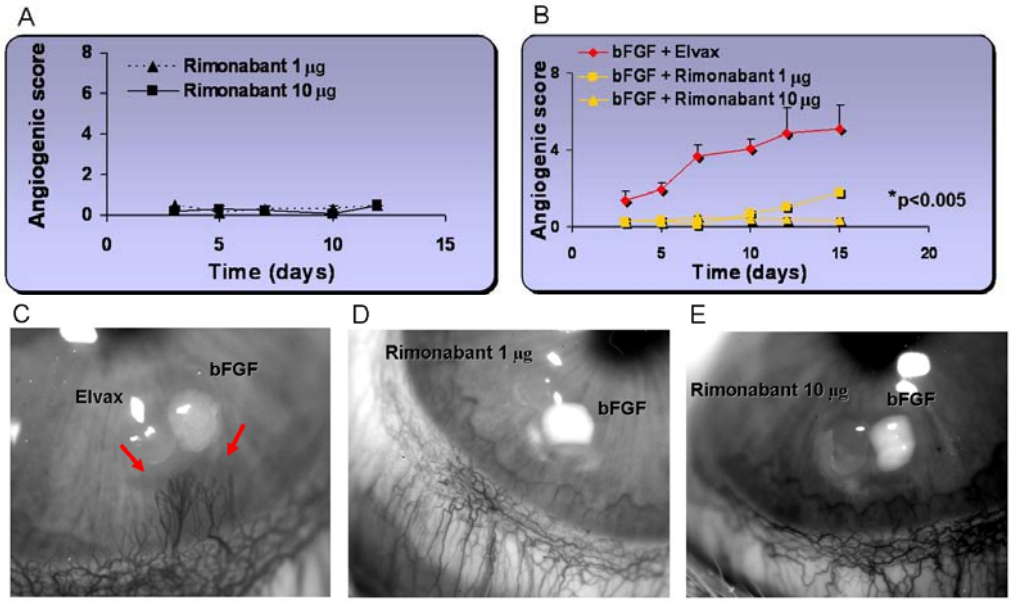


Figure 28: Rimonabant inihibits bFGF-mediated *in vivo* angiogenesis in the rabbit cornea assay. (A) Evaluation of angiogenic and inflammatory activity of rimonabant. (B) Antiangiogenic activity of rimonabant on the neovascular growth induced by bFGF (200 ng/pellet). Data are reported as angiogenic score [number of vessels x distance from the limbus] (means ± SE) during time (days) (n=4 implants). (C-E) Representative pictures of bFGF-induced angiogenesis in the absence (Elvax alone, C) and in the presence of rimonabant 1 μg/pellet (D) and 10 μg/pellet (E). Pictures were taken at the slit-lamp stereomicroscope (18X original magnification) at day 12 after implantation. Arrows indicate the newly formed vessels.

Finally, to study the properties of rimonabant in pathological angiogenesis, we used the mouse model of oxygen-induced retinopathy (OIR), a commonly used model of hypoxia-induced pathological neovascularization with consistent, quantifiable vascular changes. In the mouse OIR model, hyperoxia (75% oxygen from post-natal day P7 to P12) results in vascular obliteration. When mice are returned to normoxia (P12), retinas becomes hypoxic because of a lack of vessels, resulting in pathological neovascularization (P17). Retinas treated with both doses of rimonabant (0.7 and 3 mg/kg) displayed significantly less pathological neovascularization than retinas treated with vehicle (Fig. 29).
We recently reported that also the CB1 agonist anandamide displayed anti-angiogenic activity both *in vitro* and *in vivo* that could support its tumor-suppressor action (Pisanti et al. 2007). Since rimonabant functions as a CB1 receptor antagonist, it could be expected that it would induce, if used alone, null or opposite effects compared with the anti-angiogenic activity of anandamide. The present finding shows that also rimonabant inhibits angiogenesis, however, this is not surprising since it further proves that although the endocannabinoid system could have a role in angiogenesis, this process requires a complex interplay of multiple molecular signals and cellular

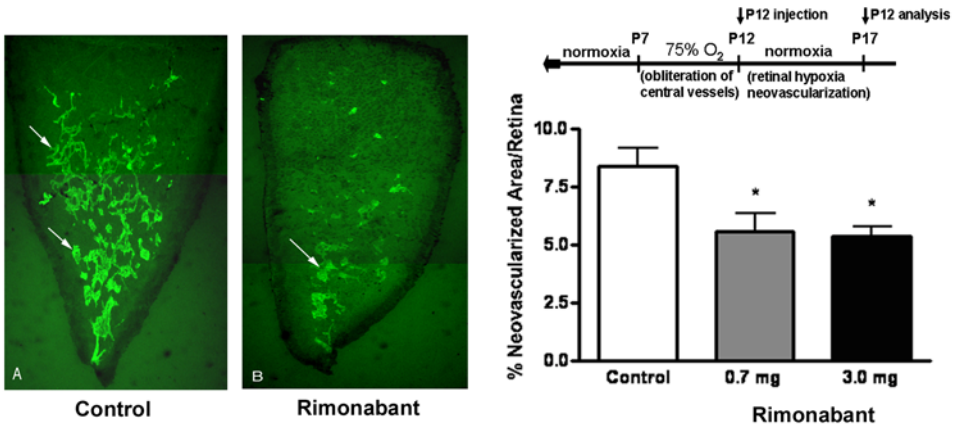


Figure 29: Rimonabant inhibits ocular pathological angiogenesis. Representative images of retinal whole mounts stained for new vessels (arrows) on the surface of the retina from untreated animals and animals treated with 3.0 mg of rimonabant per day. The retinas of animals treated with rimonabant showed significantly fewer new vessels on the surface compared to untreated animals. Values as mean ± SE for n= 3 animals for each treatment. The experiment was conducted twice. ANOVA, **P*<0.05.

players, thus providing numerous distinct or overlapping targets for anti-angiogenic intervention. Moreover, as mentioned in the previous paragraph, it is worthy of note that rimonabant, besides its antagonist properties, also functions as an inverse agonist, because it can block CB1 receptor high constitutive activity and transduction pathways of coupled RTKs. Inhibition of the MAPK pathway is a common molecular mechanism for the anti-proliferative effect of rimonabant in pre-adipocytes and hepatic myofibroblasts (Gary-Bobo et al. 2006; Teixeira-Clerc et al. 2006). In these latter rimonabant also decreased Akt phosphorylation, as we observed in the present study in endothelial cells. An interesting element in support of rimonabant inverse agonism at CB1 receptor, is represented by the finding that rimonabant does not affect endothelial cell proliferation and the others angiogenic steps without pro-angiogenic growth factor stimulation (in our study, bFGF), confirming the hypothesis that it may function blocking mitogenic and/or migratory pathways of coupled RTKs like bFGF, VEGF, EGF receptors.

Furthermore, as discussed above, both endocannabinoid related compounds and rimonabant inhibited cancer growth through the involvement of different pathways. Indeed, also in the case of angiogenesis inhibition, the molecular targets were not the same. As we reported in this thesis work anandamide, exogenously administered at pharmacological doses (10 μM), seems to be involved in vascular remodeling, through inhibition of proliferation and apoptosis induction. Indeed we showed that anandamide inhibits endothelial cell proliferation also inducing apoptosis, through the activation of p38 MAPK, a pathway reported to be downstream CB1 receptor stimulation. It has been also reported that high doses of anandamide produced by macrophages during shock condition, could play a crucial role in endothelial injury inducing

apoptosis via VR1 receptor (Yamaji et al. 2003). Noteworthy we reported that CB1 receptor activation was only partly involved in the observed effects, since either pre-treatment with rimonabant or with p38 MAPK inhibitor only partially prevented apoptosis induction (Pisanti et al. 2007). This result highlights the potential involvement of other receptors, maybe unknown, or non-receptor mediated mechanisms such as the direct activation of PPARγ, already reported in pre-adipocytes (Gary-Bobo et al. 2006), which could be responsible for the anti-tumor and anti-angiogenic effects observed.
On the contrary, rimonabant did not affect p38 MAPK and did not induce apoptosis, mainly targeting prosurvival and migratory signaling pathways. In the light of these observations, it is unlikely that the anti-angiogenic effect of rimonabant could be the consequence of endocannabinoid autocrine-paracrine signaling through an alternative non-CB1 mediated mechanism, in the case of pharmacological blockade of CB1 main receptor.

5. CONCLUSIONS

The studies on the endocannabinoid system reported in this thesis have provided strong evidence for a key tumor-suppressor action of the endocannabinoids and in particular of CB1 receptor signaling in the control of cellular pathways involved in cancer cell growth, invasion, metastases formation and angiogenesis. The CB1 antagonist/inverse agonist rimonabant and a stable analogue of the endocannabinoid anandamide, were utilized as critically important tools to explore, respectively, the basal functions of endocannabinoid system and CB1 signaling and its therapeutic exploitation in the above mentioned processes. It is noteworthy that most of the CB1 receptor antagonists developed to date, rimonabant too, have inverse agonist properties, so their effects do not necessarily reflect reversal of the tonic action of the endocannabinoids. We found an interesting potential of CB1 targeting as anti-tumor and anti-angiogenesis therapeutic strategies.

The possibility that CB1 receptor could interfere not only with breast tumor growth but also with metastatic processes, inducing a non-invasive phenotype, has been verified *in vitro* using human breast cancer cells and in animal models of metastatic infiltration, and potential molecular players (FAK, Src, Rho) have been identified. Of great interest has been the finding of the S-phase cell cycle checkpoint induction by anandamide, since it has been suggested that DNA checkpoints might become activated in the early stages of tumorigenesis, constraining tumor progression through cell cycle blockade. This further supports the proposed tumor-suppressor action of an endogenous sustained tone of endocannabinoids and highlights the therapeutic relevance of compounds aimed to augment their levels. Moreover, looking at CB1 receptor we found its association with lipid rafts/caveolae in breast cancer and obtained relevant evidence about rafts function as a useful platform to regulate the differential signaling to CB1 agonists and antagonists. We also suggested that compartmentalization of CB1 into lipid rafts, induced by rimonabant, could sequestrate key effectors signaling proteins downstream auto-activated tyrosine kinase receptors, thus determining the anti-proliferative effect observed.

Finally, a challenging research task has been the study of the endocannabinoid system in the angiogenic process, with special emphasis to tumor-induced angiogenesis and pathological neovascularization (e.g. retinopathy). In recent years it has become increasingly evident that the angiogenesis process contributes to the pathogenesis of many disorders like cancer, ocular, joint or skin disorders. Therefore characterize new physio-pathological functions of the endocannabinoid system could provide novel molecular targets for anti-angiogenic strategies. Indeed, we found that modulating the endocannabinoid system turned out to affects all the steps of angiogenesis, from the migration of endothelial cells and proliferation, to the organization in three-dimensional tube structures and neovessel stabilization.

Ongoing and future studies will improve our knowledge on this wide topic. In particular, the employment of CB1-deficient mouse-strains, will be determinant, as the use of these knock-out models in combination with receptor antagonists, in order to reinforce the putative regulatory role of endocannabinoids and of CB1 receptor signaling in tumor progression and angiogenesis and to exploit this role for therapeutic gain.

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