Anandamide, an Endogenous Ligand of Cannabinoid Receptors, Inhibits Human Breast Cancer Cell Proliferation Through a Lipid Rafts Mediated Mechanism

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Summary

The endocannabinoid system, comprising the cannabinoid receptors type 1 (CB1) and type 2 (CB2), their endogenous ligands (endocannabinoids), and the proteins that regulate endocannabinoid biosynthesis and degradation, controls several physiological and pathological functions. Indeed, recent evidence indicates that endocannabinoids influence the intracellular events controlling the proliferation and apoptosis of numerous types of cancer cells, thereby leading to both in vitro and in vivo antitumour effects. We have previously observed that the endogenous ligand arachidonoyl ethanolamide (anandamide; AEA) inhibits the proliferation of human breast cancer cells by blocking the G0/G1-S-phase transition of the cell cycle through interference with cannabinoid CB1 receptor-coupled signal-transducing events. We have also shown that a metabolically stable anandamide analogue, 2-methyl-2'-F-anandamide (Met-F-AEA), arrests the growth of K-ras-dependent tumours, induced and/or already established in vivo, and it inhibits the formation of metastatic nodules in the Lewis lung carcinoma model, these effects being largely mediated by cannabinoid CB1 receptors. However, the possibility that stimulation of CB1 receptors interferes with metastatic processes has not been fully explored. Therefore, we hypothesized that CB1 receptor activation could induce a noninvasive phenotype in breast cancer cells.

In this study we showed that, in a model of metastatic spreading in vivo, Met-F-AEA significantly reduced the number and dimension of metastatic nodes, this effect being antagonized by the selective CB1 receptor antagonist SR141716A. In MDA-MB-231 cells, a highly invasive human breast cancer cell line, and in TSA-E1 cells, a murine breast cancer cell line, Met-F-AEA inhibited in vitro adhesion and migration on type IV collagen through a decrease phosphoylation of Fak and src but without modifying integrin expression. Further, in MDA-MB-231 cell line, we showed the intracellular localization of CB1 receptor and its association with cholesterol- and sphyngolipid-enriched membrane domains (lipid rafts). Cholesterol depletion by methyl-β-cyclodextrin (MCD) treatment strongly reduced the flotation of the protein on the raft-fractions (DRMs) of sucrose density gradients suggesting that CB1 raft-association is cholesterol dependent. Interestingly, binding of Met-F-AEA also impaired DRM-association of the receptor suggesting that the membrane distribution of the receptor is dependent on rafts and is possibly regulated by the agonist binding. Finally, we found that the cholesterol depletion by MCD prevented Met-F-AEA mediated anti-proliferative and anti-migratory effects. Taken together, these results suggest that anandamide inhibits human breast cancer cell growth via a lipid rafts mediated mechanism.

Keywords: cannabinoid, cancer

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Introduction

The recreational use of Cannabis Sativa preparations is known to most people, largely as a result of the explosion in its use in the late 1960s; indeed, marijuana is still one of the most widespread illicit drugs of abuse in the world (1). However, the medicinal use of Cannabis also has a millenarian history (2), although this history has been re-examined only very recently (3). As early as 2600 BC, the Chinese emperor Huang Ti advised taking Cannabis for the relief of cramps, and rheumatic and menstrual pain; however, the great therapeutic potential of Cannabis was not scientifically assessed and publicized in the Western world until the British physician O'Shaugnessy wrote on the topic in the nineteenth century (2). This long history of Cannabis use has resulted in the development of pharmaceutical drugs, such as dronabinol (Marinol;Unimed). This preparation, prescribed in the United States as an anti-emetic and appetite-stimulants to patients with cancer and AIDS, is based on Δ^9-tetrahydrocannabinol (THC, fig.1), which in 1964 by Mechoulam and coworkers was identified as the major psychoactive component of cannabis. To date, some 60 plant terpenophenols more or less related to THC have been isolated and defined cannabinoids (4). Although the pharmacology of most of the cannabinoids is unknown, it is widely accepted that Δ^9-tetrahydrocannabinol is the most important, owing to its high potency and abundance in cannabis.

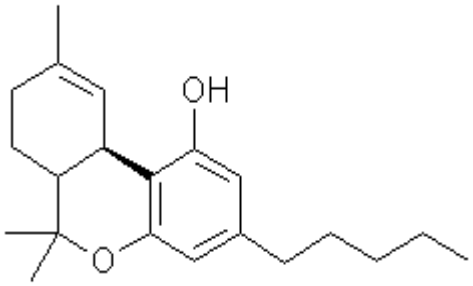

Fig. 1 Chemical structure of Δ^9-tetrahydrocannabinol

Cannabinoid receptors

So far, two cannabinoid-specific receptors have been cloned and characterized from mammalian tissues, the seven transmembrane G protein-coupled cannabinoid receptors type 1 (CB1 receptor), (5) and type 2 (CB2 receptor) (6). Both the central effects and many of the peripheral effects of cannabinoids depend on CB1 receptor activation. Expression of this receptor is abundant in the brain, particularly in the basal ganglia, cerebellum and hippocampus, which accounts for the well-known effects of cannabis on motor coordination and short term memory processing (7). The CB1 receptor is also expressed in peripheral nerve terminals and various extraneuronal sites such as the testis, eye, vascular endothelial and spleen. By contrast, the CB2 receptor is almost exclusively expressed in the immune system,

both by cells, including B and T lymphocytes and macrophages, and by tissues, including the spleen, tonsils and lymph nodes (8; 9; 10). Pharmacological evidence exists for the presence of other cannabinoid receptors, which, however, have not yet been cloned (11).

CB1 and CB2 receptors share only 44% overall identity and 68% within the transmembrane domains. Both cannabinoid receptors cloned so far are coupled to G proteins, mostly of the $G_{i/o}$ type, through whose α subunit they modulate the activity of adenylate cyclases (inhibited) and mitogen-activated protein kinases (stimulated). CB1 receptors are also coupled to modulation of voltage-activated Ca^{2+} channels (inhibited) and inwardly rectifying K^+ channels (stimulated), and activation of both phospholipase C (via the βγ subunits of the G protein) and PI-3-kinase. CB2 receptors, on the other hand, trigger a sustained activation of ceramide biosynthesis (12).

The endocannabinoid system

Several endogenous fatty-acid ligands, known as endocannabinoids, have been found. The first to discovered, in 1992, was arachidonoylethanolamide (anandamide, AEA) followed by 2-arachidonoylglycerol (2-AG). Both these compounds are derivates of arachidonic acid and are able to bind to CB1 and CB2 receptors, although with differences in affinities and activation efficacies (8). During the last few years, several other bioactive lipid mediators have described; they appear to act, through CB1 and/or CB2 receptors and confer specific pharmacological effects in vivo. Specifically, the compounds are 2-arachidonoyl-glyceryl-ether (noladin ether), o-arachidonoyl-ethanolamine (virodhamine), N- arachidonoyl-dopamine, and possibly oleamide (13; 10; 14; 15) (fig. 2).

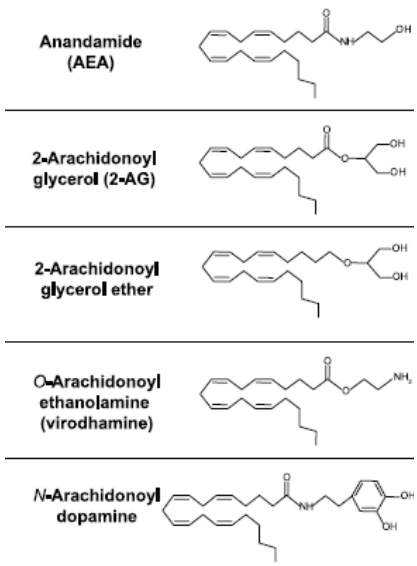

Fig. 2 Chemical structure of endogenous cannabinoid

Cannabinoid receptors, endocannabinoids and the machinery for their synthesis and degradation represent the elements of a novel endogenous signalling system (the so-called endocannabinoid system) which is involved in a plethora of physiological functions (16;17). During the last few years a remarkable amount of data has been acquired to understand the biological roles of this system in more detail.

In general, endocannabinoid system serves several functions under physiological conditions. In the CNS, endocannabinoids intervene in both short-term and long-term forms of synaptic

plasticity, including depolarization-induced suppression of both excitatory and inhibitory neurotransmission, long-term potentiation and depression, and long-term depression of inhibition (18). These actions contribute to the regulation of cognitive functions and emotions in neuronal circuits of the cortex, hippocampus, and amygdala, and to the reinforcement of substances of abuse in the mesolimbic system (19). CB1 receptors and endocannabinoids are very abundant in the basal ganglia and cerebellum, where they control movement and posture, for example by influencing dopaminergic signalling (20). The neuromodulatory actions of endocannabinoids in the sensory and autonomic nervous systems result, mostly via CB1 receptors, in the regulation of pain perception (21) and of cardiovascular (22) and gastrointestinal functions (23). Their cross-talks with steroid hormones and with hypothalamic hormones and peptides help modulate food intake, the pituitary-hypothalamus-adrenal axis, and reproduction (24). CB2 receptors, instead, are involved in cellular and particularly humoral immune response, with possible implications for (neuro)inflammation and chronic pain (25).

Finally, yet importantly, endocannabinoids are know to exert important anti-proliferative actions in tumour cells. The involvement of anandamide and 2-AG in the control of cell metabolism, differentiation, proliferation, and death via cannabinoid and non-cannabinoid receptors is suggested by data in isolated cells (26).

Apart from the possible housekeeping functions of the endocannabinoid system described above, not all of which have been shown to occur "tonically" in healthy animals, endocannabinoid signalling undergoes dramatic tissue-specific changes under pathological conditions. If these conditions are transient, then the levels of at least one endocannabinoid in the tissues involved in the disorder are elevated to help re-establish, via CB1 receptors, the normal levels of other endogenous mediators. This occurs, for example, in specific nervous system areas following insults or stressors, such as: in the hippocampus of animal model of epilepsy (27); in the hypothalamus following food deprivation (28); in the spinal cord in an animal model of multiple sclerosis (29).

Synthesis, release, uptake and degradation of endocannabinoids

Endocannabinoid are very lipophilic and thus cannot stored in vescicles like other neurotransmitters but are produced on demand by receptor-stimulated cleavage of lipid precursors. Consequently, the regulation of endocannabinoid signalling is tightly controlled by their synthesis, release, uptake and degradation (16). Several different stimuli, including membrane depolarization and increased intracellular Ca^{2+} and/or receptor stimulation, can active complex enzymatic machineries, which lead to the cleavage of membrane phospholipids

and eventually to the synthesis of endocannabinoids. Importantly, different enzymes are involved in the synthesis of distinct endocannabinoids, indicating an independent involvement of endocannabinoids in different conditions. Anandamide and its congeners (collectively referred to as N-acylethanolamines) are principally formed from their corresponding N-acyl-phosphatidylethanolamines by a phosphodiesterase of the phospholipase D-type in animal tissues (30).

It is accepted that N-acylethanolamines are principally biosynthesized in animal tissues from membrane phospholipid by two steps of enzyme reactions: 1) N-acylation of phosphatidylethanolamine (PE) to generate N-acyl-phosphatidylethanolamine (NAPE) by an acyltransferase, and 2) subsequent release of N-acylethanolamine from NAPE by a phosphodiesterase of the phospholipase D (PLD) type (31).

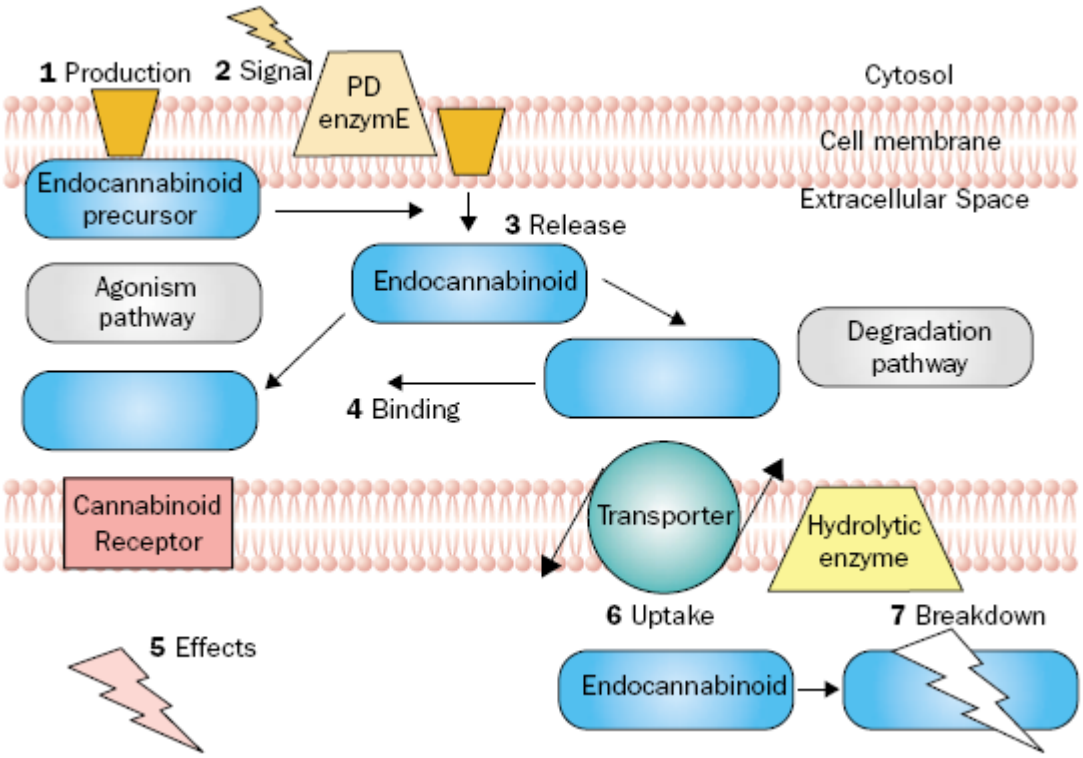

Fig. 3 Endocannabinoid agonism—degradation pathway. Endocannabinoids are formed within neurons and other cell types via multiple biosynthetic pathways (1). Rather than being stored as active molecules these are produced "on demand" from membranous fatty-acid precursors via the activity of hosphodiesterase (PDE) enzymes such as phospholipase D (anandamide) and phospholipase C (2-AG). This process occurs (2) after cellular stimulation by signals such as neuronal depolarisation (Ca^{2+} influx) to cause the extracellular release of active endocannabinoids (3). After release, the endocannabinoid can either bind cannabinoid receptors (agonism pathway) or be degraded. After receptor binding (4) the receptor signals the second messenger systems (eg, reducing adenylate cyclase and for CB1 inhibiting Ca^{2+} channels or stimulating inwardly rectifying K^+ channels) that signal the cannabimimetic activities (5). There is also a degradation pathway expressed on either receptor bearing or other cells. The endocannabinoids are degraded through reuptake by a diffusion facilitated transport molecule (6) and then hydrolytically cleaved by enzymes (7) such as fatty-acid-amide hydrolase to break down the endocannabinoids to molecules such as arachidonic acid and ethanolamine.

After synthesis, endocannabinoids can activate cannabinoid receptors, either after previous release into the extracellular space or directly moving within the cell membrane. Endocannabinoid signalling 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 molecular nature of the carrier protein(s) involved in endocannabinoid uptake 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 (32) and monoglycerol lipase for 2-AG (33), although other enzymes might be partially involved in the degradation of this last compound (34). An interesting aspect of endocannabinoid activity is the rapid induction of their synthesis, receptor activation, and degradation (16; 35). The endocannabinoid system has thus been suggested to act on demand, with a tightly regulated spatial and temporal selectivity. The system exerts its modulatory actions only when and where it is needed. This fact poses an important distinction between the physiological functions of the endocannabinoid system (selective in time and space) and the pharmacological actions of exogenous cannabinoid receptor agonists, which lack such selectivity. Concerning degradation of endocannabinoids, which represents an important regulatory aspect of the activity of the endocannabinoid system, it should also be mentioned that a recent study investigated whether endocytic processes are involved in the uptake of endocannabinoids and found that about half of the AEA uptake occurs via a caveola/lipid raft-related process (36).

Tumour progression

For most cancer cell types, the acquisition of metastatic ability leads to clinically incurable disease. Indeed, metastases rather than primary tumours are responsible for most deaths from cancer. Metastasis consists of a series of sequential steps. Briefly, these include detaching of cells from a primary tumour into the circulation, survival of the cells in the circulation, arrest in a new organ, extravasation into the surrounding tissue, initiation and maintenance of growth, and vascularization of the metastatic tumour (37). In order for malignant cells to leave the epithelium, they must overcome the adhesive interaction. There are two primary mechanism of cell adhesion: cell–cell adhesion that is affected primarily by E-cadherins and cell–extracellular matrix (ECM) adhesion mediated by integrins. Malignant cells have a reduced expression or impaired signalling of E-cadherins and produce fewer integrins capable of binding with ECM (38). After overcoming normal cell adhesion, migrating cells must break through the basement membrane by secreting enzymes called metalloproteinases (MPP) and

migrate through surrounding tissue and basement membrane mainly by the activation of pro-migratory integrins. Not only proteolytic enzymes and cell surface adhesion proteins, but also other molecular factors, such as angiogenic factors, are involved in adhesion and migration as contributing to the formation of metastases. Furthermore, changes in the organization and distribution of cytoskeleton proteins are necessary for focal adhesion formation, cell motility, and cell invasion (39). Among these molecular factors (40), the focal adhesion kinase (FAK), a non-receptor protein–tyrosine kinase that localizes to the focal adhesions, is involved in metastasis formation and development. This kinase is phosphorylated after cellular adhesion to extracellular matrix (ECM) proteins and can be activated by both growth factors and integrins, thus regulating both cell–cell and cell-matrix adhesion and signalling. Integrin clustering, actin polymerization, and actomyosin facilitate FAK autophosphorylation and activation.

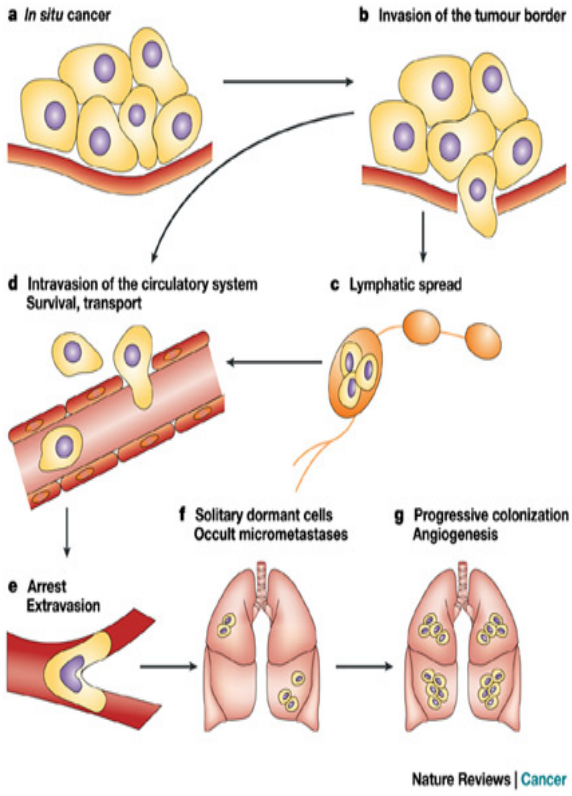

Fig. 4 A schematic of the metastatic process, beginning with (**a**) an *in situ* cancer surrounded by an intact basement membrane. (**b**) Invasion requires reversible changes in cell–cell and cell–extracellular-matrix adherence destruction of proteins in the matrix and stroma, and motility. Metastasizing cells can (**c**) enter via the lymphatics, or (**d**) directly enter the circulation. (**e**) Survival and arrest of tumour cells, and extravasation of the circulatory system follows. (**f**) Metastatic colonization of the distant site progresses through single cells, which might remain dormant for years, to occult micrometastases and (**g**) progressively growing, angiogenic metastases.

Autophosphorylated FAK recruits Src family kinases to the focal adhesion. Activated Src phosphorylates FAK on various tyrosine residues, thus creating sites for recruitment of other proteins to FAK complexes. The formation of a FAK-Src signalling complex is an initial and important event required for maximal FAK activation and cell migration. Previous studies have shown that FAK and Src are overexpressed in tumour cells, thereby providing survival

signals in breast, colon, and thyroid cancer cells (41). The integrin family represents the most important group of adhesion molecules regulating cell adhesion to the ECM and subsequent cell migration. Changes in integrin expression have been also observed in many cancers. Some integrins are either overexpressed or no longer expressed, whereas others are phosphorylated, thus affecting their cytoskeleton and extracellular ligand binding properties. The integrins have been shown to regulate anchor-independent growth of mammary tumour cells in culture (42). In general, the loss or gain of expression of particular integrins appears to be indirectly implicated in malignant transformation and directly involved in tumour progression and metastasis.

Cannabinoids and cancer

The ubiquity of the endogenous ligands of cannabinoid receptors in both vertebrate and invertebrate tissues, and their modulating activity on proteins and nuclear factors involved in cell proliferation, differentiation and apoptosis, suggest that the endocannabinoid signalling system is involved, among other effects, in the control of cell survival transformation and proliferation. The anti-proliferative properties of cannabis compounds were first reported almost 30 years ago by Munson et al., who showed that THC inhibits lung-adenocarcinoma cell growth in vitro and in vivo. Although these observations were promising, further studies in this area were not carried out until the late 1990s, when the effects of plant-derived (for example, THC and cannabidiol), synthetic (for example, WIN-55,212-2 and HU-210) and endogenous cannabinoids (for example, anandamide and 2-AG) on cancer cell proliferation and apoptosis were revisited.

In 1998, De Petrocellis et al. investigated the possible antimitogenic effects of AEA on epithelial human breast cancer cell lines EFM-19 and MCF-7. In these models, treatment with sub-micromolar concentration of AEA (as well as of 2-AG or HU-210) significantly inhibited the G1–S transition of mitotic cell cycle. Moreover, AEA inhibited the expression of prolactin receptors, induced downregulation of the brca1 gene product (43), and of trk proteins, the high-affinity neurotrophin receptors (44). 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 (45). Cannabinoids prevented the inhibition of RAF1 (caused by protein kinase A-induced Raf phosphorylation) and induced prolonged activation of the RAF1-MEK-ERK signalling cascade, leading to downregulation of PRLr and Trk (44). Mimeault et al. (46) showed that a micromolar concentration of AEA inhibited EGF-induced proliferation of DU145 and PC3 cells (androgen independent human prostate cancer cell lines

overexpressed EGF-R) as well as of androgen-stimulated LNCaP, 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 (43; 44). It is important to remark that longer AEA incubation times (5–6 days) were able to induce massive apoptosis in DU145 and PC3 cells. This effect was mediated by CB1/2 receptors via cellular ceramide accumulation, and was absent in LNCaP cells (46).

The anti-tumoural 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 proapoptotic lipid ceramide and prolonged activation of Raf1/MEK/ERK cascade. Cannabinoids induced regression of gliomas in vivo; in fact intratumour administration of THC and WIN-55,212-2 induced regression of C6-derived glioma in Wistar rats and in RAG-2-deficient mice (47).

Cannabinoid receptors could have a protective role against programmed cell death, as reported in human neuroblastoma and C6 cells, where AEA induced apoptosis, via vanilloid receptors, increasing intracellular calcium concentration, activating COX, releasing cytochrome c and activating caspase 3 (48). The mechanism through which AEA induces apoptosis in cells expressing both functional cannabinoid and vanilloid receptors is still controversial and might depend on the experimental conditions used.

Initially considered to exert their anti-tumoural actions by proliferation arrest or apoptosis, cannabinoids and their receptors are now emerging as suppressors of angiogenesis and tumour metastatic spreading. Increasing evidence suggests that antitumour effect of cannabinoid-related drugs could be at least in part ascribed to the inhibition of tumour neo-angiogenesis in animal models. The non-psychoactive CB2-agonist cannabinoid JWH-133 inhibited in vitro human umbilical vein endothelial cells (HUVEC) migration and survival (49); in vivo, JWH-133 treatment of C6 glioma- and grade IV astrocytoma-derived tumours reduced expression levels of proangiogenic factors that destabilize vessel integrity, facilitate vessel sprouting and endothelial cells growth, disrupte the extracellular matrix organization.

Casanova et al. (50) evaluated the potential antiangiogenetic power of cannabinoids in mouse skin carcinoma cell line (PDV-C57) expressing high levels of activated ras and EGF-R and showed that WIN-55,212-2 was able to arrest in vivo the growth of highly malignant PDV-C57 cells-derived tumours. In this model, cannabinoid treatment decreased the expression of proangiogenetic factors VEGF, Ang2 and placental growth factor (PlGF).

Similarly, Met-F-AEA, by inhibiting p21ras activity, prevented the growth of v-Kras-transformed rat thyroid cells both in vitro and in vivo (51). Furthermore, it inhibited growth

of already established tumours by reducing the expression of both VEGF and its receptor Flt1, and upregulating the levels of the cyclin-dependent kinase inhibitor p27kip (52). Cannabinoids' variable effects on cell migration seem to be dependent on both cellular differentiation levels and specific activation of different receptors. Song and Zhong (53) demonstrated that cannabinoid agonists (HU210, WIN 55212-2, AEA) induced migration of human embrionic kidney 293 cells. The anandamide-induced cell migration was CB1-mediated in human embrionic kidney 293 cells and it was blocked by PD98059 (MAPK inhibitor), suggesting that ERK was crucial for CB1-mediated migration. On the other hand, the antitumour effects of cannabidiol (CBD), a non-psychoactive cannabinoid, could be ascribed, beside to the antiproliferative action on U87 and U373 human glioma cells in vitro and in vivo (54), to inhibition of migration.

Moreover, Met-F-AEA was able to inhibit proliferation of a metastasis-derived thyroid cancer cell line, MPTK-6, more efficaciously than of the primary thyroid cancer-derived TK-6 cells (52). To test the in vivo effects of Met-F-AEA on induction of metastatic foci, the authors used the Lewis lung carcinoma model of metastatic spreading and demonstrated that Met-F-AEA efficaciously interfered with the formation of lung metastatic nodules by acting on CB1 receptors. In androgen-independent prostate cancer cell lines PC3 and DU145, 2-AG reduced invasion through the CB1-dependent inhibition of adenylyl cyclase, decreasing phospho-kinase A (PKA) activity (55).

Lipid rafts and caveolae

Lipid rafts are receiving increasing attention as devices that regulate membrane function in eukaryotic cells and have changed our view of membrane organization. Lipid rafts are dynamic assemblies of proteins and lipids that float freely within the liquid-disordered bilayer of cellular membranes but can also cluster to form larger, ordered platforms. Lipid rafts are planar domains in the plasma membrane that are rich in sphingolipids, cholesterol, plasmenylethanolamine and arachidonic acid. They are defined by the insolubility of their components in cold non-ionic detergents (like Triton X-100) (56; 57).

Thus, the presence of liquid-ordered microdomains in cells transforms the classical membrane fluid mosaic model of Singer and Nicholson into a more complex system, where proteins and lipid rafts diffuse laterally within a two-dimensional liquid. The raft concept has long been controversial, largely because it has been difficult to prove definitively that rafts exist in living cells. But recent studies with improved methodology have dispelled most of these doubts (58). One of the most important properties of lipid rafts is that they can include or exclude proteins

to variable extents. Proteins with raft affinity include glycosylphosphatidylinositol (GPI)-anchored proteins (59; 60), doubly acylated proteins, such as Src-family kinases or the α-subunits of heterotrimeric G proteins (61), cholesterol-linked and transmembrane proteins, particularly palmitoylated ones (59).

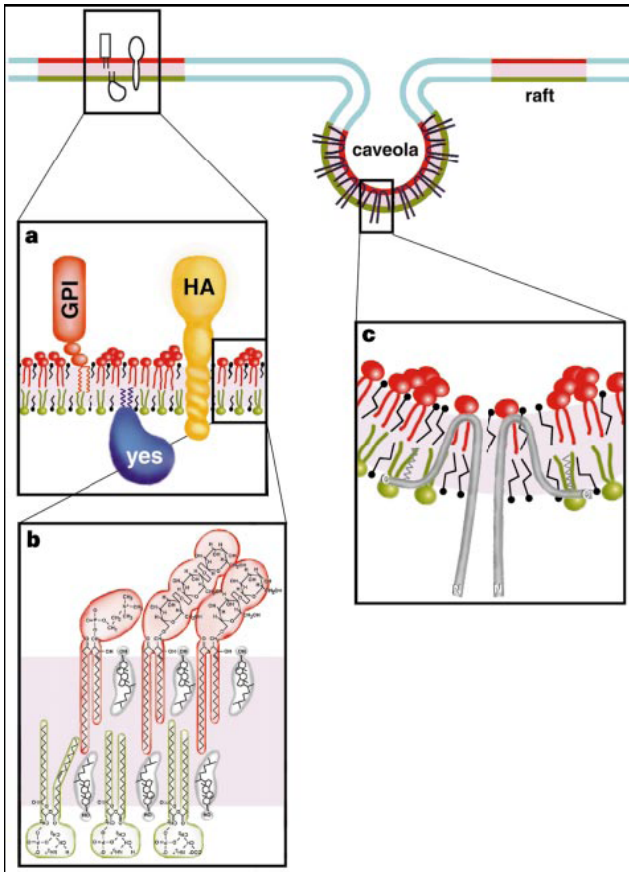

Fig.5 Model for the organization of rafts and caveolae in the plasma membrane. The rafts (red) segregate from the other regions (blue) of the bilayer in which unsaturated phosphatidylcholine is predominantly
a, Rafts contain proteins attached to the exoplasmic leaflet of the bilayer by their GPI anchors, proteins binding to the cytoplasmic leaflet by acyl tails or proteins associating through their transmembrane domains
b, The lipid bilayer in rafts is asymmetric, with sphingomyelin (red) and glycosphingolipids (red) enriched in the exoplasmic leaflet and glycerolipids (for example, phosphatidylserine and phosphatidylethanolamine; green) in the cytoplasmic leaflet. Cholesterol (grey) is present in both leaflets and fills the space under the head groups of sphingolipids or extends the interdigitating fatty acyl chain in the apposing leaflet.
c, Caveolae are formed by selfassociating caveolin molecules making a hairpin loop in the membrane. Interactions with raft lipids may be mediated by binding to cholesterol and by acylation of C-terminal cysteines

The distribution of lipid rafts over the cell surface depends on the cell type. In polarized epithelial cells and neurons, lipid rafts accumulate in the apical and axonal plasma membrane, respectively. Basolateral and somatodendritic membranes also contain rafts, but in smaller amounts (56). In lymphocytes and fibroblasts, rafts are distributed over the cell surface without obvious polarity. Raft lipids are most abundant at the plasma membrane, but can also be found in the biosynthetic and endocytic pathways. Whereas cholesterol is synthesized in the endoplasmic reticulum (ER), sphingolipid synthesis and head-group modification are completed largely in the Golgi (62). As these data predict, cholesterol–sphingolipid rafts first assemble in the Golgi. Movement of lipid rafts out of the Golgi seems to be mainly towards the plasma membrane, as vesicles going back to the ER contain little sphingomyelin and

cholesterol (63). The inclusion of proteins into rafts is important for polarized delivery to the cell surface in many cell types (56; 64; 65). Lipid raft trafficking does not end with surface delivery rafts are continuously endocytosed from the plasma membrane (66). From early endosomes, rafts either recycle directly back to the cell surface or return indirectly through recycling endosomes, which could also deliver rafts to the Golgi (67).

The most important role of rafts at the cell surface may be their function in signal transduction. It is well established that, in the case of tyrosine kinase signalling, adaptors, scaffolds and enzymes are recruited to the cytoplasmic side of the plasma membrane as a result of ligand activation (68). One way to consider rafts is that they form concentrating platforms for individual receptors, activated by ligand binding. If receptor activation takes place in a lipid raft, the signalling complex is protected from non-raft enzymes such as membrane phosphatases that otherwise could affect the signalling process. In general, raft binding recruits proteins to a new micro-environment, where the phosphorylation state can be modified by local kinases and phosphatases, resulting in downstream signalling. To highlight these principles, examples of signalling pathways that involve lipid rafts are Immunoglobulin E signalling, T-cell antigen receptor signalling and Ras signalling (58).

One subset of lipid rafts is found in cell surface invagination called caveolae. These flask-shaped plasma membrane invaginations were first identified in 1950s on the basis of their morphology. Caveolae are 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 also have been shown to play an important role in the regulation of various cellular functions including organization of cell signalling machinery such as receptor tyrosine kinases and GPCRs, cholesterol transport, potocytosis, endocytosis (56) cell polarization and migration (69; 70).

Interestingly, by using animal model approach it has been shown that caveolin 1 is a potent suppressor of mammary tumour growth and metastasis and it has been shown to regulate breast tumour growth and metastasis of breast tumour (71). However the exact functional role of caveolin 1 remains controversial.

Lipid rafts and endocannabinoid system

Recent evidence suggests that lipid rafts/caveolae play a role in the cellular processing and regulation of anandamide. Interestingly, it has been reported a role for caveolae/lipid rafts in the uptake and recycling of cannabinoid anandamide (36) and several evidences suggest a

possible involvement of lipid rafts in anandamide-induced cell death (72) and that anandamide induces apoptosis, at least in hepatoma cell line (Hep G2), interacting with cholesterol present in the cell membrane (73).

Furthermore, it has been reported that a caveolae related endocytic process is involved in the cellular accumulation of in some cell lines (RBL-2H3) and that in these cells the metabolites of anandamide are trafficked back to the plasma membrane, where they accumulate in these detergent resistant membrane domains as well (36).

Finally, it has been recently reported that detergent-resistant membrane 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 detergent-resistant membrane microdomains such as lipid rafts may play a role in the cellular regulation of anandamide inactivation and production (74).

Many studies have described the potential anti-tumour effects of endocannabinoids. However, the possibility that stimulation of CB1 receptor interferes with metastatic processes has not been fully explored. Therefore, we hypothesized that CB1 activation could induce a non-invasive phenotype in breast metastatic cells. In this study, we investigated the effects of Met-F-AEA on the signalling pathways involved in the invasiveness and metastatic capability using the highly invasive and metastatic MDA-MB-231 cells, a human breast cancer cell line that harbors constitutive ras activation and exhibits impaired p53 and oestrogen receptor expression, and the murine breast cancer cell line TSA-E1.

Moreover, although several evidences indicate a possible role of lipid rafts/caveolae in the trafficking and signalling of CB1 receptor, the molecular mechanism of anandamide uptake, the relationship with CB1 receptor and the cellular compartments involved in the signal transduction events deriving from their interaction are not yet defined and even less is known about the cellular mechanisms controlling CB1 receptor intracellular trafficking and signalling. Therefore, we studied the subcellular distribution of the CB1 receptor in basal conditions, as well as its trafficking in respons to agonist stimulation in MDA-MB-231 cell line and examined whether CB1 receptor was associated with lipid rafts.

Finally, to elucidate the mechanism of the transduction pathways in the antitumour CB1 mediated effects, we investigated the role of caveolae/lipid rafts in anti-proliferative and anti-migratory effects induced by Met-F-AEA.

Matherials and Methods

Reagents and antibodies

Met-F-AEA (2-methyl-2'-F-anandamide) was purchased from Calbiochem. The selective CB1 antagonist, SR141716A, was kindly provided by Sanofi-Aventis (Montpellier, France). Cell culture reagents were purchased from Gibco Laboratories (Grand Island, NY). The anti-CB1R antibody was from Santa Cruz Biotechnology, antiFAK and anti-src were purchased from Upstate Biotechnology (Lake Placid, NY) and anti-Cav1 antibody was from BD Biosciences. The antibodies against BiP, Giantin and LysoTracker from StressGen Biotechnologies Corp. (120-4243 Glanford Ave. Victoria, BC, Canada). The antibodies against β1, α1, α2, α3, α4, α5, α6, αvβ5, αvβ3 integrin subunits or dimers were purchased from Chemicon (Temecula, CA). MCD, type IV collagen, fibronectin, laminin and protein A/G-agarose were purchased from Sigma–Aldrich (St. Louis, MO).

Cell culture

MDA-MB-231, a human breast carcinoma cell line, was grown in RPMI 1640 medium supplemented with 10% inactivated FBS and 2 mM l-glutamine. TSA-E1 is a metastasizing mouse cell line originated from a mammary adenocarcinoma that arose spontaneously in a BALB/c female retired breeder. TSA-E1 were grown in DMEM medium supplemented with 10% inactivated FBS and 2 mM l-glutamine. T47D, a poorly invasive human breast cancer cell line, was grown in DMEM medium supplemented with 10% inactivated FBS and 2 mM l-glutamine. Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Reverse-Transcriptase Polymerase Chain Reaction

Total RNA was extracted from cell lines by guanidinium thiocyanateisopropanol 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 1X TBE.

Proliferation assay

The effects of Met-F-AEA on MDA-MB-231 and T47D proliferation were evaluated, in vitro, by [^{3}H]thymidine incorporation. $5x10^4$ cells/ml were seeded into 96-well plates and immediately treated with the drugs, incubated for 24 h at 37°C (5% CO2), then pulsed with 0.5 μCi/well of [^{3}H]thymidine and harvested 12 h later. Radioactivity was measured in a scintillation counter (Wallac, Turku, Finland). The effect of Met-F-AEA on TSA-E1 proliferation was evaluated in vitro counting cells by a hemocytometer. $5x10^4$ cells were seeded into 24-well plates and treated with the drugs for 24 h. Then cells were collected and counted.

Assessment of apoptosis by annexin V/PI double-staining assay

The effect of Met-F-AEA on the apoptosis of MDA-MB-231 was assessed by annexin V/propidium iodide double-staining assay. Briefly, cells were incubated for 24 h with Met-F-AEA 10 μM, then collected, washed with PBS, and resuspended at a concentration of $1x10^6$ 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 supravital staining with recombinant FITC (fluorescein isothiocyanate)-conjugated annexin V–antibody and propidium iodide, using the Apoptest-FITC kit (Dakocytomation) according to manufacturer's instructions. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in a Becton Dickinson FACS Calibur flow cytometer using CellQuest software.

Adhesion assay

The assay was performed in 96-well plates. The wells were coated with the appropriate dilution in PBS of type IV collagen, fibronectin, or laminin and incubated overnight at 4°C, then the plates were filled with 1% heat-denatured BSA for 1 h at 37°C. Untreated cells or cells treated for 24 h with Met-F-AEA or with Met-F-AEA and SR141716A, were pleated into coated wells ($5x10^4$ cells/well) and incubated at 37°C (5% CO_2) for 60 min. Adherent cells, fixed with 3% paraformaldehyde for 10 min, washed with 2% methanol for 10 min, were stained with 0.5% crystal violet in 20% methanol for 10 min. The stain was eluted, and the

absorbance at 540 nm was measured by a 96-well plate reader. All experiments were performed in triplicate.

Migration assay

For chemotaxis assays, Boyden chambers (8 μm Transwell polycarbonate membrane, Costar) were coated with 50 μg/ml type IV collagen and blocked with 5 mg/ml BSA. Cells ($1x10^5$ cells) treated for 24 h with Met-F-AEA or Met-F-AEA and SR141716A were added to the upper compartment and incubated (at 37°C for 4 h) in migration media (RPMI) in the presence or in the absence of FBS used as a chemotactic stimulus in the lower compartment. Chambers were washed with PBS, and migratory cells on the lower membrane surface were fixed with 3% formaldehyde (10 min). Cells were permeabilized with 0.2% Triton X-100 (5 min) and stained with Hoechst dye (5 min). The number of migrated cells was counted by a light microscope at 20× 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.

Immunoprecipitation and Western blot analysis

Cells were treated with Met-F-AEA or with Met-F-AEA and SR141716A, lysed at 4°C in RIPA buffer (500 μl) and centrifuged at 14,500 rpm. The samples (500 μg of total cell extract) were incubated for 12 h at 4°C with 3–4 μg of monoclonal antibodies directed against FAK or Src, then added with 50 μl of protein A/G-agarose and incubated overnight at 4°C. Proteins were eluted with Laemmli sample buffer (5 min at 95°C) analyzed by SDS-PAGE (7,5% polyacrylamide gels), and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk and incubated (overnight at 4°C) with monoclonal antibody against phosphorylated Tyr or with monoclonal antibodies directed against total protein (FAK or Src). Filters, washed three time with T-PBS, were incubated for 45 min at room temperature with horseradish peroxidase-conjugated goat antimouse antibody. The membranes were then washed and stained using an enhanced chemiluminescence's system (Amersham, Aylesbury, UK). Western blot analysis of CB1 was performed with a polyclonal antibody for CB1.

Integrin expression analysis

Cells, treated for 24 h with Met-F-AEA or with Met-F-AEA and SR141716A, were harvested by 10 μM EDTA-PBS and incubated with the primary monoclonal antibody for β1, α1, α2, α3, α4, α5, α6, αvβ5, αvβ3 integrin subunits or dimers, for 1 h at 4°C in 0.5% BSA-PBS, washed in the same buffer, and incubated with the secondary fluorescein-conjugated antibody for 30 min at 4°C. Cells were resuspended in PBS and analyzed by flow cytometry. Non-specific immunoglobulins of the same isotype were used as controls. The expression of each integrin was represented as the mean fluorescence index (MFI = experimental mean fluorescence/control mean fluorescence).

Experimental lung metastasis

Monocellular suspension of TSA-E1 cells containing 2.5×10^5 cells was injected into the left paw of 30-day-old C57BL/6N male mice. Animals were divided into three groups (20 animals each): Control, Met-F-AEA (0.5 mg/kg/dose) or Met-F-AEA plus SR141716A (0.7 mg/kg/dose). The drugs were injected i.p. every 72 h. Experimental metastases were evaluated 21 days after the injection. To contrast lung nodules, lungs were fixed in Bouin's fluid, and metastatic nodes were scored on dissected lung under a stereoscopic microscopy. All animal studies were conducted in accordance with the Italian regulation for the welfare of animals in experimental neoplasia.

Immunofluorescent staining

Cells were plated in 24-well plates on coverslips (Becton–Dickinson Labware). When they were 60 ± 80% confluent, they were treated with Met-F-AEA (10 μM 24 h), and/or MCD (10 mM, 15 min). After the incubation with various drugs, the cells were washed twice with PBS and fixed in 3.7% paraformaldehyde in PBS for 20 min and followed by two washes in 50 mM NH_4Cl for 10 min. Permeabilization was achieved by incubating the fixed cells in 0.1% Triton X-100 in PBS for 5 min at room temperature. The cells were then blocked in FDB buffer (1 mM $MgCl_2$, 1 mM $CaCl_2$, 5% foetal calf serum and 2% BSA in PBS) for 30 min at room temperature. All primary and secondary antibody incubations were performed in FDB buffer for 1 h at room temperature. Coverslips were mounted on 50% glycerol in PBS and examined by using a Zeiss Laser Scanning Confocal Microscope (LSCM 410 or 510).

Cholesterol determination

In order to assay cholesterol levels in the cells before and after treatment with MCD we used the following method: MDA-MB-231 cells grown in the presence or absence of MCD were washed twice with PBS, lysed with appropriate lysis buffer and Infinity Cholesterol Reagent (Sigma Chemical Co., St. Louis, MO, code number 401-25 P) was added to the lysates in the ratio 1:10 for 5 min at 37 °C (according to the suggested Sigma protocol number 401). The samples were then measured in a spectrophotometer at 550 nm.

Assays for DRM-association

OptiPREP™ density gradients: OptiPREP™ gradient analysis of TX-100-insoluble material was performed using previously published protocols (Broquet et al., 2003). Cells were grown to confluence in 100 mm dishes, washed in PBS C/M and lysed for 20 min in TNE/TX-100 1% buffer (25 mM Tris–HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% TX-100) on ice. Lysates were scraped from dishes, brought to 40% OptiPREP™, and then placed at the bottom of a centrifuge tube. A OptiPREP™ gradient (5–35% TNE) was layered on top of the lysates and the samples were centrifuged at 21 000 rpm, at 4 °C for 4 h in an ultracentrifuge (model SW41 Beckman Inst., Fullerton, CA). One-milliliter fractions were harvested from the top of the gradient. CB1R was revealed by Western blotting using the anti-CB1R antibody. As control for correct lipid rafts isolation, Cav1 was chosen as raft marker and Bip as non-raft marker.

Statistical analysis

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

Results

Anandamide inhibits breast cancer cell proliferation

In order to evaluate the effect of anandamide on the proliferation of breast cancer cells, MDA-MB-231 and T47D cell lines were treated with increasing concentrations of its synthetic analogous methanandamide (Met-F-anandamide) which is more stable to the fatty acid amide hydrolase hydrolysis than the anandamide itself, and DNA synthesis was determined by [^{3}H]-thymidine incorporation. The anandamide analogue strongly reduced the proliferation of

MDA-MB-231 in a dose-dependent manner, the reduction being statistically significant at 10 and 20 μM concentrations compared to the control (Fig. 1A). We observed that nanomolar concentrations of Met-F-AEA do not enhance MDA-MB-231 cell proliferation (data not shown). Interestingly, the inhibitory effect of Met-F-AEA on MDA-MB-231 cell proliferation was much higher than the one observed in a poorly invasive and non-metastatic cell line (T47D cells, Fig. 1B), whereas in the proliferation assay carried out on TSA-E1 cells, we found a significant decrease of cell proliferation already at 10 μM (Fig. 1C). Treatment of MDA-MB-231 with Met-F-AEA did not induce apoptosis or necrosis as revealed by a flow cytometric assay with annexin V/propidium iodide double staining (Fig. 1D).

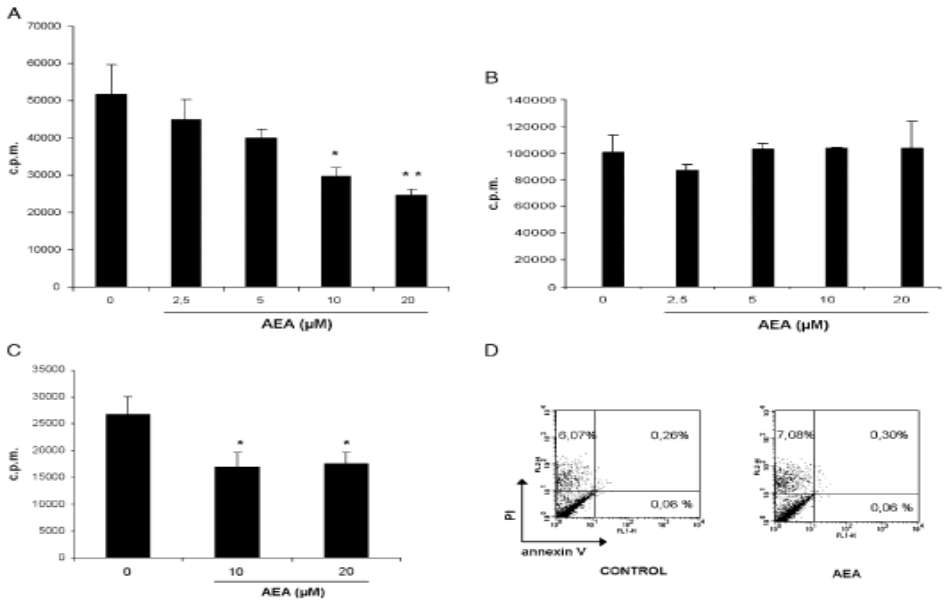

Fig. 1 Effect of Met-F-AEA (AEA) on cell proliferation. (A) Met-F-AEA (AEA) inhibits MDA-MB-231 cell proliferation. MDA-MB-231 cells (5×10^4/well) were cultured in triplicate for 24 h with concentrations of Met-F-AEA (AEA) ranging from 2.5 to 20 μM. After 18-h incubation, [^{3}H]-thymidine incorporation (0.5 μCi/well) was measured. The graph reports the mean ± SD values of three independent experiments. Inhibition of proliferation was statistically significant (one-way analysis of variance (ANOVA) $F = 15.4$, $P < 0.01$, with Student's t test $^*P < 0.05$ and $^{**}P < 0.01$ Met-F-AEA (AEA) versus control). (B) Met-F-AEA (AEA) does not inhibit T47D cell proliferation. T47D cells (5×10^4/well) were cultured in triplicate for 24 h with concentrations of Met-F-AEA (AEA) ranging from 2.5 to 20 μM. After 18-h incubation, [^{3}H]-thymidine incorporation (0.5 μCi/well) was measured. The graph reports the mean ± SD values of three independent experiments. Inhibition of proliferation was not statistically significant. (C) Met-F-AEA (AEA) inhibits TSA-E1 cell proliferation. TSA-E1 cells (5×10^4/well) were cultured in triplicate in 24-well plate for 24 h with Met-F-AEA at 10 and 20 μM. After incubation, cells were collected and counted. The graph reports the mean ± SD values of five independent experiments. Inhibition of proliferation was statistically significant (one-way ANOVA $F = 20.33$, $P < 0.001$, with Student's t test $^*P < 0.05$ Met-F-AEA (AEA) versus control). (D) Met-F-AEA (AEA) does not induce apoptosis or necrosis. MDA-MB-231 cells were incubated for 24 h with Met-F-AEA 10 μM; then cells were collected, washed with PBS, and resuspended at a concentration of 1×106 cells/ml in annexin V binding buffer. Apoptotic cell death was identified by double supravital staining with recombinant FITC (fluorescein isothiocyanate)-conjugated annexin V and propidium iodide. The results shown are representative of one of three independent experiments.

Anandamide inhibits cell adhesion and migration on collagen

The metastatic process is a complex cascade of events. Two important steps in this cascade involve the ability of cancer cells to adhere to extracellular matrix components and subsequently migrate through them. Pivotal components of the machinery promoting tumour cell invasion are adhesion molecules that modulate cell adhesion and migration. In order to determine the effect of Met-F-AEA on tumour adhesion to ECM and migration, we performed in vitro adhesion and migration assays. Moreover, we performed both western blots and RT-PCR analysis for the CB1 receptor in MDA-MB-231, T47D and TSA-E1 cells. We found that CB1 receptor was expressd in our cell systems (fig.2a) and, as expected, we did not detect mRNA expression in CHO cells (fig.2b).

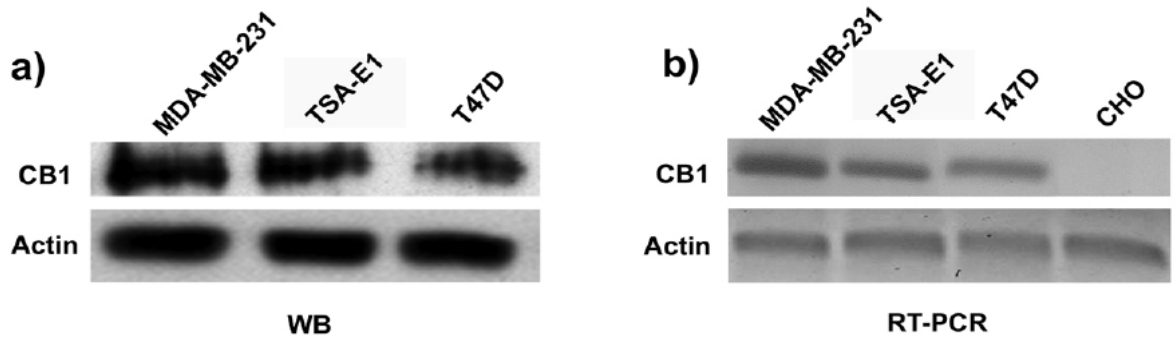

Fig. 2 CB1R expression levels in breast cancer cell lines. a) cell lysates (50 μg of total proteins) were subjected to SDS-polyacrylamide gel electrophoresis. CB1R was revealed by Western blotting on nitrocellulose and hybridization with polyclonal antiactin antibody as loading control. The experiment was repeated three times. b) for RT-PCR the cells (MDA-MB-231, TSA-E1, T47D, and CHO) were subjected to total RNA extraction, and RT was performed using Moloney murine leukemia virus reverse transcriptase, and random oligonucleotide primers for CB1R and actin were used. PCR were performed 30 s at 93°C, 1 min at 59°C, and 1 min at 69°C for 25 to 28 cycles to assess saturation of the signal.

So, we used the selective CB1 receptor antagonist SR141716A to assess the role of these receptors in the biological processes of migration and adhesion. Cell attachment assays were performed in 96-well flat-bottom microtiter plates coated with different concentrations of type IV collagen, fibronectin, or laminin (Fig. 3). The cells showed a concentration-dependent adhesion to all these substrates, reaching its maximum at about 12.5 μg/ml collagen, 5 μg/ml fibronectin, and 12.5 μg/ml laminin. Met-F-AEA-treated cells showed a significant reduced adhesion to collagen, and this effect was antagonized by treatment with CB1 receptor antagonist SR141716A (Fig. 3A). Noteworthy, Met-F-AEA treatment had no effect on cell adhesion to fibronectin and laminin (Figs. 3B and 3C). Met-F-AEA (10 μM) was unable to modify the cellular shape and spreading, and therefore, the results obtained in the adhesion assays do not reflect alterations beyond cell adhesion itself (Fig. 3D).

We then performed migration assays with type IV collagen, the most prevalent component of the basement membrane.

We observed that Met-F-AEA inhibited for at least 60% the migration of MDA-MB-231 cells on type IV collagen, and this effect was antagonized by SR141716A (Fig. 4A). Data obtained in the absence of chemotactic-factor gradient (chemokinesis) were shown in Fig. 4B. Moreover, similar results were obtained in the murine breast cancer cell line TSA-E1 where Met-F-AEA inhibited for at least 70% the migration on type IV collagen (Fig. 4B).

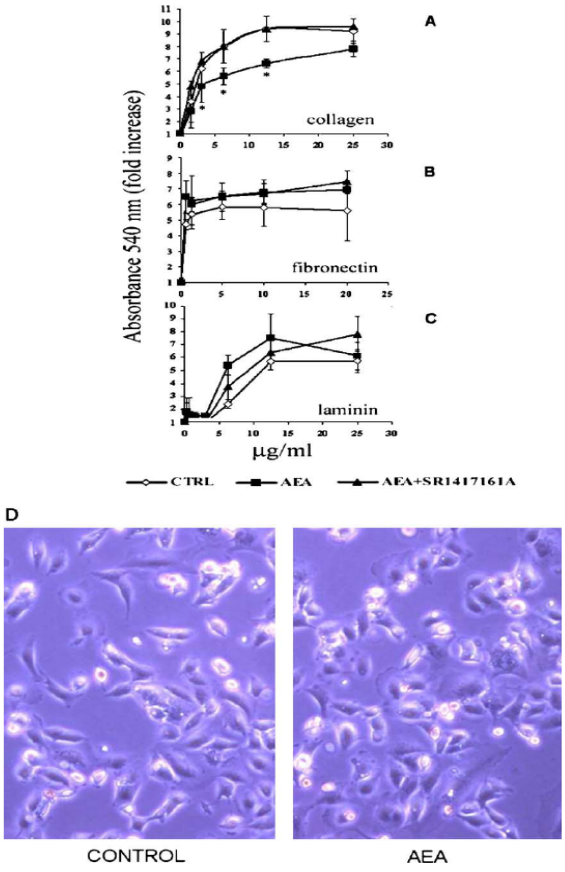

Fig. 3 Effect of Met-F-AEA (AEA) on cell adhesion. (A–C) Cells were incubated with Met-F-AEA (10 µM) or with Met-F-AEA + SR141716A (100 nM) for 24 h and plated into 96-well plates coated with type IV collagen, fibronectin, or laminin. After 1-h incubation at 37°C, the plates were washed, fixed, and stained with crystal violet. The stain was eluted, and absorbance at 540 nm was measured. The curves report the optical density versus substrate concentration and reports the mean ± SD values of three independent experiments. Reduction in adhesion to collagen induced by Met-F-AEA was statistically significant (one-way ANOVA $F = 59.4$, $P < 0.05$, with post hoc Bonferroni test, $^*P < 0.05$ Met-F-AEA (AEA) versus control and Met-F-AEA + SR141716A versus Met-F-AEA (AEA) at 0.3 µg/ml, 0.6 µg/ml, and 1.2 µg/ml collagen concentration). Met-F-AEA treatment had no statistically significant effect on cell adhesion to fibronectin and laminin. (D) Met-F-AEA (AEA) does not affect the shape and spreading of cells. MDA-MB-231 cells were incubated with Met-F-AEA (10 µM) for 24 h. Four fields, for each treatment, were acquired in light microscopy at 20× magnification and cell spreading measured. The presented results are representative of four different acquisitions with similar results.

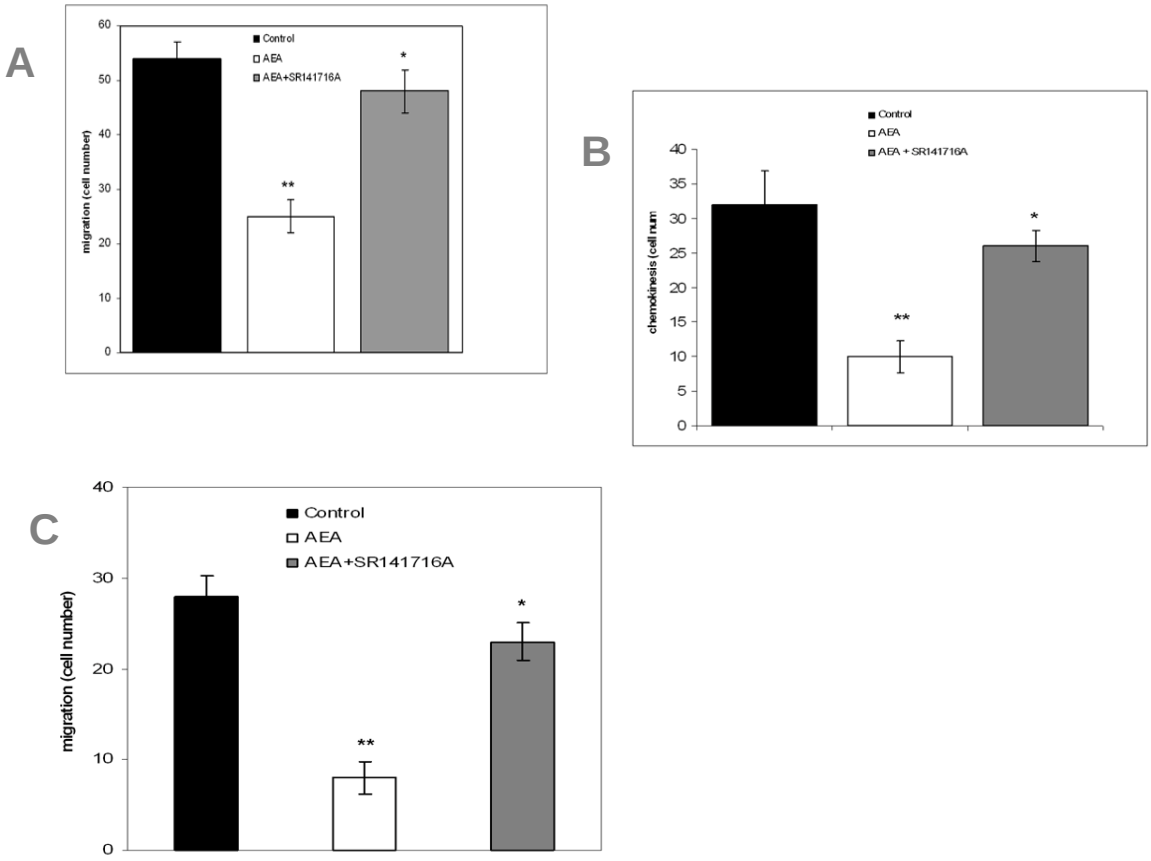

Fig. 4 Effect of Met-F-AEA (AEA) on cell migration. (A) Met-F-AEA (AEA) inhibits MDA-MB-231 migration. Cells were incubated with Met-F-AEA (10 μM) or with Met-F-AEA + SR141716A (100 nM) and plated in the upper compartment of Boyden chambers coated with type IV collagen. After 4 h at 37°C, migratory cells in the lower chamber were stained and counted under a light microscope. Shown is the mean ± SD values of triplicates from at least four independent experiments (one-way ANOVA $F = 40.14$, $P < 0.001$, with post hoc Bonferroni test, $^{**}P < 0.001$ for Met-F-AEA (AEA) versus control and $^{*}P < 0.05$ for Met-F-AEA + SR141716A versus Met-F-AEA). The background represented by the number of migrated cells in absence of the chemoattractant factor (chemokinesis, Fig. 3B) was subtracted from each experimental point (one-way ANOVA $F = 46.85$, $P < 0.001$, with post hoc Bonferroni test, $^{**}P < 0.001$ for Met-F-AEA (AEA) versus control and $^{*}P < 0.05$ for Met-F-AEA + SR141716A versus Met-F-AEA). (C) Met-F-AEA (AEA) inhibits TSA-E1 migration. Shown is the mean ± SD values of triplicates from at least four independent experiments (one-way ANOVA $F = 68.12$, $P < 0.001$, with post hoc Bonferroni test, $^{**}P < 0.001$ for Met-F-AEA (AEA) versus control and $^{*}P < 0.05$ for Met-F-AEA + SR141716A versus Met-F-AEA).

Anandamide decreases FAK and Src phosphorylation in MDA-MB-231 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. Numerous

malignant neoplasies show FAK and Src overexpression and activation, which underlie increased invasiveness and metastasis (98). In order to gain further insights into the molecular mechanisms involved in these processes and to clarify the role of the endocannabinoid system in tumour progression, we analyzed the phosphorylation of FAK and Src in Met-F-AEA-treated cells. MDA-MB-231 cells were treated for 15 min with Met-F-AEA with or without the CB1 blocker SR141716A, and the extent of FAK and Src phosphorylation was determined by Western blot with antiphospho-tyrosine in immunoprecipitates obtained with anti-FAK or anti-Src antibodies. Total FAK and Src levels were not modified by treatment of cells with Met-F-AEA (Fig. 5A), whereas incubation with Met-F-AEA induced a remarkable decrease of FAK and Src phosphorylation in a way antagonized by SR141716A (Fig. 5B).

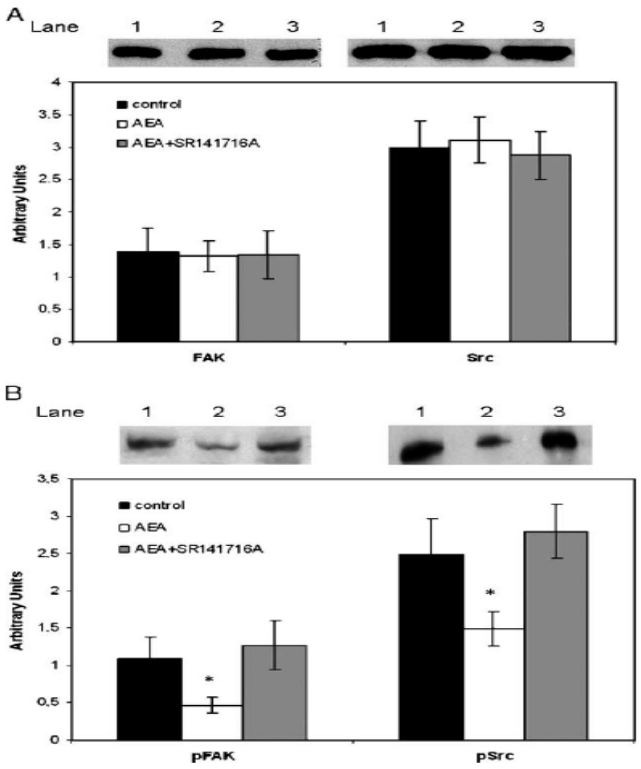

Fig. 5 Met-F-AEA (AEA) decreases FAK and Src phosphorylation in MDA-MB-231 cells. Cells were incubated with Met-F-AEA (10 μM) or with Met-F-AEA + SR141716A (100 nM) for 15 min. Cell extracts were subjected to immunoprecipitation with anti-FAK or anti-Src antibodies. (A) Immunoprecipitates were analyzed by Western blot with anti-FAK or anti-Src antibodies. (B) Immunoprecipitates were then analyzed by Western blot with antiphosphorylated tyrosine and measured by a chemiluminescent detection system. We reported the representative blots of each protein and mean ± SD values of optical density of three independent experiments (one-way ANOVA $F = 14.01$ (pFAK) and $F = 11.39$ (pSrc), $P < 0.01$, with post hoc Bonferroni test, $^*P < 0.05$ for Met-F-AEA (AEA) versus control and Met-F-AEA + SR141716A versus Met-F-AEA). (Lane 1: control; lane 2: Met-F-AEA; lane 3: Met-F-AEA + SR141716A).

Anandamide did not significantly modify the integrin expression

The integrin family represents the most important group of adhesion molecules regulating cell adhesion to the ECM and subsequent cell migration. Therefore, we analyzed the effect of Met-F-AEA on integrin expression by flow cytometry using monoclonal antibodies against single subunits or heterodimers (Fig.6). The α3β1 was the integrin receptor most expressed in MDA-MB-231 cells. As the αv subunit can dimerize with different β subunits, we measured also heterodimers by specific antibodies. The treatment of these cells with Met-F-AEA did not significantly modify the overall integrin expression.

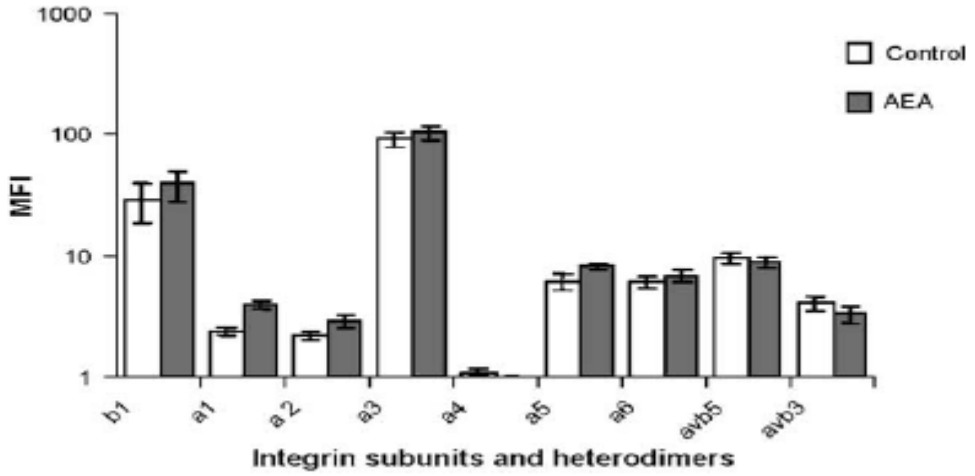

Fig. 6 Effect of Met-F-AEA (AEA) on integrin expression. MDA-MB-231 cells were incubated with or without Met-F-AEA 10 μM (AEA) for 24 h at 37°C, harvested by EDTA, and incubated with primary monoclonal antibody against integrin chains (β1, α1, α2, α3, α4, α5, α6) or dimers (αvβ5, αvβ3) for 1 h at 4°C in 0.5% BSA-PBS. Cells were finally incubated again with the secondary fluorescein-conjugated antibody for 30 min at 4°C. Cells were resuspended in BSA-PBS and analyzed by flow cytometry. The values plotted on log scale represent the mean relative fluorescence intensities (MFI). Shown is the mean ± SD values of three independent experiments.

Anandamide reduces the number of metastatic nodes in vivo

The hypothesis that CB1 receptor stimulation could interfere with metastatic processes was also verified in a model of metastatic infiltration in vivo. The murine breast cancer TSA-E1 cells were injected in syngenic C57BL/6N mice to induce lung metastasis. Animals were divided into three groups and Met-F-AEA plus vehicle (0.5 mg/kg/dose), Met-F-AEA plus SR141716A (0.7 mg/kg/dose), or the vehicle alone, were injected i.p. every 72 h. Met-F-AEA significantly reduced the number of metastatic nodes, evaluated 21 days after the injection, this effect being antagonized by SR141716A (Fig.7).

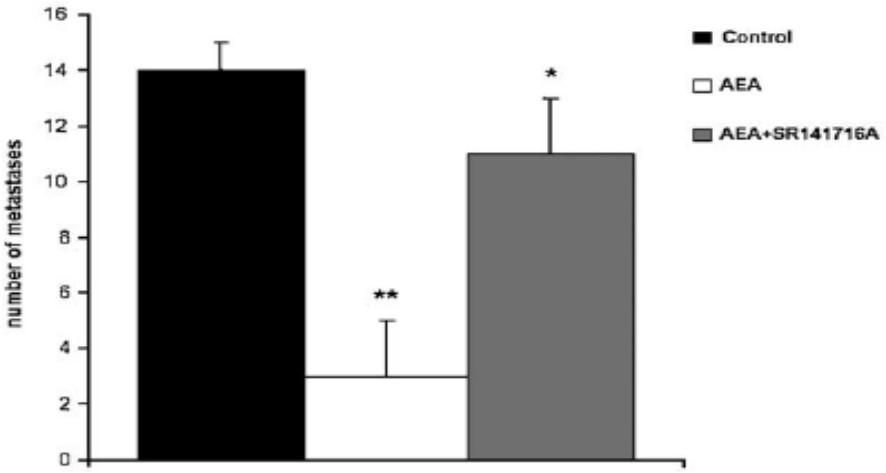

Fig. 7 Met-F-AEA (AEA) reduces the number of metastatic nodes in vivo. The murine breast cancer cells (TSA-E1) were inoculated in syngenic C57B1/6 mice. Suspension of TSA-E1 cells was injected into the left paw of 30-day-old C57B1/6 male mice. Animals were divided into three groups and Met-F-AEA (AEA, 0.5 mg/kg/dose) or Met-F-AEA + SR141716A (SR, 0.7 mg/kg/dose) were injected i.p. every 72 h. Experimental metastases were evaluated 21 days after the injection. To contrast lung nodules, lungs were fixed in Bouin's fluid, and metastatic nodes were scored with a stereoscopic microscope. Shown is the mean and SD values of each group. Differences in the number of nodules were statistically significant (one-way ANOVA $F = 32.33$, $P < 0.001$, with post hoc Bonferroni test, $^{**}P < 0.001$ for Met-F-AEA (AEA) versus control and $^{*}P < 0.05$ for Met-F-AEA + SR141716A versus Met-F-AEA). Results shown are representative of three independent experiments.

Characterization of CB1R expression and localization in MDA-MB-231 cells

We analyzed the cellular localization of CB1R by indirect immunofluorescence and confocal microscopy on cells grown on coverslips. In non-permeabilized conditions CB1R was localized on the plasma membrane of MDA-MB-231 cells mainly concentrated in large spots (Fig. 8A). In permeabilized conditions, by using markers of different intracellular compartments, such as giantin for the Golgi complex, Bip for the endoplasmic reticulum, early endosome antigen-1 for early endosomes (data not shown) and LysoTracker to label lysosomes, we found that the receptor was widely distributed in the cytoplasm and was particularly concentrated in lysosomes as shown in the merged signal from the anti-CB1R antibody and LysoTracker (Fig. 8B). Lipid rafts represent versatile devices for compartmentalizing cellular membrane processes and form large platforms involved in protein and lipid signalling, processing and transport (58; 75; 76). Because raft domains exist both at the plasma membrane and lysosomes (77), and a lysosomal degradative pathway for CB1 receptor has not yet been demonstrated, we sought to analyze whether the intracellular compartmentalization of CB1R was dependent on lipid rafts.

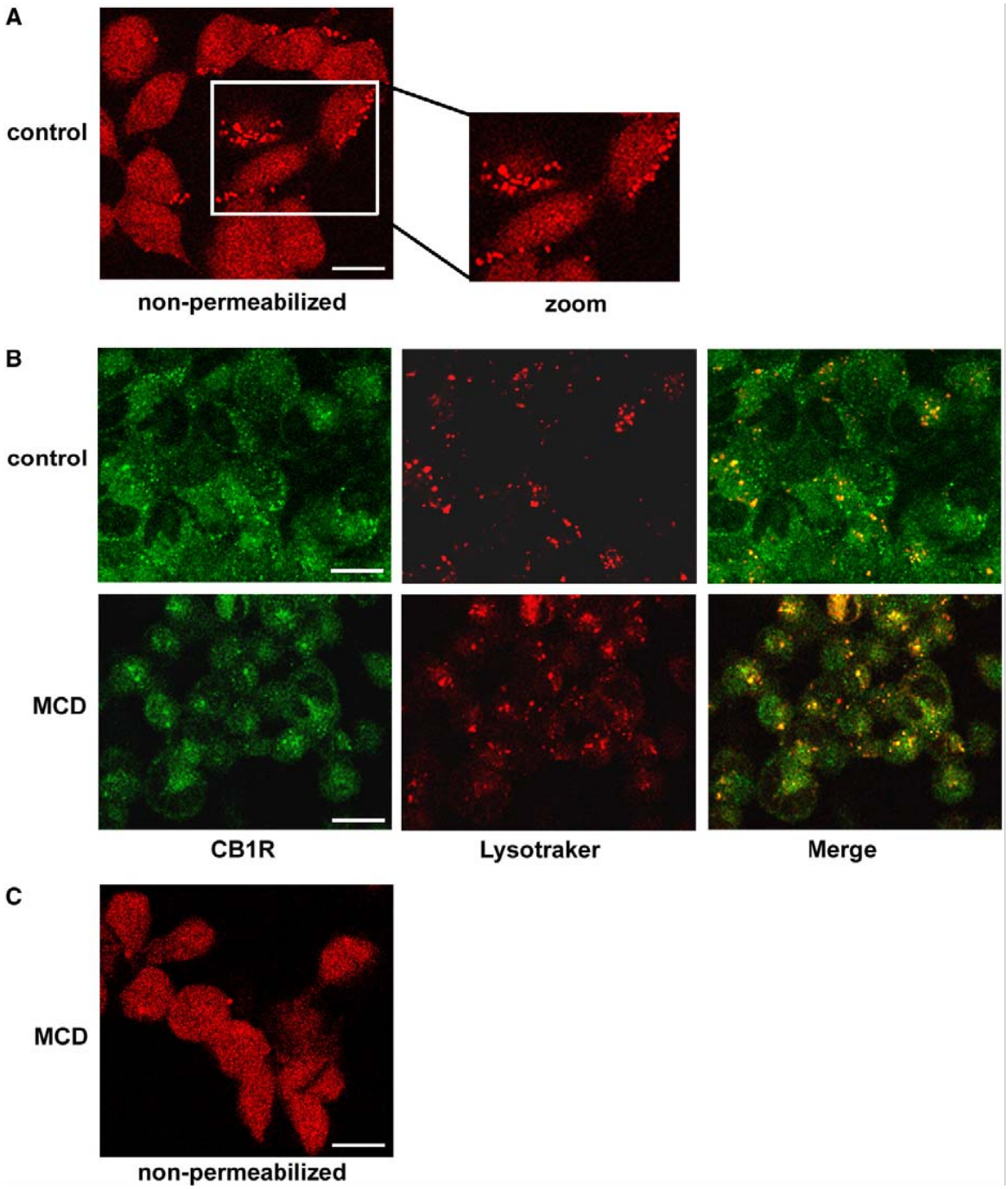

Fig. 8 Surface distribution and intracellular localization of CB1R. (A) The cells were grown on coverslips and fixed with paraformaldehyde. CB1R was revealed by incubating the cells with the first antibody and then with TRITC-conjugated secondary antibody under non-permeabilized conditions. (B) The cells were grown on coverslips without (control) or with MCD for 15 min at 37 °C and were incubated 1 h with LysoTracker 1:10 000 in complete culture medium. They were then fixed, permeabilized with TX-100 and CB1R was revealed with a FITC-conjugated secondary antibody. All the immunofluorescence samples were observed using a Laser Scan Confocal microscope. The yellow spots indicate that lysosomes and CB1R colocalize. (C) Cells were subjected to immunofluorescence analysis as in A, with the exception that here the cells were analyzed after cholesterol depletion with MCD (15 min at 37 °C).

To this aim we studied the intracellular distribution of CB1R after lipid rafts disruption by cholesterol depletion. Cholesterol is an important functional and structural component of lipid rafts and its depletion by using different drugs has been demonstrated to alter the raft composition and as a consequence the raft functions (56; 59). After incubation with MCD which extracted ~60% of the total cholesterol, we found that lysosomal localization of CB1R was not affected as shown by colocalization with LysoTracker (Fig. 8B, MCD, see merge in the right panel). On the contrary, cholesterol depletion strongly altered the cell surface CB1R localization inducing a more uniform plasma membrane distribution of the receptor (Fig. 8C). These data suggest that the plasma membrane clustering of CB1 is dependent on lipid rafts, while the receptor does not appear associate to cholesterol-dependent domains in the lysosomes. Thus, these results indicate that lipid rafts do not appear to regulate the route of CB1R to the lysosomes where presumably degradation of CB1R occurs.

CB1R localization after anandamide treatment

Because the nature of CB1R interaction with AEA, as well as the exact cellular site for their interaction are not yet known, we decided to study the effect of Met-F-AEA on CB1R intracellular localization. As shown by immunofluorescence in permeabilized conditions (Fig. 9), the presence of Met-F-AEA (10 μM, for 24 h) added to the extracellular medium did not change the intracellular distribution of CB1R (compare Fig. 9, AEA, with Fig. 8B, control). Interestingly, pre-treatment with MCD (10 mM for 15 min at 37 °C) before agonist incubation, significantly changed the intracellular distribution of the receptor which assumed a more diffuse cytoplasmic localization and lost its lysosomal localization (see Fig. 9, MCD + AEA). Thus, these data indicate that the presence of the agonist modifies the intracellular localization of the receptor when lipid rafts are perturbed by cholesterol depletion, suggesting that the intracellular pathway followed by CB1R to the lysosomes, after the binding of the ligand anandamide, is depending on lipid rafts integrity.

Characterization of CB1R-association with detergent-resistant microdomains

Association of CB1R with lipid rafts has not yet been formally demonstrated. Thus, we analyzed whether it was associated with detergent-resistant microdomains (DRMs) by performing TX-100 extraction and flotation-based assays. Indeed, resistance to non-ionic detergent extraction at 4 °C (e.g., TX-100) and association with DRMs is one of the major biochemical characteristics of lipid rafts and raft components (78; 79; 80; 81).

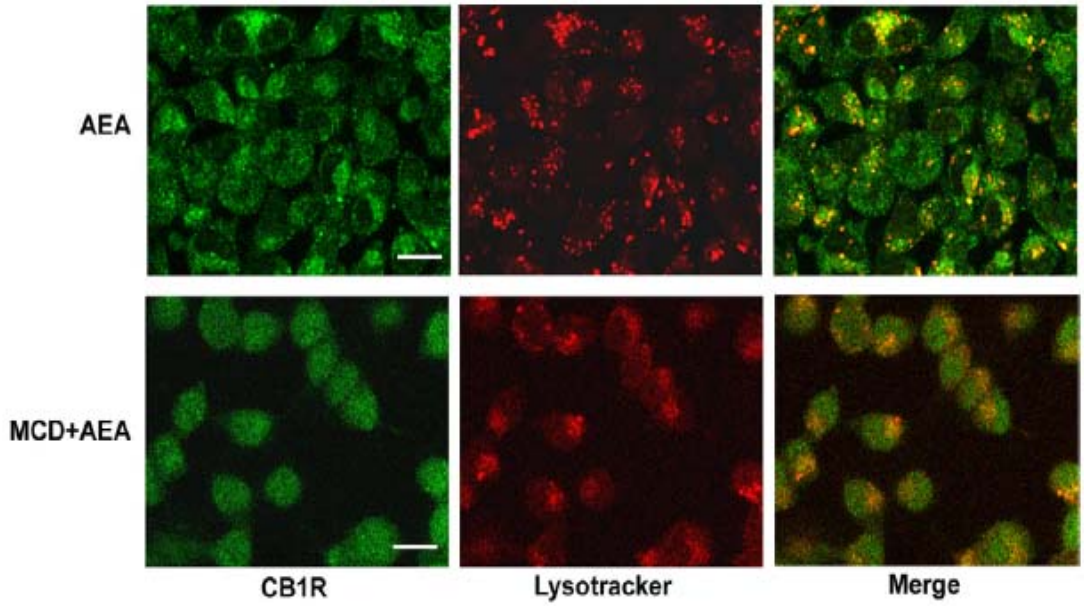

Fig. 9 Localization of CB1R after Met-F-AEA incubation in control or cholesterol depleted cells. MDA-MB-231 cells were grown on coverslips in culture medium containing Met-F-AEA (10 μM, 24 h). Where indicated, the cells were first cholesterol depleted with MCD and then treated with Met-F-AEA (see Section 2). CB1R was revealed by indirect immunofluorescence analysis as in Fig. 8B. Samples were observed using a Laser Scan Confocal microscope.

We found that CB1R was TX-100 insoluble at steady-state (not shown) and specifically ~ 80% of the receptor floated in the fractions 4–5 of the gradients, which are the typical DRMs containing fractions (Fig.10A, control), as shown by the flotation of Cav1, an extensively characterized raft marker (Fig. 10A, Cav1) (82). In particular, Western blot analysis on an aliquot of the individual fractions, confirmed an enrichment of Cav1 in fraction 5 (Fig. 10A), confirming that we had successfully isolated DRM-domains. We also verified that a typical non-raft marker, the endoplasmic reticulum resident protein Bip/GRP78, was excluded from these fractions (Fig. 10A). Of note, the prevalent enrichment of both CB1 receptor and Cav1 in the high density fraction 5 more than in fraction 4 of the gradient (Fig. 10A, control) suggested that the two proteins were distributed in lipid rafts of similar density.

Because cholesterol is an important structural and functional component of rafts (56;75), we determined whether CB1R raft-association was cholesterol-dependent. After treatment with MCD (10 mM, 15 min 37 °C), the amount of CB1R present in the lighter fractions (fractions 4 and 5) of the gradient significantly decreased, and it was estimated to be ~ 10% of the total

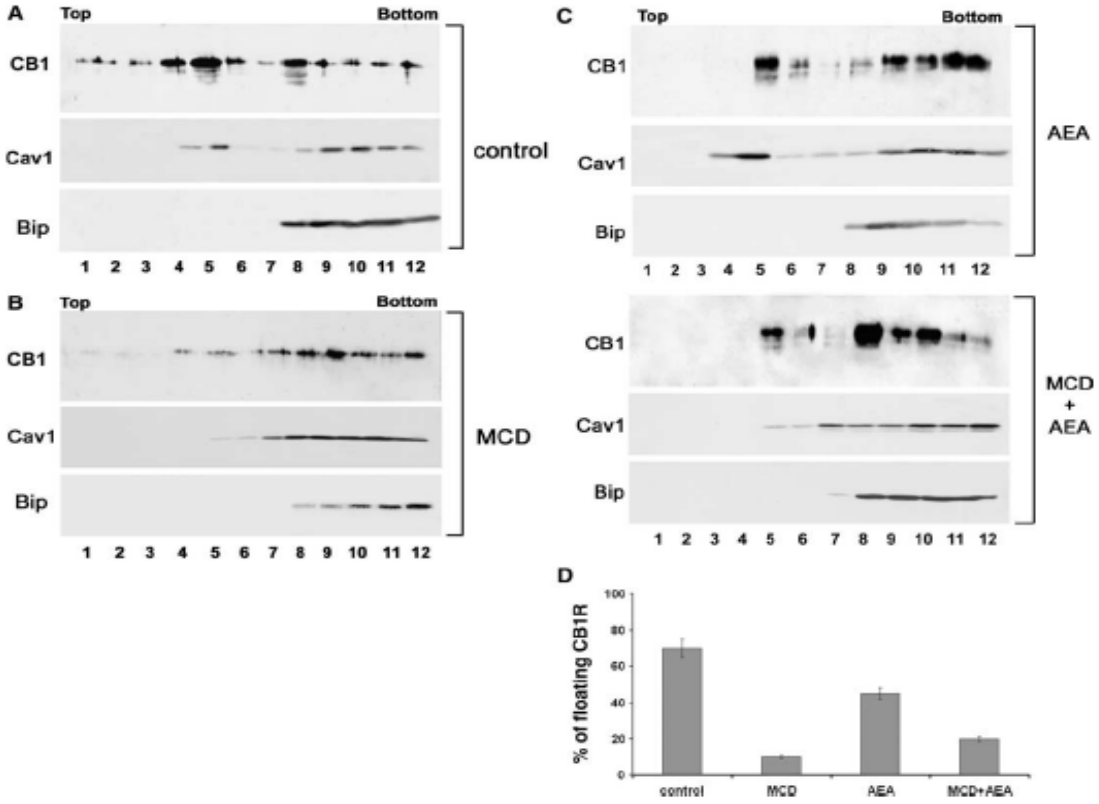

Fig. 10 Purification of CB1R on OptiPREP™ density gradients and effect of MCD and anandamide on CB1R raft-association. (A) The cells were grown in 100 mm dishes in control conditions. They were then lysed for 20 min in cold TNE/TX-100 buffer and then run through a "Two Step" OptiPREP™ gradient (5–40% gradient). One-milliliter fractions were collected from the top of the gradient after centrifugation to equilibrium. An aliquot of each fraction was loaded on 12% gel and revealed by Western blotting and ECL. The distribution of Cav1 and Bip was analyzed by Western blot, respectively, with the anti-Cav1 and anti-Bip antibody and ECL. (B) CB1R purification from the OptiPREP™ gradients was performed after cholesterol depletion with MCD. CB1R, Cav1 and Bip were revealed following the procedure described above. (C) The cells were grown in 100 mm dishes and treated with Met-F-AEA in control condition or after cholesterol extraction by MCD (MCD + AEA) and they were then subjected to purification of CB1R, Cav1 and Bip from OptiPREP™ density gradient fractions. An aliquot of each fraction was loaded on 12% gel, separated with SDS–PAGE and revealed by Western blot and ECL. (D) Quantification of the amount of CB1R in the raft fractions 4 and 5 of the gradients, in different indicated conditions, is reported as a percentage of the total amount of the protein in the gradient and was performed by a densitometric analysis of the bands by NIH image program for McIntosh. Error bars are indicated in the graph and represent the average of three independent experiments.

amount of protein distributed in the gradient (Fig. 10 and compare fractions 4–5 of the control with fractions 4–5 of the MCD treatment). As expected and previously shown in MDA-MB-231 cells (82), following cholesterol depletion there was a significant reduction of the raft marker Cav1 in the lipid raft fraction (fractions 4–5) but not of the non-raft protein

Bip (Fig. 10B). These findings suggest that both CB1R and Cav1 raft-association are dependent on cellular cholesterol levels.

Effect of anandamide on CB1R raft-association

In order to directly test the influence of the CB1R agonist on its raft-association, we included the metabolically stable anandamide analogue Met-F-AEA in the culture medium of MDA-MB-231 cells and then performed the flotation assay. We found that ~ 50% of total CB1R floated in the DRM-fractions (Fig. 10C and D, see fractions 4–5 AEA) in the presence of the ligand. Thus, differently from basal conditions in which ~ 80% of the receptor was found in the light fractions 4–5 (Fig. 10A, control), the anandamide binding lead to a redistribution of the receptor in the soluble fractions of the gradient (fractions 8–12). It is noted that after AEA treatment, Cav1 and Bip distribution in the OptiPREP™ preparation was the same of the control condition suggesting that, AEA affects specifically CB1R raft-association inducing a decrement of CB1R flotation which was exclusively related to fraction 5. Furthermore, contrarily to CB1R, we found that the flotation profile of Cav1 was not affected by AEA but exclusively dependent on cholesterol. After MCD treatment, in the presence of its agonist only ~20% of CB1R floated in the OptiPREP™ gradient and interestingly the majority of the receptor accumulated in the fraction 8 (non-raft membrane) (Fig. 10C, MCD + AEA and 4D and compare with Fig. 410A, control). These results clearly suggest that the profile of CB1R distribution in the gradient fractions is affected by its ligand. Cholesterol depletion further reduces the flotation of the receptor indicating that part of the AEA binding could occur in cholesterol sensitive domains.

Cholesterol depletion prevented anandamide-mediated antiproliferative and antimigration effect

To gain further insights into the involvement of lipid rafts in the antitumour effect induced by anandamide, MDA-MB-231 cell growth and cell migration were tested after treatment with Met-F-AEA (10 μM) in basal conditions and after perturbation of lipid raft composition with MCD (10 mM).

Thus, we performed the proliferation assay by [^{3}H]thymidine uptake into cells and in vitro migration assay on type IV collagen in basal condition and after cholesterol depletion by MCD.

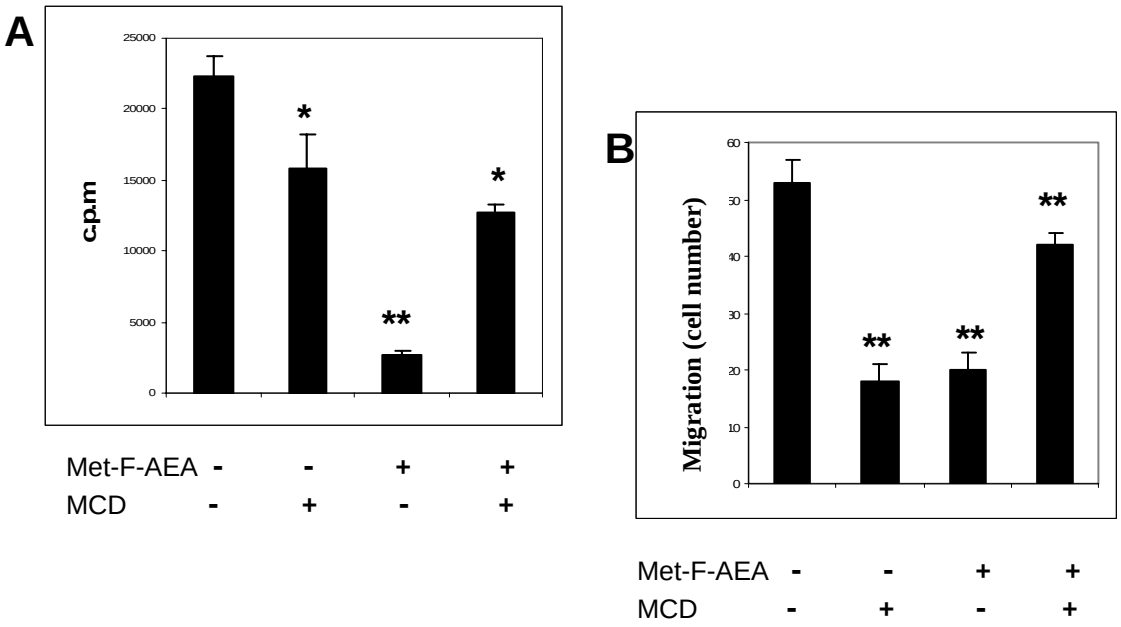

Fig. 11 Evaluation of cell proliferation and migration after lipid rafts perturbation. A) MDA-MB-231 cells were cultured in triplicate and treated with MCD for 15 min (alone or subsequently with Met-F-AEA for 24 h) or for 24 h with Met-F-AEA 10μM. After 24-h incubation, [^{3}H]thymidine incorporation (0.5 mCi/well) was measured. The graph reports the mean ±S.E. values of three independent experiments. Results were statistically significant (ANOVA, $p < 0.001$, with post hoc Bonferroni test, $p < 0.001$ for Met-F-AEA versus control, $p < 0.01$ for MCD versus control and for Met-F-AEA +MCD versus Met-F-AEA).
B) MDA-MB-231 cells were treated with MCD for 15 min (alone or subsequently with Met-F-AEA for 24 h) or for 24 h with Met-F-AEA 10μM. After 24-h incubation, cells were plated in the upper compartment of Boyden chambers coated with type IV collagen. After 4 h at 37°C, migratory cells in the lower chamber were stained and counted under a light microscope. Shown is the mean ± SD values of triplicates from at least four independent experiments. The background represented by the number of migrated cells in absence of the chemoattractant factor was subtracted from each experimental point. Results were statistically significant (ANOVA, $p < 0.001$, with post hoc Bonferroni test, $p < 0.001$ for Met-F-AEA versus control, MCD versus control, and for Met-F-AEA +MCD versus Met-F-AEA).

Interestingly, we found that the antiproliferative and antimigration effects exhibited by Met-F-AEA was partially reverted by pretreatment with MCD (Fig. 11A and 11B), indicating that the cell growth arrest and migration inhibition induced by the CB1 ligand need integrity of lipid rafts to occur. In support of our data, there is a large body of evidence that G protein coupled receptor functions depend on the lipid raft integrity (58; 83; 84).

Discussion

The endocannabinoid system is an almost ubiquitous signalling system involved in the control of cell fate. Recent studies have investigated the possibility that drugs targeting the endocannabinoid system might be used to retard or block cancer growth. The

endocannabinoids have been shown to inhibit the growth of tumour cells in culture and animal models by modulating key cell signalling pathways (85; 86). In previous studies, we reported that stimulation of cannabinoid CB1 receptors by the metabolically stable endocannabinoid analogue Met-F-AEA inhibits apex ras activity, prevents proliferation of v-K-ras-transformed rat thyroid cells both in vitro and in vivo and is also able to block the growth of already established tumours (51). Indeed, our very recent data show that Met-F-AEA significantly inhibits, in tumours as well as in transformed cells, the expression of the vascular endothelial growth factor (VEGF), an angiogenetic factor known to be up-regulated by p21ras, as well as of one of its receptors, flt-1/VEGFR-1. The levels of the cyclin-dependent kinase inhibitor p27(kip1), which is down-regulated by p21ras, were instead increased by Met-F-AEA. All these effects were antagonized by the selective CB1 receptor antagonist SR141716A. Met-F-AEA inhibited in vitro the growth of a metastasis-derived thyroid cancer cell line more potently than a primary cancer cell line (51). We showed that Met-F-AEA significantly reduced the number and size of metastatic nodes in an animal model of metastatic spreading (formation of lung nodules after inoculation of 3LL cells), in a way antagonized by SR141716A (52). Results obtained from other laboratories demonstrated the capability of cannabinoids to interfere with angiogenesis and the expression of proteins involved in this process, including VEGF and its receptors (49; 87) and to impair the migration of non-solid tumour cells (88). Therefore, we hypothesized that CB1 receptor stimulation could interfere also with the metastatic processes. Despite the promising effects of cannabinoids on cancer growth and spreading demonstrated by us and others, little data are available about the molecular mechanisms underlying their effects.

We have decided to investigate the endocannabinoids effects in human breast metastatic models and we found that Met-F-AEA has a significant and dose-dependent antiproliferative property, through the CB1R, in human breast cancer cell line, such as MDA-MB-231, highly invasive metastatic ER- cells. Interestingly, the inhibitory effect of Met-F-AEA was much higher than the one observed in a poorly invasive, non metastatic and ER+ cell line (T47D). We observed that Met-F-AEA inhibited cell adhesion and migration of the human breast carcinoma cell line (MDA-MB-231) and murine breast cancer cell line (TSA-E1), evaluated by in vitro adhesion and migration assays on type IV collagen, one of the most represented components of the basement membrane, and that these effects were antagonized by SR141716A, thus pointing to the role of cannabinoid CB1 receptors in these effects. Interestingly, the effect of Met-F-AEA on cell adhesion is restricted to collagen IV within the ECM components tested. This selective effect is not surprising as integrin affinity for their

putative ligands is modulated by several intra- and extracellular factors. Moreover, cannabinoid CB1 receptors stimulate migration in normal cells (53). Therefore, our data support the increasingly accepted notion that the endocannabinoid system very often induces opposing effects on important aspects of cell physiology in normal and transformed cells (89). 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 noninvasive phenotype in metastatic cells by acting on one of these targets. Metastatic dissemination of epithelial cells depends on tumour cell invasion of the basement membrane and then migration, survival in the circulation, and finally adhesion and invasion into ectopic tissues. These steps involve cooperation between integrins, a family of trans-membrane adhesion receptors, and matrix metalloproteinases. Integrins can exist in distinct states of activation, and these determine their affinity for ligands and functionality (90). Neoplastic transformation induces changes in integrin expression as well as in their activation state. In breast and ovarian cancer, as well as in melanoma and glioma, tumour progression is associated with expression and activation of defined integrins (90; 91; 92; 93; 94; 95). Activated αvβ3 integrin strongly promotes breast cancer metastasis (91). Integrin activation results in intracellular mechanisms that involve distinct effectors. Membrane type-1 matrix metalloproteinase is involved in the maturation of the integrin pro-αv chain into the active form (96). Also growth factors like the hepatocyte growth factor/scatter factor change the affinity and avidity of αvβ3 integrin for matrix ligands (97). Our data indicate that, although integrin expression on cell membrane surface was not significantly changed by Met-F-AEA, cancer cell affinity for collagen IV and migration were strongly reduced, thus suggesting a change of the activation state of certain integrins. Since up-stream events in the signal pathways generated by integrin clustering are FAK and Src phosphorylation, we investigated the effects of Met-F-AEA on these two events. FAK is a non-receptor protein tyrosine kinase that localizes to focal adhesions and whose activation plays an important role in integrin-mediated cell adhesion and spreading. FAK function is dynamically regulated by dephosphorylation and phosphorylation cycles during cellular locomotion. It has been suggested that FAK plays an important role in tumour cell invasion and metastasis because its expression was found to be correlated by several investigators with a more invasive phenotype (98). Furthermore, it has been observed that tyrosine dephosphorylation of FAK is associated with down-regulation of its activity, and it is both necessary and sufficient for growth factor-induced morphological changes, detachment of cells from the extracellular matrix, and increased tumour cell motility, invasiveness and

metastatic capability. Because FAK can positively regulate the growth, migration and survival of cultured cells, it is likely that its overexpression contributes to the invasive/metastatic phenotype (98). Therefore, FAK has received much attention as a point of therapeutic intervention in cancers. In addition, adhesion stabilization of malignant cells in the microcirculation is necessary for successful metastasis formation. After integrin clustering, FAK is autophosphorylated and recruits kinases of the Src family to focal adhesions. Recruitment of Src to focal adhesions has been shown to be required for integrin-mediated cell motility (41). We described here for the first time the inhibition of phosphorylation by Met-F-AEA of FAK. Interestingly, it was previously shown that anandamide increases tyrosine protein phosphorylation of several proteins including FAK in normal neurons of the rat hippocampus, by inhibiting adenylyl cyclase and PKA (99). It has been also reported that cannabinoid CB1 receptors mediate tyrosine phosphorylation of focal adhesion kinase-related non-kinase (FRNK) (100). However, these results refer to the neuronal isoform of FAK, which has different characteristics compared to the isoform expressed in all other tissues. The additional exons that are characteristic of neuronal isoforms of FAK do not alter its targeting to focal adhesion but increase its autophosphorylation and alter its phosphorylation by Src-family kinases (101). On the other hand, in a neoplastic neuroblastoma cell line, cannabinoid receptor stimulation does not induce FAK phosphorylation (100). These only apparently conflicting results are likely explained by the differential regulation of different FAK isoforms in different cell types under different conditions. Indeed, it is possible that, as shown for several other targets of CB1 receptors (89), also FAK activity is regulated in opposing ways in normal versus neoplastic cells.

So, we hypothesized that Met-F-AEA, by inhibiting cell adhesion and motility, would retard breast cancer cell invasion and metastasis. Met-F-AEA decreased tumour cells adhesion to the substrate (collagen IV) and inhibited cell migration, while concomitantly down-regulating two of the molecular events that underlay these cellular actions: FAK tyrosine phosphorylation/activation and Src phosphorylation. All these effects (a) were attenuated by SR141716A, thus strongly suggesting the participation of CB1R in the antimetastatic action of Met-F-AEA in vitro; and (b) correlated with an inhibitory effect on breast cancer cell-derived metastasis in vivo, since Met-F-AEA also significantly reduced the formation of lung metastatic nodules, in mice, in a way antagonized by SR141716A. Clearly, the metastatic process is very complex, and several steps are required to complete the formation of new metastases. Therefore, although we have clearly demonstrated that Met-F-AEA inhibits cell migration and adhesion in vitro, it is not possible to exclude that other crucial molecular steps

are concomitantly inhibited by Met-F-AEA in vivo. Furthermore, we did not show any cause–effect relationship between Met-F-AEA-induced inhibition of adhesion and motility and inhibition of FAK tyrosine phosphorylation/activation and Src phosphorylation. Therefore, these two events, although both due to CB1R stimulation and normally related, may also be unrelated to each other in this study. Nevertheless, our data show that crucial events of the metastatic process are inhibited following CB1 receptor activation.

Although cholesterol depletion experiments indicated a possible role of detergent resistant microdomains (DRMs) in the trafficking and signalling of CB1R, the molecular mechanism of anandamide uptake, the relationship with CB1R and the cellular compartments involved in the signal transduction events deriving from their interaction are not yet defined and even less is known about the cellular mechanisms controlling CB1R intracellular trafficking and signalling.

Further, we have also investigated, in MDA-MB-231 cells, the intracellular localization of CB1R and its association with lipid rafts in order to elucidate how the presence of its ligand anandamide and the intracellular cholesterol levels affect CB1R trafficking. It has been previously shown that in HEK-293 cells, as well as in LLC-PK1 epithelial cells or SHSY-5Y neuroblastoma cells, CB1R is predominantly localized in endosomes at steady-state (102), as a result of a constitutive recycling between the plasma membrane and endosomes, mediated by the small GTPases Rab5 and Rab4 (103; 104). By confocal analysis of indirect immunofluorescence we found that CB1R is expressed on the plasma membrane of MDA-MB-231 cells. However, a substantial proportion of the receptor is present in lysosomes. Thus, differently from the previous reports (103; 104), at our knowledge we provide the first evidence for a lysosomal localization of this receptor in the cells.

Lysosomes are structures with different kind of critical functions. Among these, the main lysosomes functions could be summarized as follow: (a) the lysosomes can operate enzymatic digestion of endocytosed materials; (b) can mediate events in the programmed cell death called apoptosis; (c) can play an important role in receptor-mediated endocytosis and mediate events of receptor recycling and the shutting down of events of cell communication (105). This sequence of events involves a receptor binding to its ligand followed by their uptake into coated vesicles. Hence, our finding that CB1R shows a lysosomal localization could represent fundamental basis to further studies about receptor internalization, downregulation and eventual resensitization which constitute essential steps to analyze receptors function and signalling within the cells. For several receptors, the cellular responses to effectors have been shown to be regulated in part by the compartmentalization of the receptors and their effectors

within the cells (106), therefore elucidating the mechanism underlying the trafficking of CB1R is critical for understanding the physiological response to a variety of ligands.

Interestingly, we found a significant change in CB1R cell surface distribution after cholesterol depletion by MCD. Indeed, the clustered distribution of the receptor at the cell surface was lost and it assumed a more diffuse staining thus indicating that the plasma membrane clustering of CB1R was dependent on cholesterol-enriched microdomains. It remains to be established whether the CB1 cholesterol-dependent plasma membrane localization is of any functional significance in regulating CB1-mediated signalling.

It has been reported that cholesterol depletion by MCD can affect the transport of cholera toxin from endosomes to Golgi but not from Golgi to lysosomes (107). The reason that cholesterol can regulate some transport steps but not others is unknown, but presumably depends on cholesterol levels in the compartments/vesicles that mediate transport. In addition cholesterol depletion can alter the internalization pathways of different surface receptors (108).

In the case of CB1R, at least in our cell system, cholesterol depletion alters its plasma membrane localization but not the lysosomal one, thus we propose that the receptor might exhibit a different mode of interaction with the membrane lipid bilayer of the plasma membrane respect to that of lysosomal membranes. This hypothesis is supported by the finding that different kinetics and sorting of lipid analogs out of endosomes depend exclusively on their hydrophobic chains (109). Furthermore, while on one hand cholesterol depletion did not alter the receptor lysosomal localization in control conditions, on the other hand induced a remarkable redistribution of CB1R in the cytoplasm and an impairment of the receptor lysosomal localization after extracellular addition of the agonist anandamide. These results indicate that the intracellular pathway followed by CB1R to the lysosomes after binding of the agonist might depend on lipid rafts. In order to test the hypothesis that lipid rafts might be involved in CB1R trafficking in MDA-MB-231 cell line, we analyzed the raft-association of the CB1 receptor in these cells. We found that CB1R was associated with DRMs and that cholesterol depletion by MCD decreases its raft-association, which was also differently controlled by the presence of its agonist anandamide. We found that Met-F-AEA reduces significantly CB1R DRM-association both in the absence or presence of MCD.

In this contest, our observation that cholesterol depletion, before anandamide incubation displaces CB1R from rafts and causes an intracellular redistribution, is particularly intriguing because recent evidences suggest that the cellular uptake of anandamide could be mediated by lipid rafts (36) and that MCD treated CB1R-expressing C6 cells are protected from AEA-

induced apoptosis (72; 110). Complementarily with these data, we show for the first time that CB1R is associated with DRMs and suggest that they might represent a cellular device for its intracellular trafficking as well as favorable platform to regulate CB1R signalling.

Finally, we studied in detail the molecular mechanism underlying anandamide antitumour effect and the role of lipid rafts in the cellular uptake of AEA.

While it is clear that AEA uptake has the features of a facilitated transport, and there may be indeed a site through which it diffuses (111), the molecular identity of an AEA membrane transporter still remain elusive (112; 113; 114). At any rate, the data demonstrate that lipid rafts integrity modulates endocannabinoid transport across cell membranes. Recent evidence further supports the hypothesis that AEA internalization might occur by a caveolae-related endocytic process. Bari et al showed that depletion of cholesterol, and thus detergent-resistant membrane domains/ lipid rafts, by methyl-β-cyclodextrin reduced AEA internalization by rat C6 glioma cells by ~50%. (110). In contrast to the above researchers, Sandberg and Fowler found that cholesterol depletion has no effect on AEA uptake in P19 cells (115). This could be due to the fact that detergent-resistant membrane domain mediated endocytosis, like many other factors, probably has a different role in the cellular accumulation of AEA depending on the cell type.

Thus, we examined the role that lipid rafts might play in the cellular uptake of AEA and the effect of perturbation of lipid rafts/caveolae on the antitumour effect of AEA.

MCD has also been described to have a cell growth inhibition effect in macrophage cells (116). Further, it has been shown that depletion of plasma membrane cholesterol inhibits cell migration (69; 70). Interestingly, we found that, also in MDA-MB-231 cells, MCD alone exhibits a small antiproliferative effect and an antimigratory effect comparable with that exhibited by Met-F-AEA; cholesterol depletion by MCD before Met-F-AEA incubation, causes a partial reversion of the antiproliferative and antimigration effect of Met-F-AEA, indicating that the cell growth arrest and the inhibition of migration induced by the CB1 ligand need integrity of lipid rafts to occur. In support of our data, there is a large body of evidence that G protein coupled receptor functions depend on the lipid raft integrity (58; 83; 84).

The present findings provide evidence for the role of CB1 receptor and its endogenous ligand anandamide in some events of metastatic processes. Our data also show the intracellular localization of CB1 receptor and its association with lipid rafts, and that the molecular mechanism at the basis of anandamide function might need lipid raft/caveolae integrity to occur.

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