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“Targeting the endocannabinoid system in colorectal cancer”

Giuseppina Gangemi

Università degli Studi di Napoli Federico II
Dipartimento di Medicina Molecolare e Biotecnologie Mediche

Administrative Location

Dipartimento di Biologia e Patologia Cellulare e Molecolare “L. Califano”
Università degli Studi di Napoli Federico II

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Supporting Institutions
Dipartimento di Biologia e Patologia Cellulare e Molecolare “L. Califano”, Università degli Studi di Napoli “Federico II”, Naples, Italy
Istituto di Endocrinologia ed Oncologia Sperimentale “G. Salvatore”, CNR, Naples, Italy
Istituto Superiore di Oncologia, Italy

Italian Faculty

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“Targeting the endocannabinoid system in colorectal cancer”

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LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

I. **Gangemi G**, Gazzerro P, Fiore D, Proto MC, Butini S, Gemma S, Casagni A, Laezza C, Vitale M, Ligresti A, Di Marzo V, Zisterer DM, Nathwani S, Williams DC, Campiani G, Bifulco M. PBOX-15 induces apoptosis and improves the efficacy of oxaliplatin in human colorectal cancer cell lines. *Submitted EJP-37691*. (Main body of the dissertation).

II. **Gangemi G** *et al*. Targeting the isoprenoid pathway in colorectal cancer: a novel role for N6-isopentenyladenosine. Manuscript in preparation.

III. Malfitano AM, Ciaglia E, **Gangemi G**, Gazzerro P, Laezza C, Bifulco M. Update on the endocannabinoid system as an anticancer target. Expert Opin Ther Targets 2011;15(3):297-308.

IV. Gazzerro P, Proto MC, **Gangemi G**, Malfitano AM, Ciaglia E, Pisanti S, Santoro A, Laezza C, Bifulco M. Pharmacological actions of statins: a critical appraisal in the management of cancer. Pharmacol Rev 2012;64(1):102-46.

ABBREVIATIONS

2-AG	2-arachidonoyl glycerol
5-FU	5-fluorouracil
AC	adenylate cyclase
AEA	anandamide
AKT	v-akt murine thymoma viral oncogene
AMT	anandamide membrane transporter
ATM	ataxia-telangiectasia mutated
ATR	ataxia-telangiectasia and Rad3-related
BBB	blood–brain barrier
CB1	cannabinoid receptor 1
CBD	cannabidiol
Cdc25C	cell division cycle 25C
Cdk2	cyclin-dependent kinase 2
Chk1	checkpoint kinase 1
CNR1	cannabinoid receptor 1
COX-2	cyclooxygenase-2
CRC	colorectal cancer
ECM	extracellular matrix
ECS	endocannabinoid system
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
FAAH	fatty acid amide hydrolase

FAK	focal adhesion kinase
GPCR	Gi/o protein-coupled receptor
HMG-CoA	3-hydroxy-3-methyl coenzyme A
IgSF CAMs	immunoglobulin superfamily
JNK	c-Jun N-terminal kinase
MAGL	monoacylglyceride lipase
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
mTORC1	mammalian target of rapamycin complex 1
NArPE	N-acyl-PE
NAT	N-acyltransferase
NGF	nerve growth factor
NO	nitric oxide
OEA	N-oleoylethanolamine
OXA	oxaliplatin
PARP	poly (ADP-ribose) polymerase
PE	phosphatidylethanolamine
PEA	N-palmitoylethanolamine
PLC	phospholipase C
Pol III	polymerase III
PPARγ	peroxisome proliferation activated receptor
RXRα	retinoic acid receptor alpha
SAC	spindle assembly checkpoint

SEA	N- stearoylethanolamine
THC	tetrahydrocannabinol
TIMP	tissue inhibitor of metalloproteinase
Trk	tropomyosin-receptor-kinase
TRPV1	transient receptor potential vanilloid type 1
VEGFR	vascular endothelial growth factor receptor

ABSTRACT

The endogenous cannabinoid system, comprising the cannabinoid receptors, their endogenous ligands (e.g. anandamide) and the enzymes regulating endocannabinoid biosynthesis and degradation, is an almost ubiquitous signaling system involved in the control of several physio-pathological conditions. Modulation of endocannabinoid tone has proven to hold several therapeutic promise in the treatment of a wide range of pathological processes. In this framework, given the ubiquity of the endocannabinoids and their receptors and their regulating action on proteins involved in cell fate control, there has been increasing evidence for a role of the endocannabinoid system also into neoplastic transformation and an interest to exploit it for a potential therapeutic application. However, despite several reports on endocannabinoids' properties, little is known concerning the endogenous function of the endocannabinoid system and in particular of CB1 signaling in the regulation of tumor growth. A deregulation of the endocannabinoid system occurs in colorectal cancer (CRC). Loss of cannabinoid receptor 1 expression has been associated with colon tumor progression through a mechanism of epigenetic silencing, suggesting a role for CB1 as a tumor suppressor. A stable analogue of the endocannabinoid anandamide was utilized as a critical tool to characterize the basal functions of endocannabinoid system and CB1 signaling in CRC. Anandamide emerged as suppressor of colon tumor growth, since up regulation of CB1 receptor expression based on transcriptional regulation acting on its gene promoter affected CRC proliferation. However, a rapid anandamide metabolism has been identified in CRC, thus limiting this protective mechanism. As a consequence, an indirect targeting of anandamide degradative enzyme fatty acid amide hydrolase to maintain a local endocannabinoid tone has been proved. FAAH inhibition has been demonstrated to affect CRC proliferation through cell cycle machinery deregulation, DNA damage signaling pathway activation and late programmed cell death induction. Oxaliplatin in combination with 5-Fluorouracil and leucovorin (FOLFOX) has been approved for metastatic CRC therapy. Despite clinical success, patients who initially respond to chemotherapeutics may subsequently become refractory, directing the attention towards alternative strategies, including the use of combined therapies. A combinatorial approach has proven effective since FAAH inhibition sensitizes CRC cells to targeted therapies, thus representing a valid strategy to overcome drug resistance and side effects.

1. BACKGROUND

1. 1 The endocannabinoid system (ECS)

1.1.1 Endocannabinoid receptors and signal transduction

The endogenous cannabinoid system is an almost ubiquitous signaling system involved in the control of several physio-pathological conditions. It comprises the cannabinoid CB1 and CB2 receptors, their endogenous ligands (endocannabinoids) –e.g. N-arachidonoylethanolamine (anandamide) and 2-arachidonoyl glycerol (2-AG) - and the enzymes regulating endocannabinoid biosynthesis and degradation (Di Marzo et al. 2004).
To date, two subtypes of receptors for endocannabinoids have been cloned: cannabinoid receptor 1 (CB1), originally named 'central' receptor (Matsuda et al. 1990) and cannabinoid receptor 2 (CB2), also known as 'peripheral' receptor (Munro et al. 1993). CB1 and CB2 receptors share only 44% sequence homology and 35% to 82% within the transmembrane domains, which are thought to contain the binding sites for cannabinoids (Mackie 2006). Both CB1 and CB2 genes encode a seven-transmembrane-domain protein belonging to the Gi/o protein-coupled receptor (GPCR) family (Munro et al. 1993).
Several non-CB1/CB2 pharmacological effects have been also reported (Brown 2007). They are mediated by transient receptor potential vanilloid type 1 ion channel (TRPV1), activated by various lipids including anandamide (Zygmunt et al. 1999) and, more recently discovered, by the orphan receptors GPR55 (Ryberg et al. 2007), thought to be a novel "type-3 (CB3)" cannabinoid receptor (Moriconi et al. 2010), playing a physiological role in lipid or vascular biology (Baker et al. 2006), and GPR119 for oleoylethanolamide, originally identified in genome-sequencing efforts and expressed predominantly in the pancreas and gastrointestinal tract (Fredriksson et al. 2003; Balenga et al. 2011).
CB1 receptors were found to efficiently couple and activate both G_i and G_o, whereas CB2 only G_o, thus showing an agonist-selective G protein signaling (Glass and Northup 1999). The signal transduction pathway downstream cannabinoid receptors includes inhibition of adenylate cyclase (Howlett et al. 1986), stimulation of mitogen-activated protein kinase (MAPK) (Bouaboula et al. 1995) and phosphatidylinositol-3-kinase pathway (Gomez Del Pulgar et al. 2002) and, for CB1, direct modulation of cell membrane ion channels, activating inward-rectifying K^+ channels (Kir) and inhibiting N-, P/Q- and L-type voltage-gated Ca^{2+} channels, mostly by direct $G\beta\gamma$ interaction (Howlett et al. 2002; Mackie and Hille 1992) (Figure 1). The generation of nitric oxide (NO) upon CB1 stimulation has also been documented in various cell types (Jones et al. 2008; Prevot et al. 1998) while in others, CB1 reduces NO levels (Cabral et al. 2001; Hillard et al. 1999).

Additionally, the activation of GPR55, has been linked to intracellular Ca^{2+} increase (Lauckner et al. 2008); activation of the small GTPase proteins RhoA, Rac and Cdc42 (Henstridge et al. 2009), and ERK phosphorylation (Oka et al. 2009). Moreover, by triggering PPARs, ECS exert a variety of long-term effects via genomic ad nongenomic mechanisms, which are opposite to those evoked by activation of "classical" surface cannabinoid receptors (Pistis and Melis 2010).

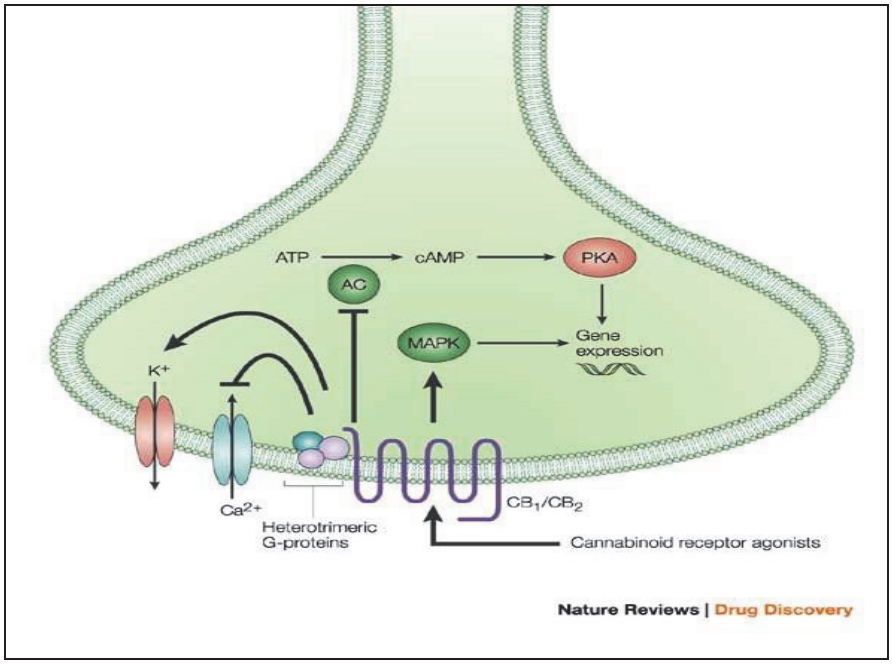


Figure 1. The major signal transduction pathways downstream cannabinoid receptors (Di Marzo et al. 2004).

The central and most of the peripheral effects of cannabinoids rely on CB1 activation (Malfitano et al. 2011). Since it was originally considered to be mainly a CNS receptor, CB1 was named 'central' receptor (Pacher et al. 2006), preferentially expressed in the central nervous system, in several brain areas where it mediates cannabinoid psychoactive effects, in the globus pallidus, in the substantia nigra, in the cerebellum, hippocampus, caudate nucleus, putamen, hypothalamus and amygdala with presynaptic functions (Pertwee 2005). However, CB1 receptors are also present in peripheral nerve terminals, as well as in extra-neural tissues such as testis, uterus, vascular endothelium, eye, spleen, ileum, adipocyte and in several tumors (Matsuda et al. 1990; Munro et al. 1993; Bifulco et al. 2006). The CB2 receptor is known as 'peripheral' receptor and it is mainly

expressed in immune cells, unrelated to cannabinoid psychoactive effects (Basu and Dittel 2011; Bouaboula et al. 1993; Castaneda et al. 2013). CB2 is normally expressed in areas enriched of B lymphocytes, that, when activated, can affect the release of chemical messengers and, as a consequence, modulate immune cell trafficking (Zhang et al. 2005; Castaneda et al. 2013) and in natural killer (NK) cells, macrophages, neutrophils, $CD8^+$ and $CD4^+$ (Mackie 2006).
However, CB2 expression was also found in the brain at the cerebellum, cerebral cortex and brainstem response (Mackie 2006), in non-parenchymal cells of the cirrhotic liver (Julien et al. 2005), in the endocrine pancreas (Juan-Pico et al. 2005) and in the bone (Ofek et al. 2006).

1.1.2 Cannabinoid Receptor 1 Gene Structure

In human, mouse and rat, the CB1 coding region is contained within one exon and shows significant homology across species, variating only for the 5'untranslated region (UTR) length (Miller et al. 2011). Three additional upstream exons in human CB1 have been identified in hippocampus, giving a large 5'UTR, characteristic of neuronally expressed genes (Zhang et al. 2004). This 5'UTR can be alternatively spliced (CB1 A–D) or transcribed at different sites (CB1A–D *versus* CB1E) to yield five possible transcripts with region-specific expression in brain (Figure 2). The promoter activity is identified in 3-kb sequence upstream from the exon 1 transcription start site in various CB1-expressing neuroblastoma cell lines. Consistent with the lower expression of transcript CB1E, 5'flanking sequences of exon 3 yielded much lower promoter activity. Mouse CB1R gene structure cloned from striatum contains a shorter 5'UTR, with only one additional exon located upstream of the coding region, containing multiple transcription start sites (McCaw et al. 2004).

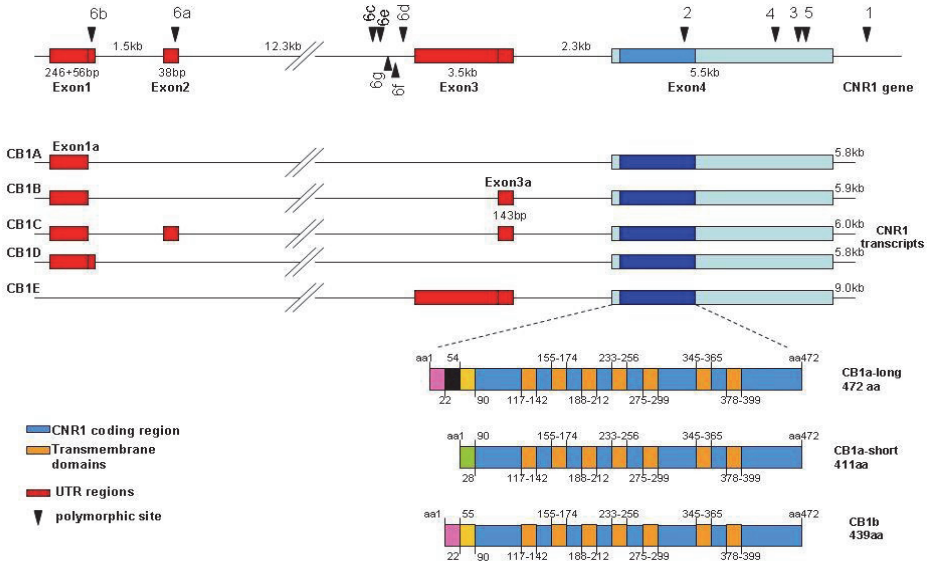


Figure 2. Structure of *CNR1* gene. The emerging putative genetic structure of *CNR1* gene (Zhang et al. 2004).

1.1.3 The discovery of endogenous ligands

The discovery of cannabinoid receptors suggested the existence of endogenous ligands capable of activating them, the so-called 'endocannabinoids'. Endocannabinoids are lipid molecules containing long-chain polyunsaturated fatty acids, amides, ethers and esters, with different selectivity for the two receptor subtypes (Mcallister and Glass 2002). The best studied are anandamide (AEA), isolated from porcine brain (Devane et al. 1992) and 2-arachidonoylglycerol (2-AG) (Sugiura et al. 1995). During the last few years, several other bioactive lipid mediators from the non-oxidative metabolism of arachidonic acid have been described: 2-arachidonoyl-glyceryl-ether (noladin), o-arachidonoyl-ethanolamine (virodhamine), N- arachidonoyl-dopamine and oleamide (Hanus et al. 2001). Moreover, several compounds called 'endocannabinoid-like', N-palmitoylethanolamine (PEA), N-oleoylethanolamine (OEA) and N-stearoylethanolamine (SEA) are present in human, rat and mouse brain (Di Marzo 1998) (Figure 3).
Anandamide is a partial or full agonist of CB1 receptors, depending on the tissue and biological response measured. Since its structure is very flexible, it can assume U-shape or J-or extended conformations based on the surrounding

environment. In the phospholipid bilayer, AEA assumes the extended form, with the polar group at the level of the phospholipidic polar heads and, by which, through lateral diffusion, it quickly reaches the active site of the CB1 receptors (Tian et al. 2005). Although it also binds CB2 receptors, it has very low efficacy as CB2 agonist (Devane et al. 1992), instead, 2-AG activates both CB1 and CB2 receptors (Mechoulam et al. 2001). Unlike other neurotransmitters, endocannabinoids are very lipophilic and thus they cannot be stored and released in vesicles, but they are biosynthesized and released by the cells 'on demand' in response to certain physiological and pathological stimuli (Leung et al. 2006; Liu et al. 2006; Pertwee 2005; Di Marzo et al. 2004). Stimuli include membrane depolarization and increased intracellular Ca^{2+} and/or metabotropic receptor stimulation, activating complex enzymatic machineries, which lead to the cleavage of membrane phospholipids and eventually to the biosynthesis of endocannabinoids (Bisogno et al. 2005).

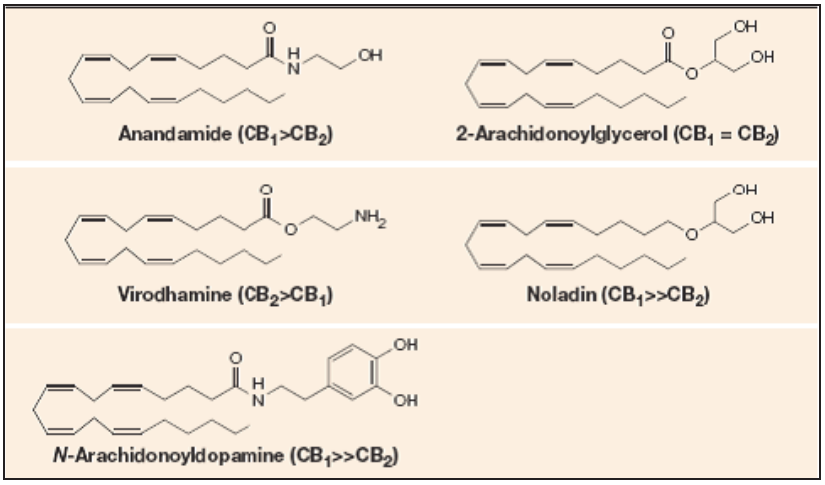


Figure 3. Main 'endocannabinoids' and their specificity (Di Marzo et al. 2004).

1.1.4 Endocannabinoid biosynthesis

Endocannabinoid metabolism includes altogether biosynthetic, hydrolytic and oxidative enzymes that exert a metabolic control of the endogenous tone, and hence the biological activity of AEA, 2-AG and their congeners, together with their target receptors and metabolic enzymes, purported EMT and intracellular transporters, form the ECS (Figure 4).

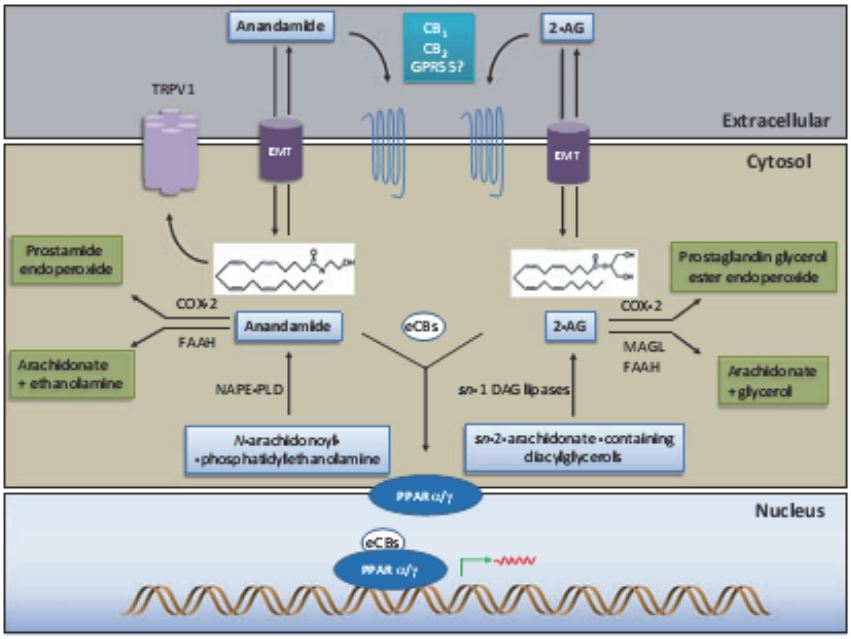


Figure 4. Endocannabinoid metabolism: Biosynthesis, degradation and target receptors of anandamide (AEA) and 2-arachidonoylglycerol (2-AG). CB1/2, type 1/2 cannabinoid receptors; COX-2, cyclooxygenase-2; DAG, diacylglycerol; eCBs, endocannabinoids; EMT, endocannabinoid membrane transporter; FAAH, fatty acid amide hydrolase; GPR55, G-protein-coupled receptor 55; MAGL, monoacylglycerol lipase; NAPE-PLD,N-acylphosphatidylethanolamine-selective phospholipase D; PPAR, peroxisome-proliferator-activated receptor; TRPV1, transient receptor potential vanilloid 1 channel (D'Addario et al. 2013).

The biosynthesis of NAE occurs in a"two stages" enzymatic process: as a first step, a N-acyltransferase (NAT), Ca^{2+}-dependent and enhanced by the presence of cAMP, transfers an acyl group from position sn-1 of a phospholipid to the amino group of a phosphatidylethanolamine (PE) generating the N-acyl-PE (NArPE). The NArPE intermediate is converted by phospholipase D (NAPE-PLD), belonging to the zinc-metallo-hydrolase family of the β-lactamase fold (Ueda et al. 1993) in NAE and phosphatidic acid (Leung et al. 2006). As a result, AEA *in vivo* biosynthesis is believed to occur through the enzymatic hydrolysis catalysed by NAPE-PLD of its membrane lipid precursor N-arachidonyl phosphatidylethanolamine (NArPE). In turn, N-arachidonylphosphatidylethanolamine is derived from the transfer of arachidonic acid from position sn-1 of phosphatidylcholine to the group head of phosphatidylethanolamine through NAT (Liu et al. 2006; Di Marzo et al. 2004). Pharmacological and electrophysiological reports have demonstrated that AEA

biosynthesis can be induced both through the entry of calcium ions into the cell, following its depolarization and the activation of metabotropic receptors, such as group I glutamate, muscarinic acetylcholine and D2 dopamine receptors, independently or cooperatively (Piomelli 2003). Alternative biosynthetic pathways have been proposed, involving NAPE hydrolysis into phosphoranandamide catalyzed by a phospholipase C (PLC), followed by dephosphorylation through a phosphatase (Liu et al. 2006) or NAPE double-deacylation and subsequent hydrolysis of the resulting glycerophosphate-NAE into glycerol-3-phosphate and anandamide (Simon et al. 2006). 2-AG is generated from diacylglycerol (DAG) by a DAG lipase selective for the sn-1 position (Bisogno et al. 2005) (Figure 5).

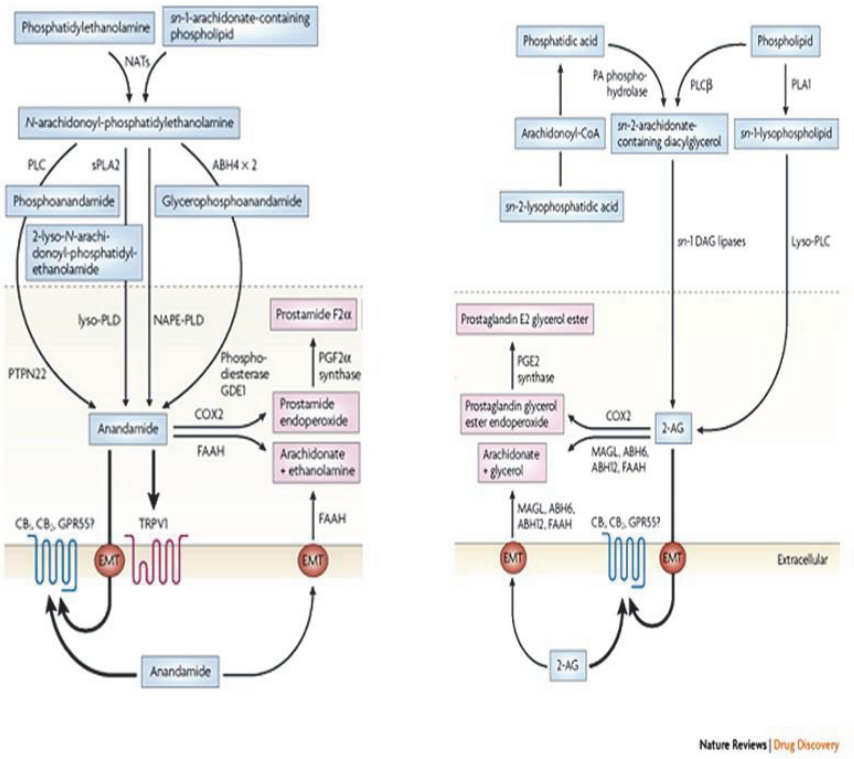


Figure 5. Proteins and pathways for the biosynthesis of anandamide and 2-AG. Abh4, alpha/beta-hydrolase 4; lyso-PLD, lysophospholipase D; PTPN22, protein tyrosine phosphatase N22 (Di Marzo 2008).

After biosynthesis, endocannabinoids are released into the extracellular space or directly move within the cell membrane (Fowler 2013). There are at least three

models proposed for anandamide uptake by cells: i. a facilitated diffusion process across the plasma membrane (Hillard et al. 1999), driven by transmembrane concentration gradient, saturable (Sandberg and Fowler 2005) and temperature-dependent (Thors and Fowler 2006), followed by intracellular carrier-mediated transport to effector molecules; ii. activation of carrier protein(s) known as "anandamide membrane transporter, AMT" or more generally "endocannabinoid membrane transporter, EMT", with a plasma membrane localization, involved in endocannabinoid uptake (Rang et al. 2012) and translocation from one side of the membrane to the other (Ligresti et al. 2004); iii. endocytosis mechanism through caveolae/lipid rafts (Navarrete and Araque 2008) (Figure 6).

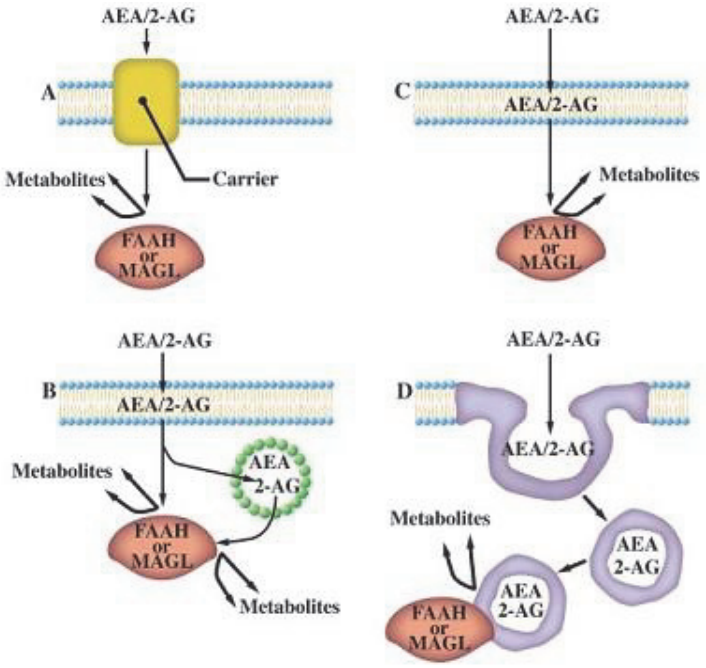


Figure 6. Various proposed models for endocannabinoid transport. (A) a transmembrane carrier protein assists in the translocation of endocannabinoids across the plasma membrane; (B) endocannabinoids passively diffuse across the plasma membrane along a catabolism-driven concentration gradient but are sequestered in an intracellular compartment or by binding to an intracellular binding protein before metabolism; (C) endocannabinoids passively diffuse across the plasma membrane along a concentration gradient that is driven by their rapid metabolism; (D) endocannabinoids are transported into cells via a protein carrier-mediated caveolae-related endocytic event (Yates and Barker 2009).

The compartmentalization of endocannabinoids into lipid rafts, specialized membrane domains enriched in cholesterol and sphingolipids, has been described (McFarland et al. 2004). Various physiological roles have been attributed to lipid

rafts, including endocytosis and trafficking of signaling molecules (Allen et al. 2007). One subset of organized raft domains is represented by caveolae, flask-shaped invaginations of the membrane formed through polymerization of caveolins, a family of integral membrane proteins tightly binding cholesterol (Allen et al. 2007). Interestingly, it has been reported a caveolae/lipid raft-mediated uptake and recycling of anandamide (McFarland et al. 2004) and intracellular trafficking and regulation of CB1-mediated signal transduction (Bari et al. 2005) (Figure 7). Moreover, DGLα, 2-AG, and its precursor arachidonoyl-containing diacylglycerol were found to be localized to lipid rafts in a dorsal root ganglion cell line (Rimmerman et al. 2008).

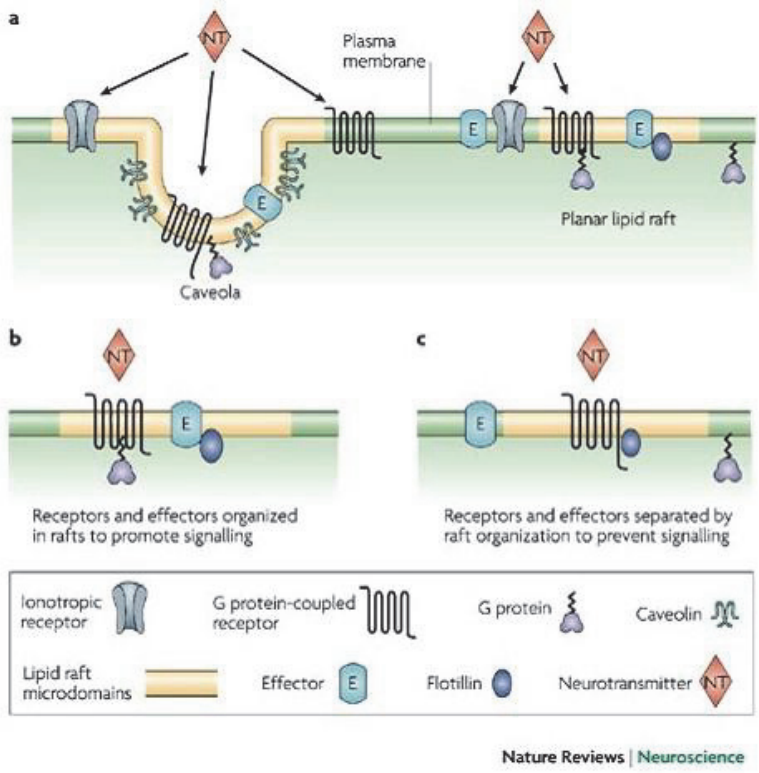


Figure 7. Lipid rafts in the organization of the endocannabinoid system. a. There are two common raft domains in mammalian cells: planar lipid rafts and caveolae. **b.** The lipid raft signaling hypothesis proposes that these microdomains spatially organize signaling molecules at the membrane, perhaps in complexes, to promote kinetically favorable interactions necessary for signal transduction. **c.** Alternatively, lipid raft microdomains might inhibit interactions by separating signaling molecules and thereby dampening signaling responses (Allen et al. 2007).

1.1.5 Endocannabinoid degradation

Adiposomes have been proposed as a potential platform for the metabolic control of AEA (Maccarrone et al. 2010). Once internalized in the cell, AEA can be trafficked to adiposomes *via* HSP70, which might dynamically shuttle it from the inner leaflet of the plasma membrane to the phospholipid monolayer of the adiposomes (Oddi et al. 2009) where it can be degraded. The enzymes able to degrade endocannabinoids are quite well characterized. They are fatty acid amide hydrolase (FAAH) for anandamide and related compounds (Giang and Cravatt 1997) and monoacylglyceride lipase (MAGL) for 2-AG (Dinh et al. 2002). Recently, another membrane-associated FAAH was identified and named FAAH-2, present in several species including human and primates, but absent in murids (Wei et al. 2006). The role of FAAH in disrupting anandamide signaling was indicated by the phenotype of FAAH knockout mice ($FAAH^{-/-}$), which displayed super sensitivity to exogenous anandamide and elevated levels of anandamide across the brain resulting in CB1 receptor-mediated hypoalgesia (Cravatt et al. 1999). Transiently-associated (FAAH-1) or permanently-associated (FAAH-2) fatty acid amide hydrolases are distributed, to different extents, between lipid bodies and ER membranes (Masanobu et al. 2009). Both FAAHs can degrade AEA into arachidonic acid and ethanolamine on the adiposome surface or at the ER, instead FAAH-2 could oriented, as emerged from *in silico* studies, the active site towards the luminal compartment of the cell (Wei et al. 2006). In this way, adiposomes could represent specialized sites for AEA storage and accumulation, thus forming a dynamic reservoir for long-lasting activities, and for effects that require lipid high concentrations (Maccarrone et al. 2010) (Figure 8).
Furthermore, when MAGL or FAAH activity is suppressed, both AEA and 2-AG might become substrates for cyclooxygenase-2 and give rise to the corresponding hydroperoxy derivatives (Rouzer and Marnett 2011). These metabolites show different activity at CB1/2 receptors (van der Stelt et al. 2002) or appear to act at novel binding sites. Overall, the physiological relevance of the oxidative pathways of ECS still needs to be clarified (Rouzer and Marnett 2011).

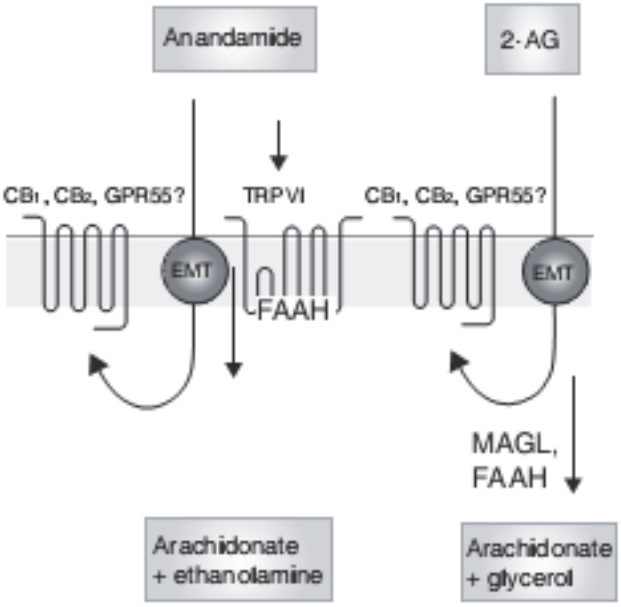


Figure 8. Proteins and pathways for the degradation of endocannabinoids (Malfitano et al. 2011).

1.2 The endocannabinoid system in health and disease

The quantification of endocannabinoids and cannabinoid receptor levels in tissues and biological fluids lead to understand the biological role of the endocannabinoid system and its regulatory functions in health and disease (Di Marzo et al. 2004). The discovery of ECS as implicated in a large number of physiological functions of the nervous system and peripheral organs lead to hold several therapeutic promise in the treatment of a wide range of diseases and pathological conditions (Pacher et al. 2006).

In particular, the endocannabinoid system is implicated in mood and anxiety disorders (Hill and Gorzalka 2005; Garcia-Gutierrez and Manzanares 2010); in psychosis diseases (Leweke et al. 2012); in neuroprotection both in acute neuronal injury such as traumatic brain injury, epilepsy and stroke (Sinor et al. 2000), in chronic neurodegenerative disorders, such as multiple sclerosis (Baker et al. 2001), Parkinson's (Di Marzo et al. 2000), Alzheimer's (Pazos et al. 2004; Karl et al. 2012) and Huntington's (Van et al. 2010) and in cardiovascular diseases (Montecucco and Di Marzo 2012) including atherosclerosis (Mach et al. 2008) and myocardial heart attack (Wagner et al. 2003). In these disorders, upregulation of the endocannabinoid system may cause a reduction in the severity of symptoms or

a slowing of disease progression. The endocannabinoid system is also known to play a crucial role in energy balance and substrate metabolism, regulating food intake and metabolic factors (Christopoulou and Kiortsis 2011), through peripheral CB1 receptors located at multiple sites throughout the body (Engeli 2008). The activation of the endocannabinoid system promotes excessive food intake and fat accumulation with obesity-related diseases, such as type 2 diabetes, hypertension and dyslipidemia (Okamoto et al. 2006), suggesting that this system itself sometimes may mediate pathological event development. A pivotal role has also been suggested in male and female reproduction, concerning fertilization, oviductal transport, implantation, embryo development and maintenance of early pregnancy (Taylor et al. 2007) and in bone metabolism by regulating bone mass, bone loss and bone cell function (Idris et al. 2012).

1.3 The endocannabinoid system in cancer

1.3.1 Endocannabinoid levels in cancer

The endocannabinoid system is highly conserved among species and shows regulating activity on proteins and nuclear factors involved in cell differentiation, proliferation and survival. Endocannabinoid levels are finely modulated under physiological and pathological conditions. A transient physiological increment appears to be an adaptive reaction to restore cell homeostasis after perturbation. As an example, high AEA levels were found in placenta, umbilical vein and plasma from maternal circulation as a consequence of pregnancy maintenance and parturition (Marczylo et al. 2010). However, altered endocannabinoid levels have been reported in several tumors compared to the respective healthy tissue (Schmid et al. 2002), in pituitary adenoma (Pagotto et al. 2001), glioblastoma and meningioma (Petersen et al. 2005), prostate carcinoma (Nithipatikom et al. 2004) and endometrial sarcoma (Schmid et al. 2002).

1.3.2 Cannabinoid receptors and cancer

Moreover, several evidence has suggested that cannabinoid receptor expression is altered during carcinogenesis (Malfitano et al. 2011). Levels of cannabinoid receptors are differently regulated in normal *versus* malignant cells, as a consequence of a protection mechanism of normal cells from pro-apoptotic and anti-proliferative effects of cannabinoid agonists (Bifulco et al. 2006) and have a prognostic value alone or in association with other recognized prognostic

markers (Caffarel et al. 2006) since they correlate with the degree of tumor malignancy (Marincsak et al. 2009). The association of CB receptor expression with tumor malignancy and disease outcome in cancer has been studied in several settings and is dependent on the specific cancer type. Altered levels of endocannabinoids and CB receptors in malignant *vs* normal cells are summarized in Table 1.

Table 1. Levels of endocannabinoids and CB receptors in malignant *vs* normal cells (Malfitano et al. 2011).

Levels of CB receptors	Tumor type	Levels of endocannabinoids
↑ **CB1 and CB2 receptors**	Mantle cell lymphoma	↑ AEA
	Acute myeloid leukemia	↑ AEA
	Well-differentiated hepatocellular carcinoma	↑ AEA
	Prostate carcinoma	↑ 2-AG; ↓ AEA
	Malignant astrocytoma	↑ AEA
	Pancreatic carcinoma	= AEA and 2-AG
↓ **CB1 and CB2 receptors**	Poorly differentiated hepatocellular carcinoma	↑ AEA
CB1 and CB2 receptors similar to control levels	Kaposi's sarcoma	
	Non-melanoma skin cancer	
	Astroglial carcinoma	↑ AEA
	Pituitary adenoma	↑ AEA and 2-AG
↑ **CB2 receptors**	Glioblastoma	↑ AEA
	Meningioma	↑ 2-AG
	Estrogen receptor-negative primary breast carcinoma	↑ AEA
	Endometrial carcinoma	↑ 2-AG
	Colon carcinoma	↑ AEA and 2-AG
↑ **CB1 receptors**	Rhabdomyosarcoma	
	Gastrocarcinoma	↓ AEA
↓ **CB1 receptors**	Primary breast carcinoma	↑ AEA
↑ **TRPV1 receptors**	Prostate carcinoma	↑ 2-AG; ↓ AEA
	Squamous cell carcinoma of the human tongue	

2-AG: 2-Arachidonoylglycerol; AEA: Anandamide; CB: Cannabinoid.

1.3.3 Endocannabinoid degrading system and cancer

A local endocannabinoid tone is an important factor to control the malignancy of different cancer cells. Thus, modulation of endocannabinoid levels, by use of inhibitors of endocannabinoid synthesis or metabolism, results in a change of the invasive properties of cancer cells in a manner consistent with a protective effect of endocannabinoids. MAGL has been recently found highly expressed in aggressive human cancer cells and primary tumors, including melanoma, ovarian and breast cancer, where it regulates a lipid network enriched in protumorigenic signaling molecules (Nomura et al. 2010). A correlation

between FAAH and cancer has been primarily investigated in prostate adenocarcinomas, where tumor epithelial FAAH immunoreactivity has been recently associated with prostate cancer severity and outcome (Thors et al. 2010; Endsley et al. 2008) and in pancreatic ductal adenocarcinomas where a correlation of high FAAH/MAGL levels and survival has been observed (Michalski et al. 2008).
A controversial question is opened (Velasco et al. 2012). On one hand, the endocannabinoid system may be over activated in cancer and it may be considered pro-tumorigenic. In support of this hypothesis, in murine models of cancer, both CB1 and CB2 knockdown resulted in reduced ultraviolet light-induced skin carcinogenesis (Zheng et al. 2008) and CB2 receptor overexpression enhances leukaemia predisposition after virus infection (Joosten et al. 2002). Conversely, and in line with evidence that the pharmacological activation of cannabinoid receptors reduces tumour growth (Sarfaraz et al. 2008), the up regulation of endocannabinoid-degrading enzymes has been observed in aggressive human tumours and cancer cell lines (Thors et al. 2010), indicating that endocannabinoid signaling can also have a tumour-suppressive role. In support of this, CB1 deletion accelerates intestinal tumour growth in a genetic murine model of colon cancer (Wang et al. 2008); increased endocannabinoid levels diminish azoxymethane-induced precancerous lesions in the mouse colon (Izzo et al. 2008); and a reduced MAGL expression reduces tumour growth in xenograft mice (Nomura et al. 2010).

1.3.4 Antitumor mechanisms of endocannabinoids and their derivates

Since the late 1990s, a large body of evidence has accumulated, demonstrating that various cannabinoids exert antitumor actions in a wide variety of experimental models of cancer, ranging from cancer cell lines in culture to genetically engineered mice. Various cannabinoids, including Δ9-THC and cannabidiol, and several endocannabinoid mediators have been shown to inhibit tumor growth and progression of several types of cancers (Malfitano et al. 2011). The proposed mechanisms are complex and may involve anti-proliferative and pro-apoptotic action, anti-metastatic effect through inhibition of neo-angiogenesis and tumor cell migration (Bifulco et al. 2006) and could be ascribed to a CB1, CB2 or TRPV1 receptor-dependent or independent mechanism (Alpini and DeMorrow 2009).
Endocannabinoids are able to inhibit the proliferation of various tumor cells, including cholangiocarcinoma (DeMorrow et al. 2008), thyroid (Cozzolino et al. 2010) and hepatocellular carcinoma (Giuliano et al. 2009), COX-2 overexpressing non-melanoma skin cancer (Rukiyah et al. 2009) and breast cancer (Caffarel et al. 2010). The anti-proliferative effects occur through different pathways including

activation of cAMP/PKA/MAPK signaling (Portella et al. 2003), decreased expression of prolactin receptor (De Petrocellis et al. 1998) and both trk and NGF receptor (Melck et al. 2000), blockage of cell cycle progression by activation of checkpoint kinase Chk1 or cyclin-dependent kinase inhibitor p27/kip1 (Portella et al. 2003) and suppression of Cdk2 activity (Laezza et al. 2006), reduction of EGFR expression (Mimeault et al. 2003). Recently, HMG-CoA reductase has been suggested as a new target of antitumor effect of AEA. In particular, the decreased activity of this enzyme is responsible for the inhibition of Ras oncogenic protein farnesylation involved in human breast cell proliferation (Laezza et al. 2010).
Cannabinoid-induced apoptosis can involve caspase-dependent and -independent pathways (Gallotta et al. 2010), as well as CB1- and CB2-dependent stimulation of *de novo* synthesis of the pro-apoptotic sphingolipid ceramide (Gomez del Pulgar et al. 2002; Galve-Roperh et al. 2000). For instance, AEA can induce growth suppressive/pro-apoptotic effects in cholangiocarcinoma cells via stabilization of the lipid raft structures within the plasma membrane, increased production of ceramide and subsequent recruitment of death receptor complex components into the lipid raft structures through activation of the non-canonical Wnt signaling pathway (DeMorrow et al. 2008). After ceramide production, the cannabinoid THC upregulates in glioma cells the expression of the stress-regulated protein p8, which is a transcriptional regulator controlling tumorigenesis and tumour progression (Encinar et al. 2001), together with several of its downstream targets such as the endoplasmic reticulum stress-related transcription factors ATF4 and CHOP, as well as the pseudokinase tribbles-homologue 3 (TRIB3) (Carracedo et al. 2006). THC-triggered stimulation of the p8-regulated pathway induces the inhibitory interaction of TRIB3 with the pro-survival kinase, AKT, which leads to the mammalian target of rapamycin complex 1 (mTORC1) inhibition and the subsequent stimulation of autophagy-mediated cell death (Salazar et al. 2009; Vara et al. 2011). Modulation of cancer cell invasion has recently emerged as a topic of increasing interest. Endocannabinoids have been reported to block invasion and metastasis through modulation of MMPs (Blazquez et al. 2008) and their tissue inhibitors of (TIMPs) (Ramer et al. 2008). Indeed, a critical factor in tumor growth, migration and metastasis is represented by the adhesive interaction of tumor cells with the surrounding microenvironment. Matrix proteins such as integrins, cadherins, selectins and cell adhesion molecules of the immunoglobulin superfamily (IgSF CAMs) are responsable of tumor cell adhesion to the extracellular matrix (ECM). Cannabinoids regulate the adhesion of tumors cells to the ECM through suppression of phosphorylation of the focal adhesion kinase (FAK) and the pro-oncogenic tyrosine kinase Src (Grimaldi et al. 2006). Moreover, several studies suggest that angiogenesis is also regulated by cannabinoids (Pisanti et al. 2011; Freimuth et al. 2010). AEA has been shown to reduce sprout number and length of endothelial cell spheroids, inhibit capillary-

like tube formation and suppress angiogenesis in the *in vivo* chick chorioallantoic membrane model (Pisanti et al. 2007) through VEGFR downregulation (Portella et al. 2003) (Figure 9).

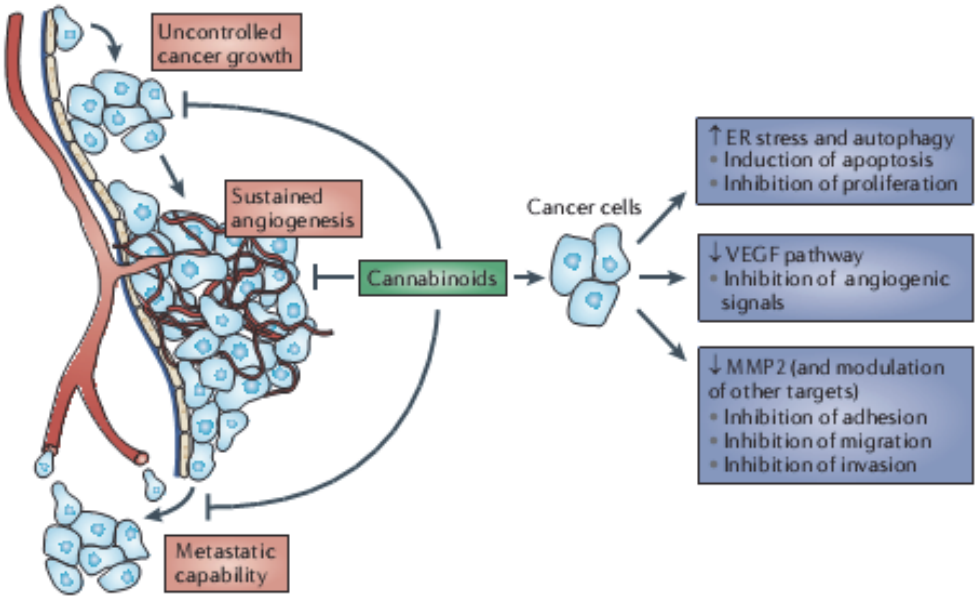


Figure 9. General mechanisms of cannabinoid antitumour action. Cannabinoids block tumour progression by targeting several hallmarks of cancer. They impair uncontrolled cancer cell growth inducing cancer cell death by apoptosis and by inhibiting cancer cell proliferation. They also hamper tumour angiogenesis by downregulating the vascular endothelial growth factor (VEGF) pathway in cancer cells. Finally, cannabinoids hinder metastasis by inhibiting cancer cell adhesion and migration/invasiveness through the modulation of matrix metalloproteinase 2 (MMP2) and tissue inhibitor of matrix metalloproteinases 1 (TIMP1) (Velasco et al. 2012).

1.4 Targeting endocannabinoid system in cancer: pharmacological strategies and therapeutic opportunities

The potentiality of targeting the endocannabinoid system in cancer therapy is increasingly intriguing (Pisanti and Bifulco 2009). Cannabinoids appear to be well tolerated in animal studies and do not produce generalized toxic effects on normal tissues that limit agents used in conventional chemotherapy (Bifulco and Di Marzo 2002). Even if a further preclinical research is needed to confirm their safety and efficacy, cannabinoids show several advantages compared to current

anti-cancer therapies: i. viability of non-transformed cells is unaffected or, under certain conditions, even favored by cannabinoid challenge (Salazar et al. 2009; Galve-Roperh et al. 2008); ii. the systemic administration of selective inhibitors of endocannabinoid degradation may be effective in those tissues where endocannabinoid levels are pathologically altered; iii. cannabinoids may represent an effective alternative to NSAIDs (Nonsteroidal Anti-inflammatory Drugs) for therapy of tumors displaying a resistance to apoptosis induction because of COX-2 overexpression (Bifulco et al. 2006).

1.4.1 Targeting peripheral CB1

The anti-proliferative properties of the active principle of marijuana, Δ9-THC, have been vastly explored (Parolaro et al. 2002). However, in addition to exogenous cannabinoids, recent attention has been focused on patho-physiological effects of some CB1 agonists, including regulation of cell growth and differentiation. Anandamide has been shown to inhibit the *in vitro* growth of human breast and prostate cancer cells (De Petrocellis et al. 2000) through growth arrest at the G1/S cell cycle transition and activation of CB1 receptors. In turn, activation of CB1 triggered simultaneous inhibition of AC and activation of the Raf1/extracellular signal-regulated kinase (ERK) transduction pathway (Melck et al. 1999), leading to inhibition of mitogenic properties of nerve growth factor (NGF) and TrkA and thus interfering with autocrine and paracrine mitogenic stimuli CB1-mediated. Moreover, Raf1/ERK activation has been also associated to a programmed death in glioma cells through activation of CB1 (Galve-Roperh et al. 2000), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK). Overall, it may be speculated that anandamide binding to CB1 receptors modulates the balance among ERK, JNK and p38 MAPK, thus regulating the cellular choice between proliferation and death.
The antitumor efficacy of anandamide has been also demonstrated *in vivo*, where it appears to inhibit the activity of the K-ras oncogene product p21 ras, thereby leading to the inhibition of the ras cascade-dependent tumor growth (Bifulco et al. 2001). Furthermore, a recent report has shown that activation of CB receptors prevents the growth of murine and human skin tumors by inducing apoptosis and blocking angiogenesis (Casanova et al. 2003).

1.4.2 Targeting Degradation Enzymes

Pharmacologically blocking anandamide metabolism results in increased endocannabinoid levels and, as a consequence, in enhanced beneficial effects in

cancer treatment (Bifulco and Di Marzo 2002). It has been reported that palmitoylethanolamide treatment enhances the anti-proliferative action of AEA in a human breast and melanoma (Hamtiaux et al. 2012) cancer model (Di Marzo et al. 2001) through a downregulation of FAAH expression up to 30–40% and a subsequent accumulation of AEA (Di Marzo et al. 2001). Moreover, athymic mice with thyroid tumor xenografts treated with a selective blocker of endocannabinoid hydrolysis or a selective inhibitor of endocannabinoid cellular reuptake increased intratumoral levels of anandamide and significantly decreased tumor volume (Bifulco et al. 2004). The anti-tumor efficacy of FAAH enzyme inhibitors has been also observed in neuroblastoma (Hamtiaux et al. 2011) and prostate carcinoma (Endsley et al. 2008) and could be only partly affected by a CB1 receptor antagonist, suggesting that endocannabinoids tonically control tumor growth *in vivo* by both a CB1-mediated and a CB1-indipendent mechanisms (Bifulco et al. 2004). Although undeniable beneficial effects of cannabinoid receptor agonists in tumor therapy, it is now generally described cannabinoid-induced unwanted effects, caused by activation of CB1 receptors located within the brain (Pertwee 2012).
The side effects of CB1-selective agonists might be overcome, at least in principle, by using one or a combination of the following strategies: i. intra-tumoral application of cannabinoids which results in little undesired 'central' effects in mice (Bifulco and Di Marzo 2002); ii. partial agonists of cannabinoid receptors employment, capable of activating vanilloid receptors, such as the synthetic compound arvanil, more efficacious than the 'pure' agonist (Melck et al. 1999); iii. CB1 receptor agonists utilization which do not cross the blood–brain barrier (BBB) and thus are deprived of psychotropic and immunosuppressive effects; iv. combination use of all these substances with non-psychotropic compounds, which lower the threshold of concentrations necessary to observe CB1 receptor-mediated tumor suppressing effects *in vitro* (Di Marzo et al. 2001).

1.4.3 Combinational therapies

The use of combinational anticancer therapies has several advantages compared to single-agent-based regimen, since they allow the concomitant targeting of tumour growth, progression and spreading. Several evidence has reported combinational anticancer therapies based on the co-administration of cannabinoid analogs and chemotherapeutic drugs, in order to reduce doses and avoid chemo resistance. For instance, the co-administration of THC and temozolomide, the chemotherapeutics for the management of glioblastoma, results in a strong anti-tumour action in glioma xenografts, also evident in temozolomide-resistant tumours (Torres et al. 2011). Recently, it has been demonstated that

gemcitabine in combination with different cannabinoid agonists synergistically reduces the viability of pancreatic cancer cells (Donadelli et al. 2011). Moreover, HU-210 and anandamide may enhance, respectively, the anticancer activity of 5-fluorouracil (Gustafsson et al. 2009) and paclitaxel (Miyato et al. 2009) and anaplastic thyroid carcinoma was sensitized to paclitaxel-based-chemotherapy through CB2 receptor expression (Shi et al. 2008). An effective additional approach consists in the combination of THC with CBD (Torres et al. 2011; Marcu et al. 2010). Therefore, the combination of THC and CBD with temozolomide reduces glioma xenografts growth (Torres et al. 2011). Importantly, CBD has also been shown to alleviate some of the undesired effects of THC administration, such as discoordination, convulsions and psychotic events, and, therefore, improves the tolerability of cannabis-based medicines (Pisanti et al. 2009; Pertwee et al. 2009). In addition, a small number of preclinical studies have recently demonstrated that cannabinoids attenuate chemotherapy-induced side effects including neuropathic pain. Indeed, direct agonists such as WIN55,212-2, a mixed CB1 and CB2 agonist, attenuates mechanical allodynia in models of paclitaxel (Pascual et al. 2005), vincristine (Rahn et al. 2007) and cisplatin (Vera et al. 2007)-induced neuropathy. Moreover, CB2 agonists also alleviate mechanical allodynia in paclitaxel- (Naguib et al. 2008; Xu et al. 2010) and vincristine-induced neuropathy (Rahn et al. 2007). In addition, as an alternative approach to the use of direct cannabinoid agonists, FAAH inhibition in combination with cisplatin administration reverses established cisplatin-induced side effect of peripheral neuropathic pain by elevating endocannabinoid levels.

2. AIMS OF THE STUDY

Modulation of endocannabinoid system has proven to hold several therapeutic promise in the treatment of a wide range of pathological processes. In this framework, given the ubiquity of the endocannabinoids and their receptors and their regulating action on proteins involved in cell fate control, there has been increasing evidence for a role of the endocannabinoid system also into neoplastic transformation and an interest to exploit it for a potential therapeutic application.
However, despite several reports on endocannabinoids' properties, little is known concerning the endogenous function of the endocannabinoid system and in particular of CB1 signaling in the regulation of tumor growth. Colorectal cancer (CRC) is the leading cause of mortality in Western countries (Wolpin et al. 2008). Loss of CB1 expression has been associated with tumor progression in human II and III degree carcinomas and in human CRC cell lines and takes place by a mechanism of epigenetic silencing acting on gene promoter *CNR1*. Moreover, CB1 knockdown in murine models Apc $^{Min/+}$ accelerates intestinal polyps growth, suggesting a role for CB1 as a tumor suppressor. Oxaliplatin in combination with 5-Fluorouracil and leucovorin (FOLFOX) has been approved for metastatic CRC therapy. Despite clinical success, patients who initially respond to chemotherapeutics may subsequently become refractory (Cassidy et al. 2004), directing the attention towards alternative strategies, including the use of combined therapies.
Therefore, the main aims of this doctorate thesis were:

1. to provide key information on the involvement of the endocannabinoid system in CRC growth control;
2. to characterize the specific role of CB1 receptor into the above process, focusing on transcriptional regulation acting on CB1/CNR1 gene promoter;
3. to explore the possibility of direct and indirect targeting of endocannabinoid system for CRC treatment;
4. to identify novel therapeutic strategies based on combinatorial approach.

3. MATHERIALS AND METHODS

3.1 Chemicals

2-Methyl-2'-fluoro anandamide (Met-F-AEA) (Sigma–Aldrich, Inc., St. Louis, MO) was dissolved in ethanol. AM251 [N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide], purchased from Cayman Chemical Co. (Ann Arbor, MI), FI-15 synthesized as previously described (Campiani et al. 1996) and the chemotherapeutic agents oxaliplatin, kindly provided by Sanofi-Aventis Research (Montpellier, France) and 5-Fluorouracil (Sigma–Aldrich, Inc., St. Louis, MO) were dissolved in dimethylsulfoxide (DMSO) to a final concentration of 10 mM and stored at -20°C .

3.2 Cell Culture

Human colorectal cancer cell line DLD-1 (Dexter et al. 1979; Interlab Cell Line Collection ICLC no. HTL95011) derived from a colorectal adenocarcinoma of type C according to Dukes classification, was grown in RPMI 1640 medium (Lonza, Belgium) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (P/S; Euroclone) at 37°C in a humidified 5% CO_2 air atmosphere. Human colorectal cancer cell line SW620 (Leibovitz et al. 1976; ATCC no. CCL-227™) derived from a lymph node metastatic site of a colorectal adenocarcinoma, was grown in Dulbecco's Modified Eagle's Medium (Lonza) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (P/S; Euroclone) at 37°C in a humidified 5% CO_2 air atmosphere.

3.3 Cell Transfection

To obtain CB1-silenced cells, at 24 h before transfection, SW620 cells were seeded into six-well plates at a concentration of $2x10^5$ cells/ml to obtain 30-60% confluence at the time of transfection. Then, CRC cells were transfected with a specific short interfering oligoribonucleotide (siRNA) or with a non-silencing oligoribonucleotide (NS RNA) as a control, at a final concentration of 50 nM using Lipofectamine (Invitrogen; Carlsbad, California) method according to the manufacturer's recommendations. The NS RNA and siRNA corresponding to human cDNA sequence for CB1 was purchased from Qiagen (Germantown, Philadelphia, PA, USA). Expression of CB1 in the transfected cells was evaluated by Western Blot analysis using an anti-CB1 antibody (AbCam).

3.4 Immunoblotting assay

For protein extraction, CRC cells were washed twice in ice-cold phospate-buffered saline (PBS) and lysed in buffer A (50 mM Tris-HCl pH 8.0 buffer containing 150 mM NaCl, 1% Nonidet P-40, 2 mg/ml aprotinin, 1 mg/ml pepstatin, 2 mg/ml leupeptin, 1 mM Na_3VO_4). Protein concentration was determined by the Bradford assay using bovine serum albumin as standard. Cell lysates were subjected to 10% SDS-PAGE. Gels were electroblotted into nitrocellulose membranes (Millipore Co., Bedford, MA) and filters were probed with the indicated primary antibodies: anti-CB1 and CB2 (AbCam), anti-FAAH (AbCam), anti-full length and cleaved caspase-3 (Asp175), anti-full length and cleaved caspase-9 (Asp330), anti-full length and cleaved PARP (Asp214) (Cell Signaling), anti-ERK 1/2 (Cell Signaling), anti-pERK1/2 (Cell Signaling), anti-phospho-CDK1 (Thr161) (Sigma), anti-CDK1 (Sigma), anti-Cyclin B (Cell Signaling), anti-CDC25c (Cell Signaling), anti-phospho ATR (Ser428) (Cell Signaling), anti-phospho p53 (Ser15) (Cell Signaling), anti-phospho-CHK1 (Ser345) and CHK1 (Abcam), anti-phospho-CHK2 (Thr68) and CHK2 (Cell Signaling), anti-Aurora B (Cell Signaling), anti-phospho-BubR1 and anti-BuBR1 (AbCam), anti-phospho survivin (Thr34) and anti-survivin (AbCam), anti-phospho-p38 (Cell Signaling) and anti-β-actin (Sigma, St. Louis, MO). Proteins were visualized with peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence system (Amersham-Pharmacia Biosciences LTD, Uppsala, Sweden).

3.5 RNA extraction and Reverse-transcriptase polymerase chain reaction

Total RNA was extracted from cell lines by guanidinium thiocyanate isopropanol method (Chomczynski, 1987). To measure mRNA expression, reverse transcription (RT) was performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen, San Giuliano Milanese, Milan, Italy) and random oligonucleotide primers. The generated cDNAs were amplified by PCR, using the correspondent primers. The first-strand cDNA was amplified using the sense primer CB1-F (5'-GATGTCTTTGGGA-AGATGAACAAGC-3) and the anti-sense primer CB1-R (5'-GAC-GTGTCTGTGGACACAGACATGG-3). The primers used to amplify the β2-microglobulin were the sense β2M1 (5'-CCTGGATTGCTATGTGTCTGGGTTTCATCC-3) and the anti-sense β2M2 (5'-GGAGCAACCTGCTCAGATAC-ATCAAACATG-3).

3.6 Chromatin immunoprecipitation (ChIP)

For chromatin immunoprecipitation (ChIP) assays, SW620 cells were grown to 95% confluence in phenol red-free DMEM supplemented with 10% charcoal dextran-stripped fetal bovine serum for at least 3 days. ChIP assay was performed according to a modified version of the protocol previously reported by Villa et al. (2007). Briefly, following the addition of anadamide at various time points, cells were cross-linked with 0.8% of formaldehyde at room temperature for 6 minutes and the reaction was stopped by the addition of glycine (0.125M). Cells were rinsed twice with cold PBS, resuspended in lysis buffer (1% SDS, 10 mM EDTA pH 8, 50 mM Tris HCl pH 8, PI) and sonicated. Lysates were diluted 10 times with the IP buffer (1% TritonX-100, 150 mM NaCl, 2 mM EDTA pH 8, 20 mM Tris HCl pH 8) and then incubated overnight with 5 mg of each antibody. 40 microliters of protein A sepharose beads saturated with salmon sperm (Upstate, Billerica, MA) were added to the lysates for 2 hours and then washed four times with Wash buffer 1 (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA pH 8, 20mM Tris HCl pH 8) and once with Wash buffer 2 (1% Triton X-100, 500 mM NaCl, 2 mM EDTA pH 8, 20 mM Tris HCl pH 8). The immunoprecipitated DNA was analyzed by PCR. We used the sense primer prCB1-F: 5'-TTTCGTTCTAGCGGACA-ACCA-3 and the anti-sense primer prCB1-R: 5'-GGAATAAGA-ACTCCACGAAGGAC-3. The specific antibodies used were anti-H3, anti-H3K9, anti-H3K27, purchased from Abcam; anti-pPol, anti-PolII, anti-RXRα, and anti-PPARγ, purchased from Santa Cruz (Santa Cruz, CA); anti-AcH3 and anti-AcH4 from Upstate.

3.7 Fatty acid amide hydrolase assay on CRC cell homogenates

In order to detect the presence of anandamide enzymatic hydrolysis, glass tubes containing increasing amounts of cell homogenates obtained from SW620 and DLD1 cells (165 ml, 10mM Tris-HCl, 1mM EDTA, pH 7.4) and 10 ml of DMSO were incubated for 10 min at 37°C with the radiolabeled substrate [3H]-anandamide (50000 dpm). Reaction was stopped by rapidly placing the tubes in ice-cold water followed by the addition of cold chloroform-methanol (1:1 v/v, 400ml). After centrifugation (850g, 5 min, 4°C), the radioactivity in the aqueous phase (200 ml) was counted by liquid scintillation (UltimaGold from Perkin-Elmer). As control for chemical hydrolysis, dpm values obtained for tubes containing buffer instead of proteins were systematically subtracted. The inhibitor activity was expressed as the concentration exerting 50% inhibition of AEA hydrolysis (IC_{50}), calculated by GraphPad®. Data are reported as means of n=3 experiments.

3.8 Cell proliferation ELISA

Cell proliferation was evaluated, *in vitro*, by measuring Bromodeoxyuridine (BrdU) incorporation during DNA synthesis through a colorimetric ELISA kit (Roche Diagnostics GmbH). In brief, DLD-1 and SW620 cells were seeded in triplicate into 96-well plates. Cells (1 x 10^4/ 0.32 cm plate) were treated with the drugs and incubated for 24 or 48 hours. According to the protocol provided by the manufacturer, at the end of treatment, cells were first fixed with a denaturation solution for 30 minutes and then incubated with anti-BrdU antibody peroxidase conjugate solution (anti-BrdU-POD) for about 4 hours followed by incubation with substrate solution for 20 minutes. The colorimetric reaction was measured through a microplate reader (Molecular Devices) at 370 nm. The blank was performed in each experimental setup. The absorbance value of blank was subtracted from other experimental values and cell proliferation was expressed as the percentage of absorbance values of treated samples to untreated controls.

3.9 FACS analysis

To assess cell cycle progression, after treatment with vehicle or compounds for 24 and 48 hours, CRC cells (250 000 per 6 cm plate) were resuspended with Trypsin-EDTA, washed once in ice-cold phospate-buffered saline (PBS), fixed in 70% ethanol and resuspended in 1 ml of PBS containing 1mg/ml RNase (Roche) and 50µg/ml propidium iodide (Sigma). After 4 hours of incubation at room temperature, propidium iodide incorporation in CRC cells was analyzed by a FACSCalibur flow cytometer (Becton Dickinson).

3.10 DNA fragmentation analysis

After treatment, adherent CRC cells were harvested, washed once with ice-cold PBS and incubated with lysis buffer (5mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5% Triton X-100) on ice for 20 minutes. Then, cell lysates were clarified by centrifugation at 12000 rpm for 20 min. The obtained supernatant was treated with RNase A (1 mg/ml) at 37°C for 60 minutes and Proteinase K (8 mg/ml) at 65°C for 15 minutes and subsequent incubation at 37°C for 12 hours. The total DNA, extracted by purification with phenol/chloroform/isoamyl alcohol and ethanol precipitation, was dissolved in 20 µl TE buffer (10 mM Tris-HCl , 1 mM EDTA), analyzed by 1.8% agarose gel electrophoresis. Approximately 20 µl DNA was loaded, stained by ethidium bromide and visualized under UV light.

3.11 Drug combination assay

The combination index (CI) was calculated by the CalcuSyn software based on the Chou-Talalay equation (Chou and Talalay 1984) which takes into account both potency (Dm or IC50) and shape of the dose-effect curve. Briefly, CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect and antagonism, respectively. Dose reduction index (DRI) representing the measure of how much the dose of each drug in a combination may be reduced at a given effect level compared with the doses of each drug alone (Takahashi et al. 2002).
To explore the relative contribution of each agent to the anti-proliferative effect, combinations with 1:10 FI-15/Oxaliplatin and 1:100 FI-15/5-FluoroUracil molar ratios, were tested in DLD1 cell line. Assessment of drug interaction was performed calculating the Combinatorial Index (CI). CI/fractional effect curves represent the CI *versus* the fraction of cells affected/killed by oxaliplatin or 5-FU and FI-15 in combination.

3.12 Statistical analysis

Statistical differences between the treatments and the control were evaluated by one-way analysis of variance (ANOVA). In the case of a significant result in the ANOVA, Student's t test was performed for all experiments. A p value less than 0.05 was considered statistically significant.

4. RESULTS

4.1. Anandamide as emerging suppressor of colon tumor growth

In order to determine the role of CB1 receptor into the control of CRC tumor growth, the ability of a selective CB1 agonist, 2-Methyl-2-fluoro anandamide (Met-F-AEA), a stable analogue of the natural endocannabinoid anandamide, to affect proliferation of human CRC cell lines was examined. To this end, we utilized as *in vitro* models two highly invasive and metastatic human colon cancer cell lines, SW620 and DLD1, expressing different CB1 receptor levels, as assessed by RT-PCR (Figure 10).

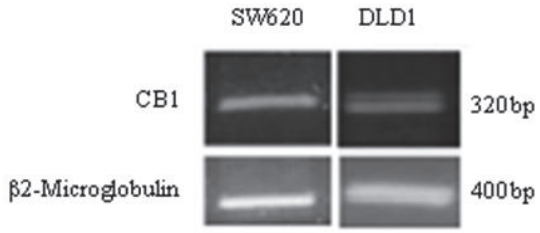


Figure 10. CB1 expression in human CRC cells.
cDNA obtained from SW620 and DLD1 cells was analyzed for CB1 through RT-PCR. To confirm equal loading, mRNA expression level was normalized for the housekeeping gene β2-microglobulin. Results shown are representative of at least three independent experiments.

As observed, the exogenous administration of increasing doses of anandamide for 24 and 48 hours significantly inhibited CRC cellular proliferation in a dose- and time-dependent manner, as evaluated by BrdU incorporation ELISA assay. In particular, SW620 cells expressing high CB1 levels were more sensitive to anandamide-induced proliferation inhibition (Figure 11A), instead DLD1 cells expressing lower CB1 levels were more refractory to treatment (Figure 11B). As observed, 10 μM was the most effective dose and was chosen for all following experiments. The proliferation index (PI) obtained through dividing the absorbance of treated CRC cells *versus* untreated was determined (Figure 11C, D).

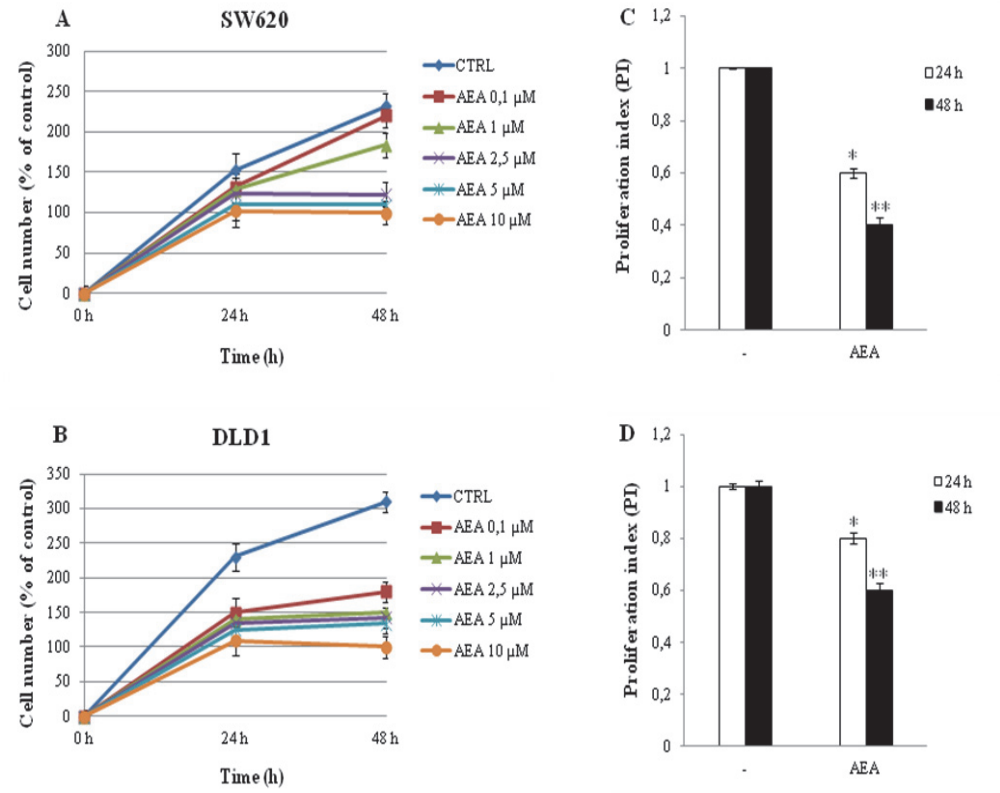


Figure 11. Anandamide affects human CRC cell lines proliferation.
(A,B) SW620 and DLD1 cells (1 x 10^4/ 0.32 cm plate) were incubated with increasing doses of AEA for 24 and 48 hours. Cell proliferation was evaluated by measuring BrdU incorporation during DNA synthesis through a colorimetric ELISA assay. Results are shown as the mean ± SD from triplicate cultures. In *(A,B)*, error bars depict means ± SD (n = 3). *(C,D)* Proliferation index (PI) was determined as a fold change of the absorbance of CRC treated cells *versus* untreated. Error bars depict means ± SD (n = 3) (ANOVA *vs* control, *p < .05, **p < .01).

In order to investigate CB1 receptor functional involvement into the cellular response to anandamide, the consequences of its pharmacologic and genetic inactivation, respectively by blocking CB1 receptor with the selective antagonist AM251 or through RNA interference, were examined in CRC cells.
A pharmacologic approach, blocking CB1 receptor in CRC cells through its selective antagonist AM251, was performed. To this aim, SW620 cells were pretreated for 30 minutes with AM251 (3 µM) before exposure to anandamide (10 µM, 24 and 48 hours) and CRC cell proliferation was evaluated through BrdU incorporation ELISA assay. As observed in Figure 12A, pharmacological inactivation of CB1 receptor reverted anandamide-induced anti-proliferative

effects in human CRC cells. Similar results were obtained also with SR141716 (0.3 μM), a different CB1 antagonist, indicating that the observed effect was due to CB1 antagonism independently from the antagonist used (Figure 12B) and specifically because of CB1 blockade, because the selective CB2 antagonist SR144528 failed to alter CRC proliferation (data not shown).

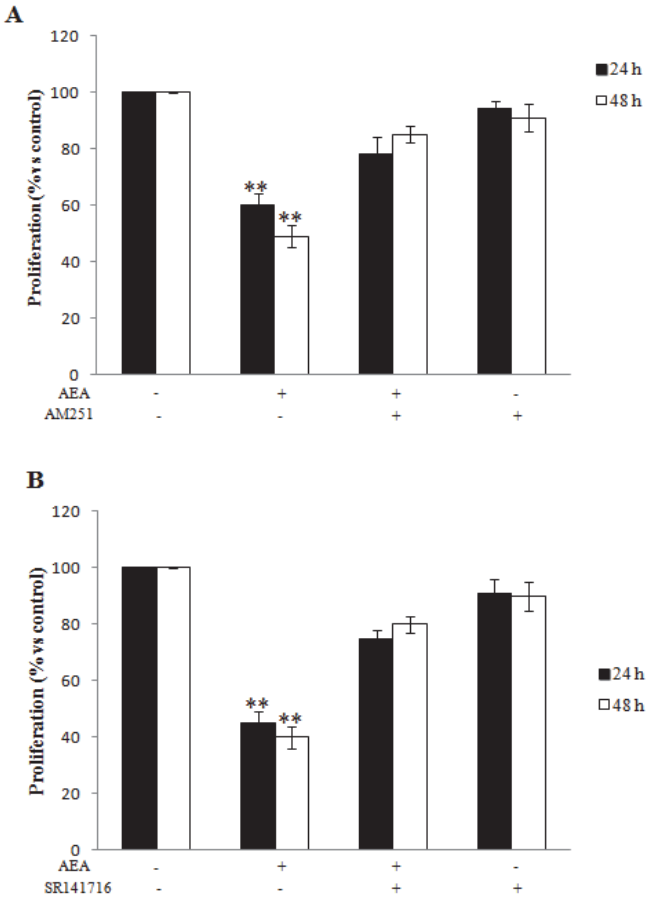
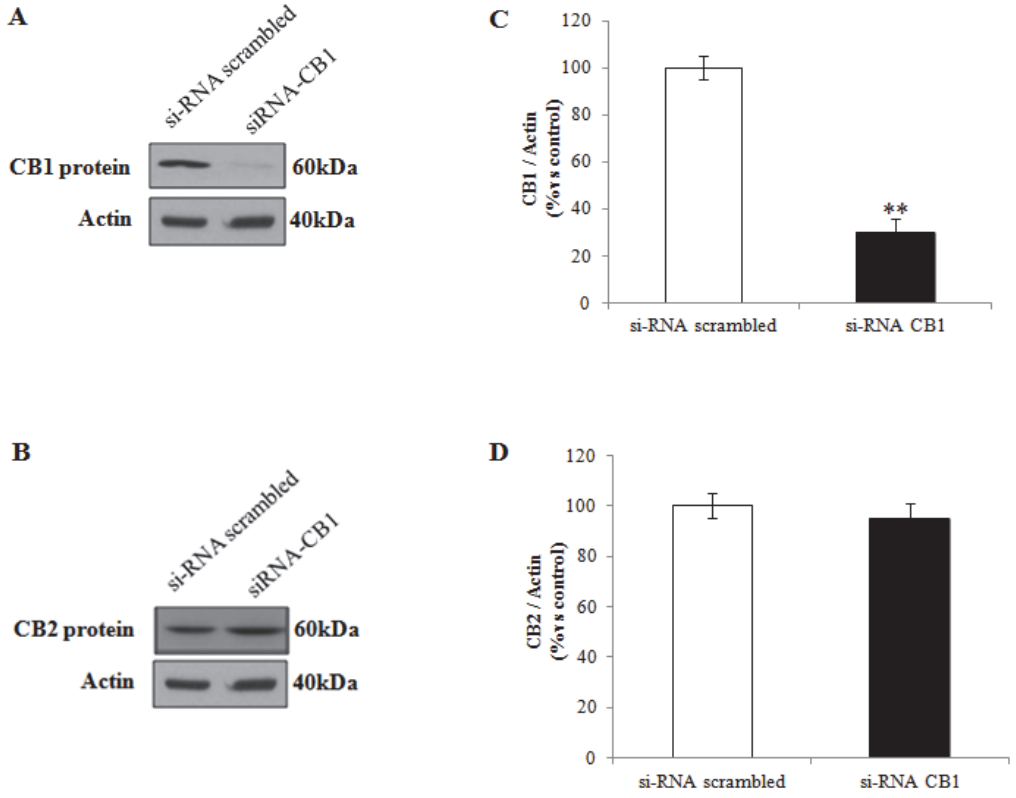


Figure 12. CB1 pharmacological inactivation reverted anandamide-induced anti-proliferative effects in human CRC cells.
(A) SW620 cells (1 x 10^4/ 0.32 cm plate) were pretreated for 30 minutes with AM251 (3 μM) before exposure to anandamide (10 μM, 24 and 48 hours) and CRC cell proliferation was evaluated through BrdU incorporation ELISA assay.The results are shown as the mean ± SD from triplicate cultures. *(B)* SW620 cells (1 x 10^4/ 0.32 cm plate) were pretreated for 30 minutes with SR141716 (0.3 μM) before exposure to anandamide (10 μM, 24 and 48 hours) and CRC cell proliferation was evaluated through BrdU incorporation ELISA assay. Results are shown as the mean ± SD from triplicate cultures. In *(A,B)*, error bars depict means ± SD (n = 3) (ANOVA *vs* control, *p < .05, **p< .01).

To confirm this result, the effect of CB1 knockdown was evaluated in CRC cells transiently trasfected with a specific short interfering (si)RNA. First of all, the efficacy of a CB1 siRNA in inducing CB1 ablation was tested. CB1 siRNA silenced CB1 protein to 70% at 24 h after transfection, as evaluated through Western Blot analysis, whereas a control scrambled siRNA had no effect (Figure 13A, C). Moreover, CB2 expression was not affected, thus ruling out siRNA off-target action (Figure 13B, D). As expected, anandamide failed to induce growth inhibition in CB1-silenced SW620 cells, as measured by BrdU incorporation ELISA assay (Figure 13E).



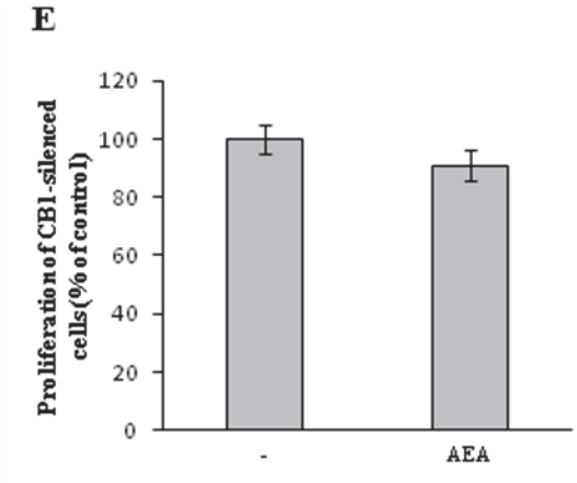


Figure 13. CB1 genetic inactivation reverted anandamide-induced anti-proliferative effects in human CRC cells.
(A,B) SW620 cells were transfected with either control scrambled siRNA or CB1-siRNA for 24 hours and the lysates were analyzed by Western blot for CB1 and CB2 expression. To confirm equal loading the filters were stripped and reprobed with anti β-actin antibodies, as indicated. *(C,D)* Histograms show densitometric analysis of CB1 and CB2 protein expression, reported as percentage of control (mean± SD of 3 independent experiments). *(E)* SW620 cells were transfected with CB1-siRNA for 24 hours and stimulated with AEA (10 μM) for 24 hours. Cell proliferation was evaluated through BrdU incorporation ELISA assay. Results are shown as the mean ± SD from triplicate cultures. In *(C, D, E)* error bars depict means ± SD (n = 3) (ANOVA *vs* control, **p< .01).

These results show that the pharmacological and genetic inactivation of CB1 receptor is capable of reversing the growth inhibition induced by the administration of anandamide and clearly suggest that inhibition of CRC growth mediated by anandamide is CB1 receptor-dependent.

4.2 Anandamide-induced upregulation of CB1 receptor in human CRC cells

In order to dissect the contribution of cannabinoid receptor 1 into the anti-proliferative effect observed and to assess the modulation, if any, of CB1 expression by anandamide following its activation, the levels of expression of CB1 after treatment of CRC cells with anandamide were determined. To this aim, DLD1 and SW620 cells were treated with anandamide (10 μM) for increased times and CB1 expression levels were measured through Western blot analysis. As observed in Figure 14A-C, CB1 protein expression was increased of about 40% in

anandamide-treated SW620 cells starting from 2 hours and until 24 hours compared to untreated control cells, as appreciated by means of densitometric analysis of the blots. The amount of CB1 increase after 2 hours was higher in DLD1 cells, reaching 50% compared to untreated control cells, consistent with low basic protein expression (Figure 14B, D).

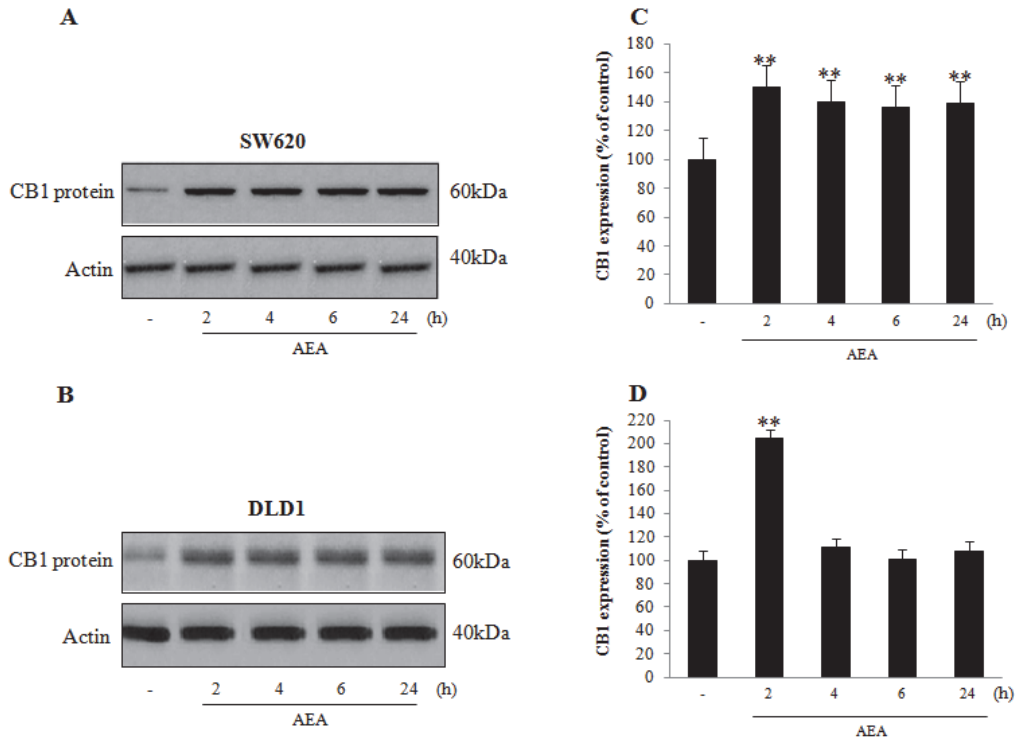


Figure 14. CB1 protein upregulation induced by anandamide in human CRC cells.
(A,B) Lysates from SW620 and DLD1 cells, left untreated (-) or treated with AEA (10 μM) for the indicated incubation times (2, 4,6, 24 hours), were immunoblotted with anti-CB1 antibody. To confirm equal loading, the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments. *(C,D)* Histograms show densitometric analysis of CB1 protein expression, reported as percentage of control (mean± SD of 3 independent experiments) (ANOVA *vs* control, **p< .01).

In order to further characterize CB1 expression upon activation, quantification of messenger RNA (mRNA) levels of the receptor in anandamide-stimulated CRC cells was performed. Incubation of both cell lines with anandamide (10 μM) for increased times resulted also in a drastic increase of mRNA for the CB1 gene, as

evaluated by RT-PCR. The pattern of CB1 mRNA was similar to that observed at protein level for both CRC cells. This result suggested that anandamide-induced CB1 protein expression was consistent with increased levels of the corresponding CB1 mRNA (Figure 15) and that a transcriptional mechanism was involved in CB1 modulation after anandamide exposure.

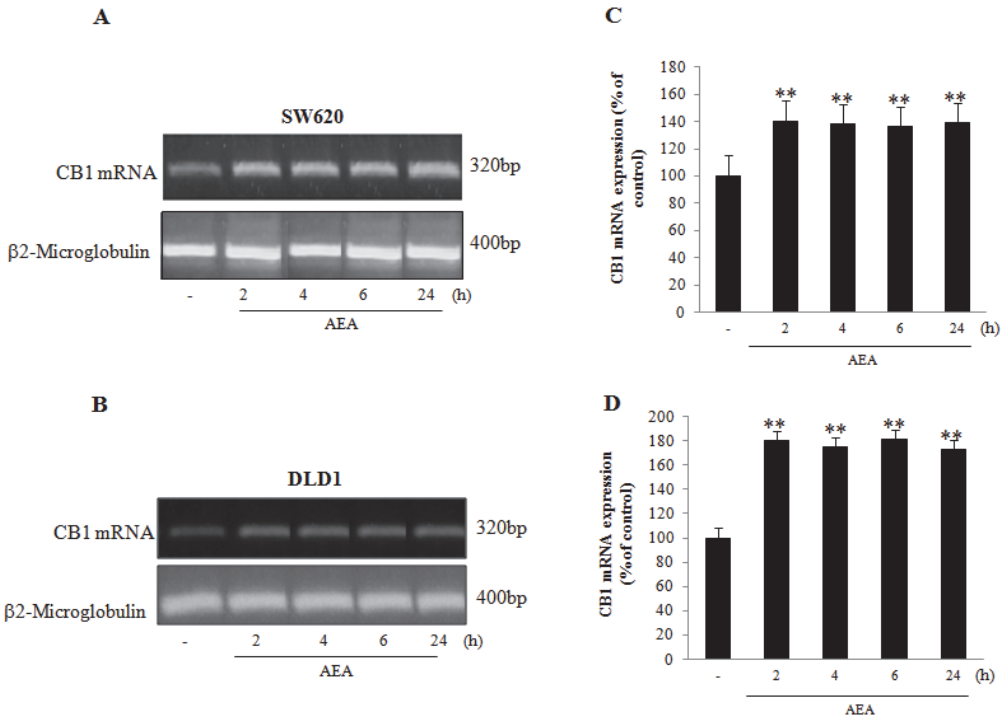


Figure 15. CB1 mRNA upregulation induced by anandamide in human CRC cells.
(A,B) cDNA from SW620 and DLD1 cells, left untreated (-) or treated with AEA (10 μM) for the indicated incubation times (2, 4, 6, 24 hours), was analyzed for CB1 through RT-PCR. To confirm equal loading, mRNA expression level was normalized for the housekeeping gene β2-microglobulin. Results shown are representative of at least three independent experiments. *(C,D)* Histograms show densitometric analysis of CB1 mRNA expression, reported as percentage of control (mean± SD of 3 independent experiments) (ANOVA *vs* control, **p< .01).

4.3 Transcriptional regulation of CB1 through anandamide-induced activation of its gene promoter

With the aim to verify whether the induction of CB1 expression in CRC cells was due to an anandamide-mediated transcriptional regulation acting on *CNR1* gene promoter, chromatin immunoprecipitation assays (ChIP) were performed in SW620 cells. Histone H3 and H4 modifications as well as polymerase status and occupancy at *CNR1* promoter region after exposure to anandamide (10 μM) for increasing times were evaluated on exon I. To this aim, SW620 cells were cross-linked and protein-DNA complexes were immunoprecipitated using antibodies recognizing normal rabbit IgG or anti-AcH3, -AcH4, -H3K27, -H3K9, -H3, -phospho-Pol III, -Pol III. As shown in Figure 16, since after 90 minutes and until 20 hours of treatment with anandamide, an increase in the amount of acetylated Histone H3 and H4 and a concomitant decrease in methylation at Lysine 9 and 27 of Histone H3 were observed. Interestingly, the phosphorylated amount of polymerase III was increased compared to total polymerase III expression, suggesting that recruitment and activation of polymerase also occurred for transcriptional activity. As a positive control, non-precipitated input DNA, from both treated and untreated cells, was used as a template for PCR. Using this input DNA as template, PCR produced a distinct band and, as expected, anandamide did not affect the level of PCR product. As a negative control, IgG antibodies did not immunoprecipitate the *CNR1* promoter region and gave no PCR product. These results show that histone modifications and polymerase activation observed are consistent with a transcriptional regulation of CB1 through anandamide-induced activation of its gene promoter.

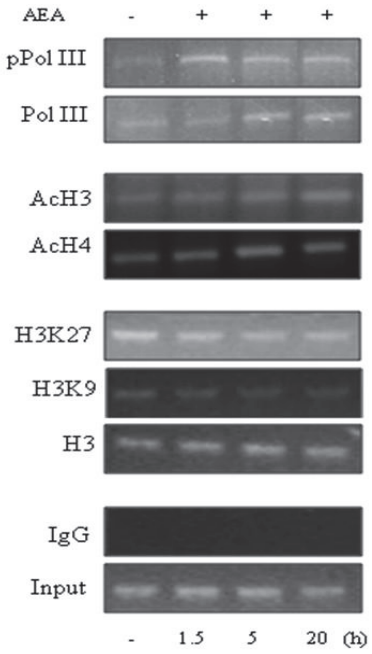


Figure 16. Anandamide-induced CB1 expression through transcriptional activation of its gene promoter.
Chromatin from SW620 cells, left untreated (-) or treated with AEA (10 μM) for the indicated incubation times (1.5, 5, 20 hours), was analyzed through ChIP assays with the indicated specific monoclonal antibodies. The input confirms the comparable strength of the primer pairs specific for promoter region. As a positive control, non-precipitated input DNA, from both treated and untreated cells, was used as a template for PCR. As a negative control, IgG antibodies did not immunoprecipitate the CNR1 promoter region and gave no PCR product. Results shown are representative of at least three independent experiments.

At this point, the involvement of transcription factors in anandamide-mediated induction of CB1 gene was evaluated. The promoter region of CB1 gene is known as the binding site of RARγ, responsible for the induction of CB1 expression induced by RA and 2-AG in mouse hepatocytes (Mukhopadhyay et al. 2010). In addition, the peroxisome proliferator-activated receptor gamma (PPAR-γ) dimerizes with retinoid receptors and binds to DNA sequences called PPAR-γ responsive elements, leading to the transcription activation of target genes (Ziouzenkova and Plutzky 2008). Anandamide is reported to bind directly PPAR-γ, activate its transcriptional activity and stimulate the differentiation of fibroblasts into adipocytes (Gasperi et al. 2007). Since anandamide is a direct ligand of PPAR-γ, the association of the transcriptional factors PPAR-γ and RXRα on *CNR1* promoter was evaluated through ChIP. To this aim, SW620 cells were

cross-linked after exposure to anandamide for increasing times starting from 1.5 to 20 hours and protein-DNA complexes were immunoprecipitated using antibodies recognizing normal rabbit IgG or anti-RXRα and PPARγ. As shown in Figure 17, both the transcriptional factors were found associated to *CNR1* gene promoter 5 hours after anandamide exposure.

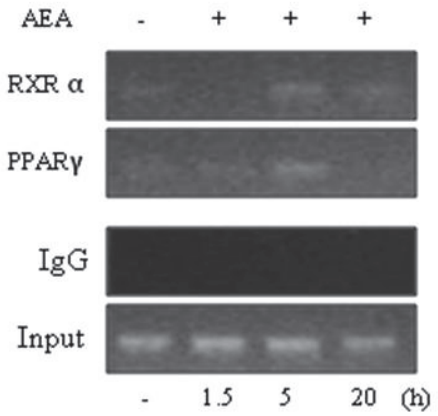


Figure 17. Anandamide induced RXRα and PPARγ recruitment at the *CNR1* promoter.
Chromatin from SW620 cells, left untreated (-) or treated with AEA (10 μM) for the indicated incubation times (1.5, 5, 20 hours), was analyzed through ChIP assays with the indicated specific monoclonal antibodies. The input confirms the comparable strength of the primer pairs specific for promoter region. As a positive control, non-precipitated input DNA, from both treated and untreated cells, was used as a template for PCR. As a negative control, IgG antibodies did not immunoprecipitate the CNR1 promoter region and gave no PCR product. Results shown are representative of at least three independent experiments.

4.4 Endocannabinoid metabolism: anandamide enzymatic degradation

Since the goal of this work was to characterize endocannabinoid system involvement in CRC growth control in order to explore the possibility of a direct and indirect targeting, endocannabinoid metabolism in CRC cells has been explored. To this aim, the rate of hydrolysis of anandamide in human colon cancer cells was primarily determined. The expression of the enzyme responsible for anandamide metabolism, FAAH, was evaluated in CRC cell lines. Western blot analysis revealed that FAAH enzyme was highly expressed in CRC cells, suggesting a role for the enzyme overexpression in the deregulation of the endocannabinoid levels in CRC tumors (Figure 18).

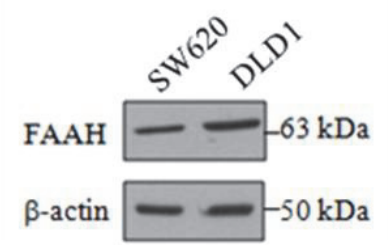


Figure 18. FAAH expression in human CRC cells.
Lysates from SW620 and DLD1 cells were immunoblotted with anti-FAAH antibody. To confirm equal loading, the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments.

Anandamide degradation, occurring uniquely through FAAH enzyme, produces arachidonic acid and ethanolamine (Wei et al. 2006). Thus, using [3H]-AEA incubation, the enzymatic activity of FAAH on cell homogenates obtained from SW620 and DLD1 cells was valued, through counting the amount of ethanolamine produced. The rate of hydrolysis following incubation of increasing doses of cell homogenate was elevated, as revealed by the high amount of ethanolamine detected (dpm) in both SW620 (Figure 19A) and DLD1 (Figure 19B) cells.

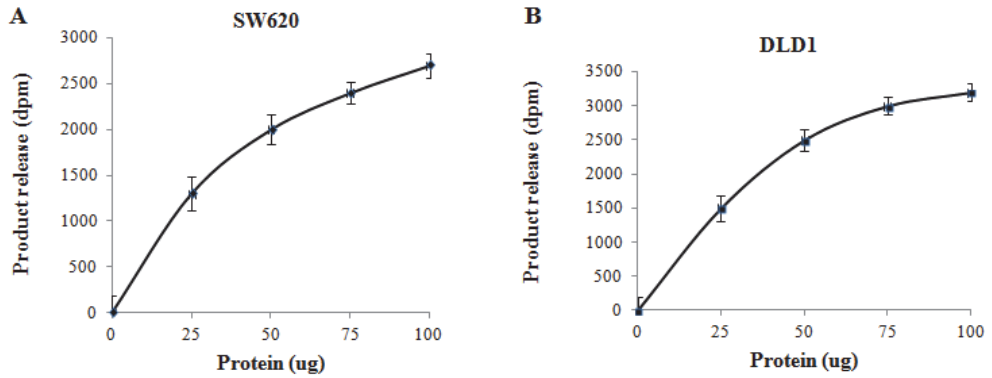


Figure 19. Human CRC cells efficiently hydrolyze anandamide.
(A,B) Enzymatic activity for AEA hydrolysis was measured in SW620 and DLD1 cell homogenates using [3H]-AEA. Data are the mean of three experiments performed in duplicate.

To test whether the increased rate of endocannabinoid hydrolysis was due to an altered FAAH expression, enzyme levels were evaluated after anandamide

exposure (10 μM) in CRC cells through Western Blot analysis. Consistent with increased enzymatic activity, anandamide induced an increase of FAAH amount of about 50% after 2 hours and prolonged until 24 hours in both CRC cells (Figure 20).

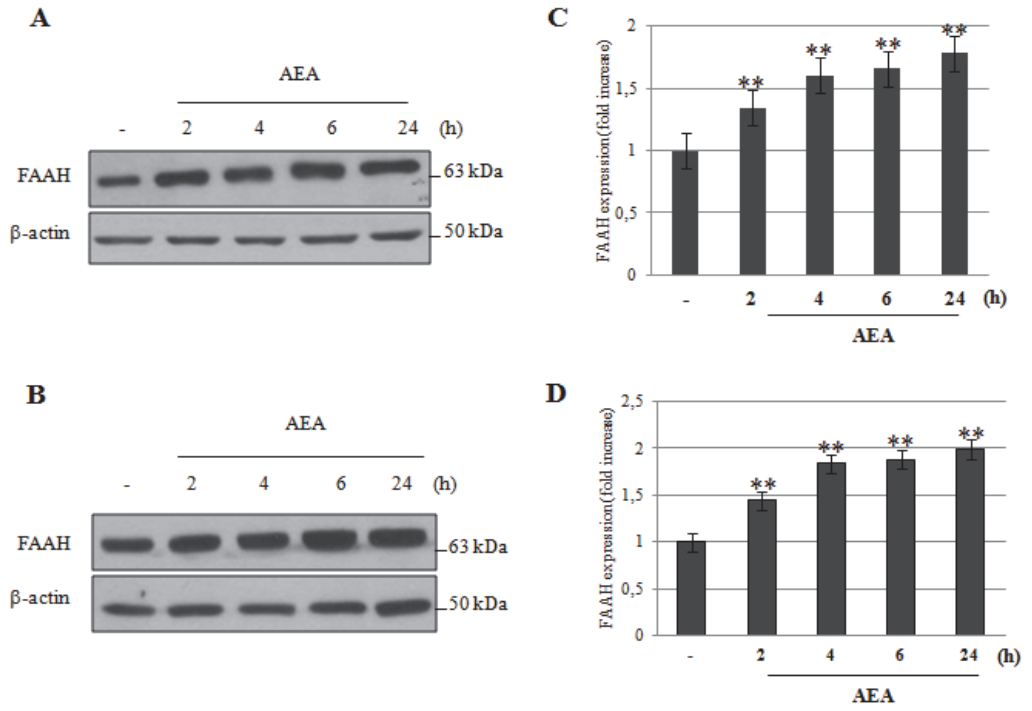


Figure 20. FAAH upregulation induced by anandamide in human CRC cells.
(A,B) Lysates from SW620 and DLD1 cells, left untreated (-) or treated with AEA (10 μM) for the indicated incubation times (2, 4,6, 24 hours), were immunoblotted with anti-FAAH antibody. To confirm equal loading, the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments. *(C,D)* Histograms show densitometric analysis of FAAH protein expression, reported as percentage of control (mean± SD of 3 independent experiments) (ANOVA *vs* control, **p< .01).

4.5 Targeting Anandamide Degradation: FAAH enzyme inhibition

Since the protective mechanism of anandamide in CRC is made incomplete by its FAAH-mediated rapid degradation, a potential approach to increase the local concentration of the endocannabinoid could consist in delaying its inactivation through FAAH pharmacologically blockage (Bifulco and Di Marzo 2002). In

order to explore this possibility, the enzymatic activity of a series of compounds synthesized as FAAH inhibitors (FI) was tested. After a systematic screening, FI-15 was identified as a selective inhibitor of FAAH enzyme, since it was found able to inhibit FAAH activity with a submicromolar potency (IC_{50} = 843.3 nM).

4.6 Exploring FAAH inhibition in CRC: molecular aspects

Anti-tumor potential has been described for the CB1 receptor agonist. However, the effect on CRC growth control of FAAH inhibition has not been proved, yet. The identification of a compound that binds to and inhibits FAAH enzymatic activity raises the obvious question of whether this compound could affect cellular target proliferation. Thus, the anti-proliferative effect of increasing doses of FI-15 was evaluated through a BrDU incorporation ELISA assay on CRC FAAH-positive cells, DLD1 and SW620 cells. As a consequence of anandamide increase, the exogenous administration of FI-15 for 24 and 48 hours significantly inhibited CRC cell proliferation in a dose- and time-dependent manner (Figure 21). Interestingly, in accordance with no expression of FAAH enzyme in Hela cells, FI-15 did not affect cell proliferation (data not shown). As observed, FI-15 (1 μM) reduced FAAH-positive CRC cell proliferation of about 70% compared to untreated cells (Figure 21A, B) and, as the lowest effective dose, was chosen for all following experiments.

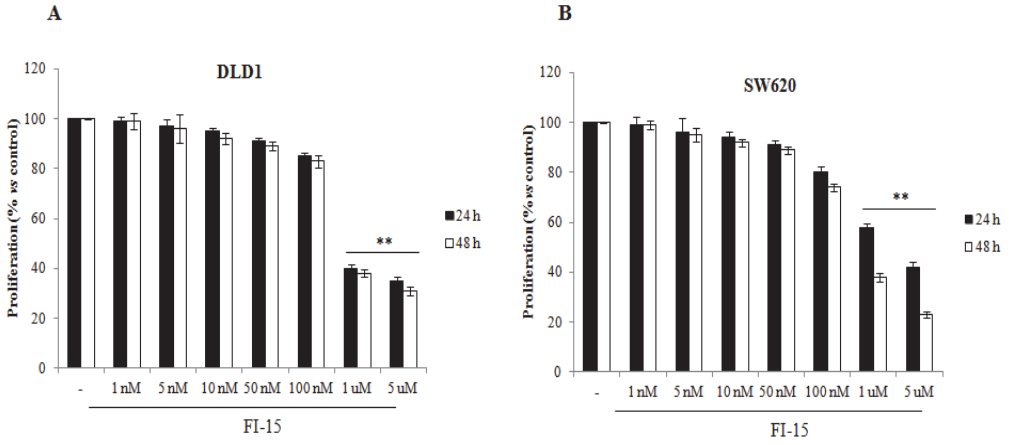
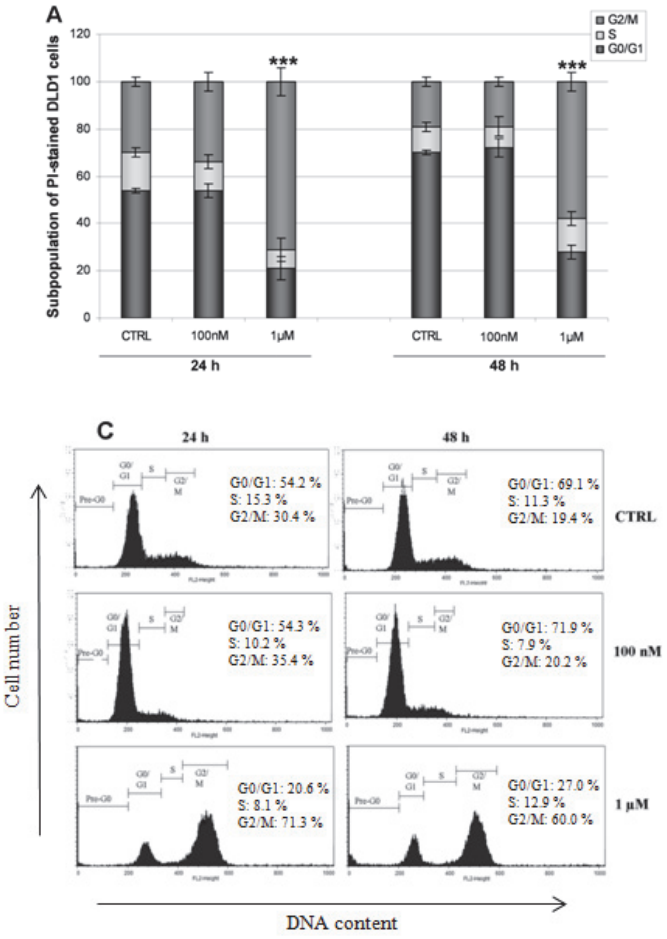


Figure 21. FAAH inhibition affected human CRC cell lines proliferation.
(A,B) DLD1 and SW620 cells (1 x 10^4/ 0.32 cm plate) were incubated with increasing doses of FI-15 for 24 and 48 hours. Cell proliferation was evaluated by measuring BrdU incorporation during DNA synthesis through a colorimetric ELISA assay. Results are shown as the mean ± SD from triplicate cultures. In *(A,B)*, error bars depict means ± SD (n = 3) (ANOVA *vs* control, *p < .05, **p < .01).

In order to investigate whether FI-15-mediated CRC growth inhibition may occur through cell cycle deregulation in CRC cells, cell cycle phase distribution of propidium iodide (PI)-stained DLD-1 cells after exposure to FI-15 (1 μM) for 24 and 48 hours was carried out through flow cytometry. FACScan analysis revealed an altered cell cycle profile since a delay in G2/M phase progression occurred through a G2/M phase accumulation of about 70% compared to exponentially growing untreated CRC control cells (Figure 22C). Accordingly, the amount of DLD1 cells in G0/G1 phase decreases of about 30%, compared to untreated cells (Figure 22A). Similarly to DLD-1, SW620 cells showed an increased accumulation in G2/M phase upon exposure to FI-15 (1 μM) (Figure 22B, D), suggesting that cell cycle modulation induced by the FAAH inhibitor in human CRC cells was not cell line-selective. As expected, FI-15 (100 nM), unable to affect CRC cell proliferation, failed to significantly alter percentage distribution of treated cells in different phases of the cell cycle.



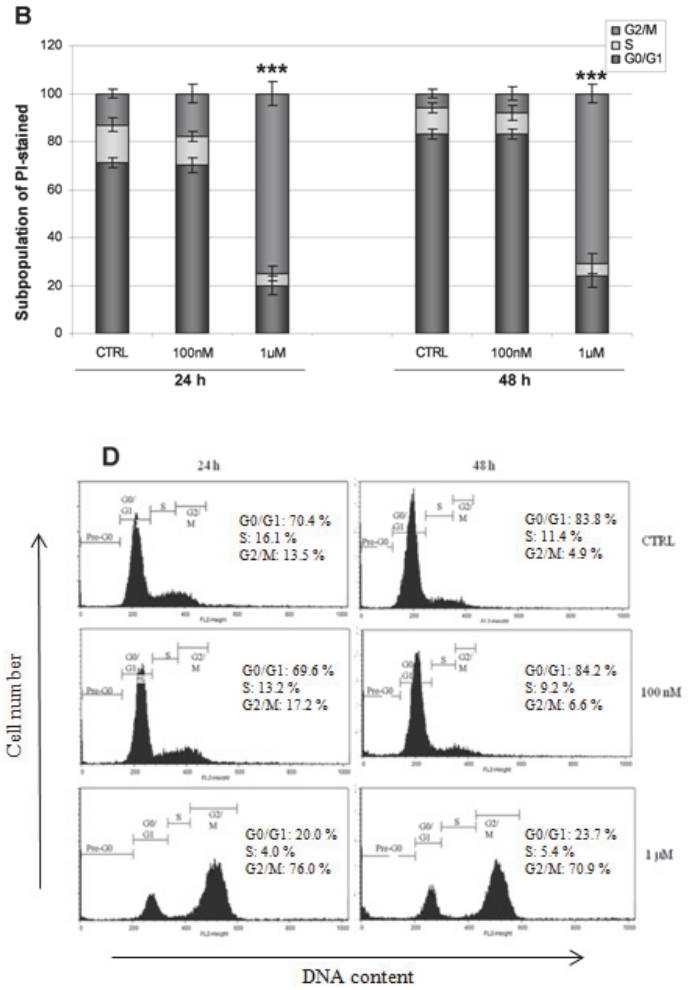


Figure 22. FI-15 affected CRC proliferation through cell cycle deregulation.
(A,B) Changes in the percentage of cells in G0/G1, S and G2/M phases after treatment of DLD1 (*A*) or SW620 (*B*) cells with FI-15 (100 nM and 1 μM) for 24 and 48 hours are normalized to the cell cycle distribution of exponentially growing untreated control cells. Each value is the mean ± SD of 3 separate experiments performed in duplicates (***$p<0.01$). *(C, D)* DLD1 and SW620 cells cultured in the presence and in the absence of FI-15 (100 nM or 1 μM) were collected after 24 and 48 hours of incubation, stained with propidium iodide (PI) and analysed by flow cytometry. The cell cycle profile of a single experiment representative of three is reported in the Figure.

Cell cycle progression from the G2 to M phase is regulated by activation of CDK1, whose activity is dependent upon coordination with cyclin B (Malumbres and Barbacid 2009; Lapenna and Giordano 2009). The activation of the CDK1/cyclin B complex (MPF, Maturation promoting factor) is maintained

through phosphorylation at Thr161 and dephosphorylation at Thr14 and Tyr15 of CDK1 (Malumbres and Barbacid 2009; Lapenna and Giordano 2009). Dephosphorylation of the Thr14 and Tyr15 residues in CDK1 is catalyzed by the phosphatase Cdc25C. It is thought of as a rate-limiting step for G2 entry into mitosis (Malumbres and Barbacid 2009; FitzGerald et al. 2009). Considering the role of the CDK1/cyclin B complex and Cdc25C in regulating G2 to M phase transition, levels of expression of cell cycle machinery proteins were evaluated in CRC cells after exposure to FI-15 for 24 hours compared to untreated cells through Western blot analysis. FI-15 (1 μM) exposure altered the expression levels of cyclin B1 and Cdc25C, as well as the amount of CDK1 phosphorylation. As expected, the amount of phosphorylated CDK1 protein at Thr161 was reduced compared to CDK1 total expression as well as the levels of cyclin B1 and Cdc25C expression in cells treated with FI-15 (Figure 23A, B). These results indicate that FI-15 exposure induced a typical G2/M cell cycle arrest in CRC cells by regulating cyclin B1 and Cdc25C expression and CDK1 phosphorylation.

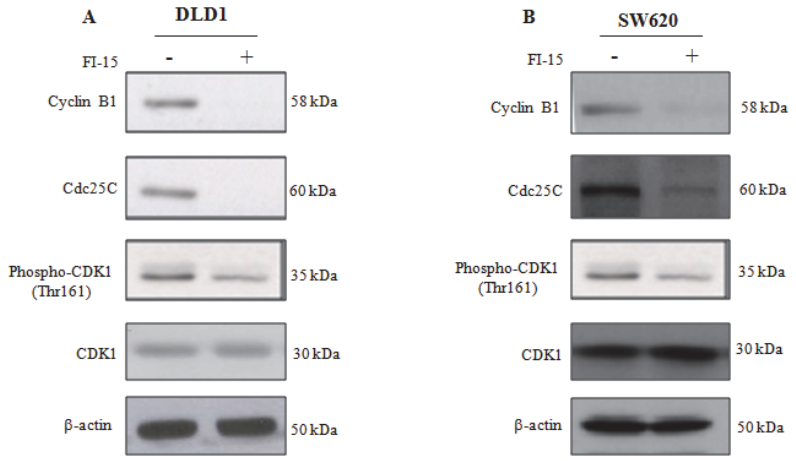


Figure 23. FAAH inhibition altered G2/M cell cycle machinery.
(A,B) Lysates from DLD1 and SW620 cells, left untreated (-) or treated with FI-15 (1 μM) for 24 hours were immunoblotted with the indicated antibodies. To confirm equal loading, the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments.

The G2/M checkpoint is an important quality control measure that ensures the proper sequence of cell cycle events and allows cells to respond to DNA damage. As a part of the cell cycle surveillance system, the DNA damage and spindle checkpoints protect cells from genomic instability (Matsuoka et al. 2000; Bucher

and Britten 2008). In order to verify whether the FI-15-induced G2/M cell cycle arrest was due to the activation of a DNA damage signaling, expression levels of checkpoint proteins involved in response to DNA damage are evaluated by Western blot analysis after exposure to the FAAH inhibitor. The G2/M DNA damage checkpoint involves the activation of ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR) proteins (Bucher and Britten 2008). Both ATM and ATR activate p53 in response to DNA damage by phosphorylation of Ser15, which is required for inhibiting expression of the key regulators of the G2/M transition, CDK1 and cyclin B (Talos and Moll 2010). As observed in Figure 24A-B, the amount of phosphorylated ATR (Ser428) and p53 (Ser15) was increased in FI-15-exposed CRC cells after 24 hours.

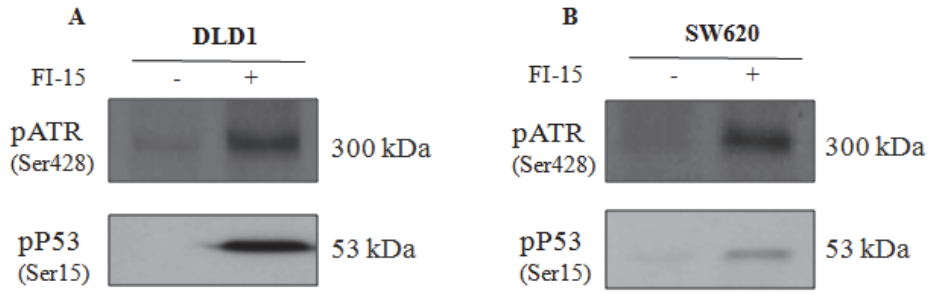


Figure 24. FAAH inhibition activated DNA damage signaling.
(A,B) Lysates from DLD1 and SW620 cells, left untreated (-) or treated with FI-15 (1 μM) for 24 hours were immunoblotted with the indicated antibodies. To confirm equal loading, the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments.

Moreover, the mitotic spindle checkpoint proteins BubR1 and Bub3 have been shown to monitor tension across attached kinetochores and initiate mitotic arrest in response to loss of microtubule tension (Elowe et al. 2007). Western blot analysis was used to determine whether both proteins were associated with FI-induced G2/M cell cycle arrest in DLD1 cells exposed to FI-15 for increasing times. The FAAH inhibitor induced the iper-phosphorylation of BubR1, that occurs from 24 hours, slightly reducing after 48 hours of treatment (Figure 25). A regulator of the spindle checkpoint signaling is checkpoint kinase 1 (Chk1), a substrate of ATR together with Chk2, which is required for optimal regulation of Aurora B and BubR1 (Zachos et al. 2007). As a consequence of ATR activation, an increase in Chk1 phosphorylation amount at Ser345 was detected from 24 hours compared to total Chk1 expression. The increase was persistent until 72 hours of treatment, when a downregulation was observed consistent with a concomitant decrease of

Aurora B expression and BubR1 phosphorylation. No changes in the amount of phosphorylated Chk2 as well as of total Chk1 or Chk2 were assessed (Figure 25). Since Chk1 inhibition has been associated to the abolition of the spindle assembly checkpoint (SAC), premature entry in mitosis, catastrophic mitotic events, as well induction of apoptosis (Parsels et al. 2011), Chk1 inhibition occurring after 72 hours of FI-15-treatment strongly suggested the activation of apoptotic program in CRC cells.

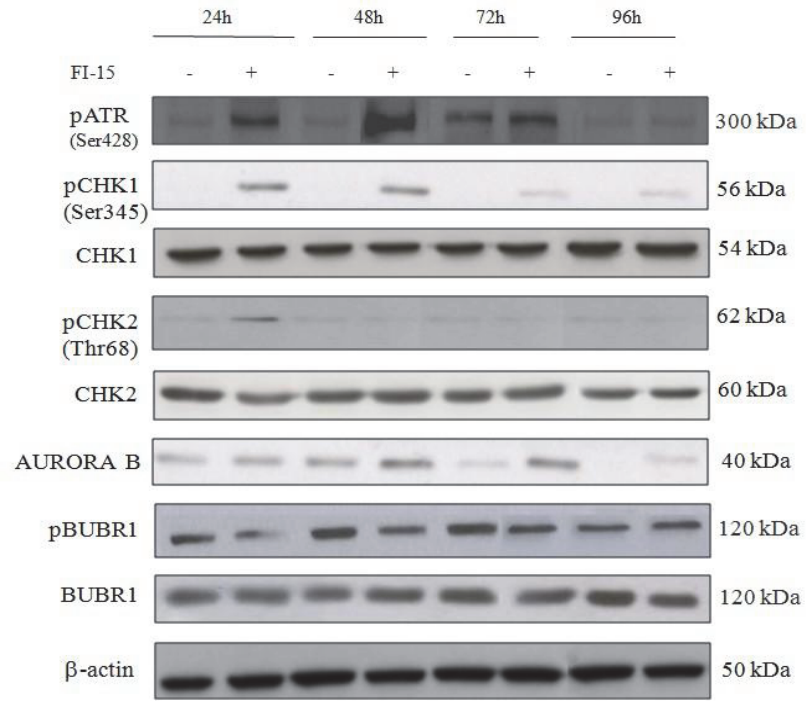


Figure 25. FAAH inhibition activated G2/M spindle checkpoint signaling.
Lysates from DLD1 cells, left untreated (-) or treated with FI-15 (1 μM) for increasing times (24, 48, 72, 96 hours) were immunoblotted with the indicated antibodies. To confirm equal loading, the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments.

In order to dissect whether FI-15-induced prolonged cell cycle arrest resulted into the activation of a programmed cell death, a TUNEL assay on CRC cells was performed. To this aim, total DNA obtained from CRC cells after exposure to FI-15 (1 μM) for increasing times was analyzed. A FAAH inhibitor-induced strong DNA degradation was assessed in both CRC cells as displayed through the appearance of typical DNA ladder patterns (Figure 26). In particular, DNA fragmentation occured in DLD-1 cells after a 48hours-exposure (Figure 26A),

instead, SW620 cells required prolonged time treatment to 96 hours (Figure 26B), suggesting a FI-15-mediated induction of a late apoptosis.

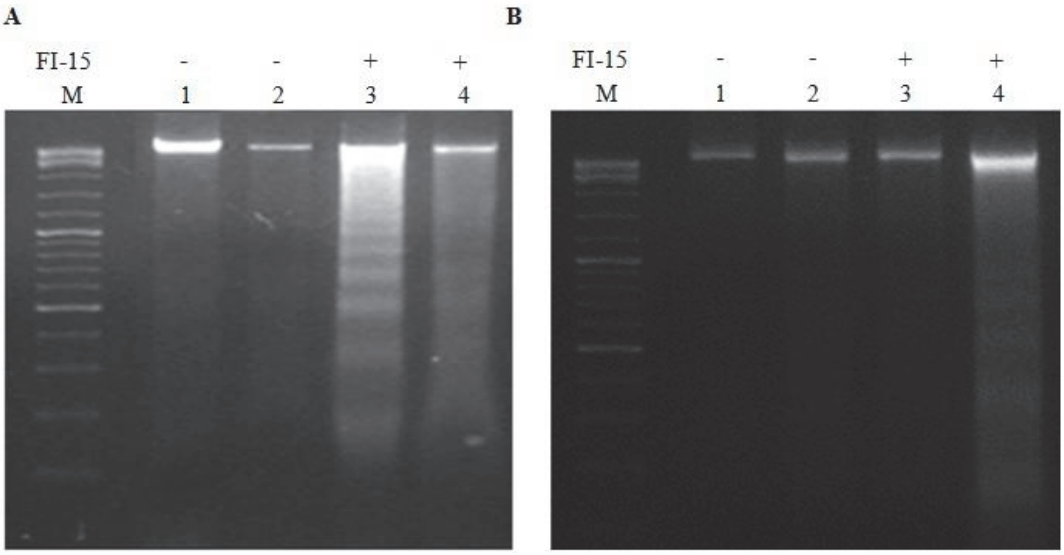


Figure 26. FAAH inhibition induced a programmed cell death in CRC cells.
(A,B) DNA was prepared from DLD1 and SW620 untreated cells (lanes 1 and 2) or CRC cells treated respectively for 48 (lane 3) or 96 hours (lane 4) with FI-15 (1 μM) and analyzed in a 1.6% agarose gel. Lane M is the 100-bp DNA ladder.

Since caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis (Jänicke et al. 1998), in order to determine whether FI-15-induced late apoptosis in CRC FAAH-positive cells occured through caspase regulation, total and cleaved amount of executioner caspase 3, initiators caspase 8 and 9 and Poly (ADP-ribose) polymerase (PARP) were detected through Western blot analysis. As shown, FI-15 exposure resulted in a strong activation of caspase-3 through cleavage, particularly evident following 72 hours in DLD1 (Figure 27A) and SW620 (Figure 27B) cells; cleavage of Poly (ADP-ribose) polymerase (PARP) also correlated with caspase-3 activation in both CRC cells (Figure 27A, B). Caspase-9, the initiator protease of the intrinsic apoptotic pathway (Figure 27A, B), and not caspase-8 (data not shown) activation, also occurred after FI-15 treatment. No effect was observed in the presence of the vehicle negative control and FI-15 (100 nM) (Figure 27A, B).
These data, taken together, indicate that FI-15 interferes with CRC proliferation by activation of the DNA damage pathway accompanied by G2/M cell cycle arrest and induction of a late intrinsic apoptotic pathway.

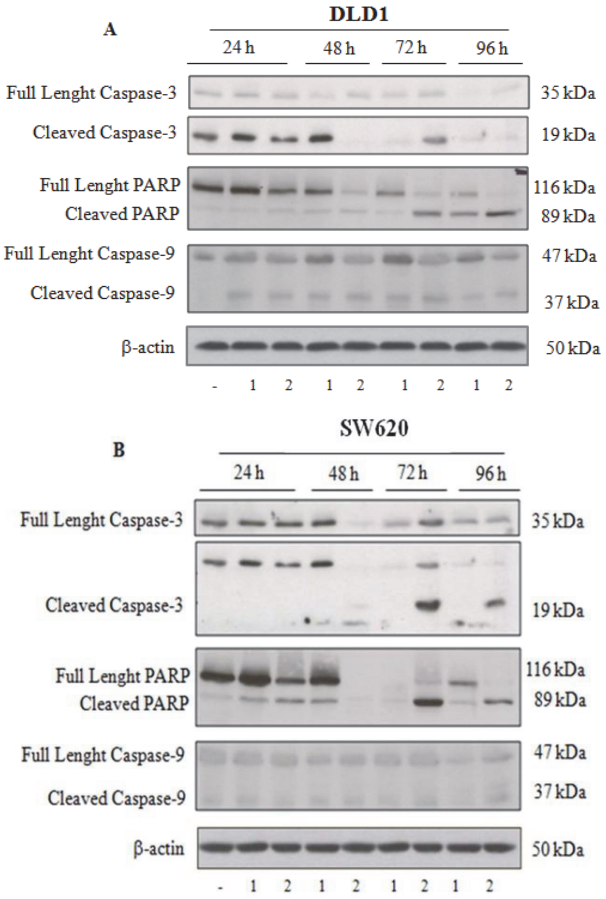


Figure 27. FAAH inhibition induces a late intrinsic apoptotic pathway in CRC cells.
(A,B) Lysates from DLD1 and SW620 cells, left untreated (-) or treated with FI-15 for the indicated incubation times at the concentrations of 100 nM (lane 1) and 1 μM (lane 2), were immunoblotted with the indicated antibodies. To confirm equal loading the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments.

4.7 Combinatorial approaches with oxaliplatin and 5-fluouracil through survivin modulation

Oxaliplatin in combination with 5-Fluorouracil and leucovorin (FOLFOX) has been approved for metastatic CRC therapy (Mayer 2012). Despite clinical success, patients who initially respond to chemotherapeutics may subsequently become refractory (Cassidy et al. 2004). Thus, the attention has been directed

towards alternative strategies, including the use of combined therapies. As a consequence, a combinatorial approach based on the combination of FI-15 and the two chemotherapeutics oxaliplatin and 5-fluorouracil was exploited in DLD1 cells. First of all, the characterization of chemo resistance phenotype of CRC cells to the two agents was performed in dose-dependent experiments. Cell proliferation rate was evaluated through BrDU incorporation ELISA assay in DLD1 cells exposed to increasing doses of oxaliplatin (0.08 µM to 10 µM) and 5-fluorouracil (3 µM to 100 µM) for 72 hours. CRC cells were found sensitive to oxaliplatin (10 µM) and 5-fluorouracil (50 µM) since the affected fraction (FA), obtained through dividing the killed CRC cell amount *versus* total cell number was about 15% and 13%, respectively (Figure 28). Low doses of both drugs were unable to have a cytotoxic effect and are chosen for following experiments.

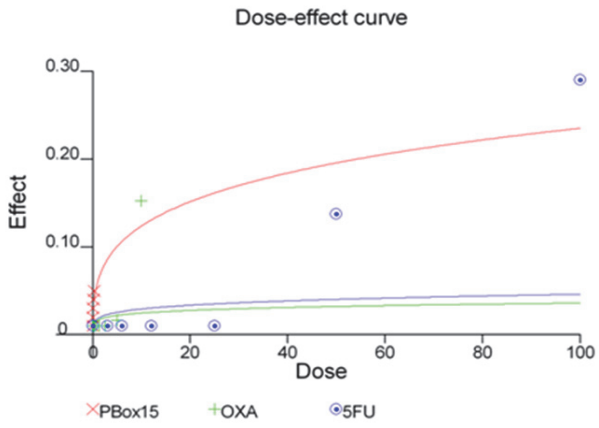


Figure 28. Characterization of chemo resistance phenotype of CRC cells.
DLD1 cells were incubated with increasing doses of FI-15 (ranging from 0.005 µM to 0.3 µM), oxaliplatin (ranging from 0.08 µM to 10 µM) or 5-FU (ranging from 3 µM to 100 µM) for 72 hours. Cell proliferation was evaluated by measuring BrdU incorporation during DNA synthesis through a colorimetric ELISA assay. The dose-effect curve was obtained through application of CalcuSyn software, showing the dose of the drug *versus* the fraction of cells affected/killed by FI-15, oxaliplatin or 5-fluorouracil used alone (mean± SD of 3 independent experiments).

Interestingly, the combined treatment of FI-15 with oxaliplatin (1:10 molar ratio) (Figure 29A) or 5-fluorouracil (1:100 molar ratio) (Figure 29B) reduced DLD1 cell proliferation more effectively than the treatment with each single agent alone. The combination index (CI), obtained through the application of CalcuSyn

software, was under 1, thus indicating a synergic interactions between FI-15 and the chemotherapeutics (Table 2).

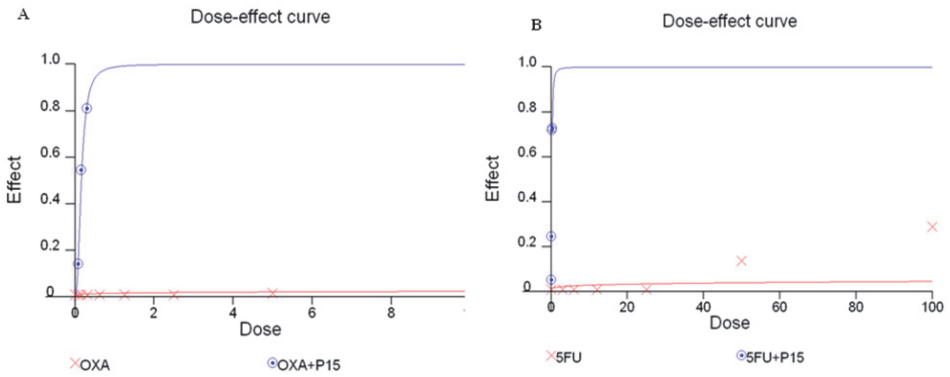


Figure 29. FAAH inhibition sensitizes cells to targeted CRC therapies.
(A) DLD1 cells were incubated with FI-15 in combination with oxaliplatin (1:10 molar ratio) or *(B)* 5-Fluorouracil (1:100 molar ratio) at the concentrations indicated in the Table 1. Cell proliferation was evaluated by measuring BrdU incorporation during DNA synthesis through a colorimetric ELISA assay. The dose-effect curve was obtained through application of CalcuSyn software (mean± SD of 3 independent experiments).

Table 2. Combination index (CI), Fraction affected (FA) and Dose Reduction Index (DRI) for FI-15 and OXA/5-Fluorouracil in DLD1 cells.

FI-15 (μM)	OXA (μM)	CI	FA FI-15 + OXA	DRI FI-15	OXA
0.075	0.75	0.005	0.1413	2.08	7.79
0.15	1.5	2.66e-005	0.5450	3.76	4.37
0.3	3	1.22e-006	0.8105	8.19	3.79
FI-15 (μM)	5-FU (μM)	CI	FA FI-15 + 5-FU	DRI FI-15	5-FU
0.075	7.5	0.001	0.2455	1.57	1.53
0.15	15	2.76e-006	0.7200	3.62	1.92
0.3	30	4.91e-006	0.7279	2.04	1.16

There is not much evidence about the molecular mechanism of oxaliplatin. However, recent studies have reported survivin as a key target for oxaliplatin (Baek 2010) and its downregulation as one of the drug mechanism in CRC cells (Fujie et al. 2005).
In order to dissect the molecular mechanism(s) underlying the synergic action of FI-15 and oxaliplatin in CRC cells, the protein expression levels of survivin were evaluated through Western Blot analysis after exposure for 72 hours of DLD1 cells to oxaliplatin (3 μM) alone or in combination with FI-15 (300 nM). As expected, oxaliplatin down regulated survivin expression of about 60% compared to untreated cells reaching about 80% when it was used in combination with FI-15 (Figure 30A, B). Moreover, survivin downregulation occurs also after oxaliplatin exposure in combination with AEA (10 μM), although with a less extent.

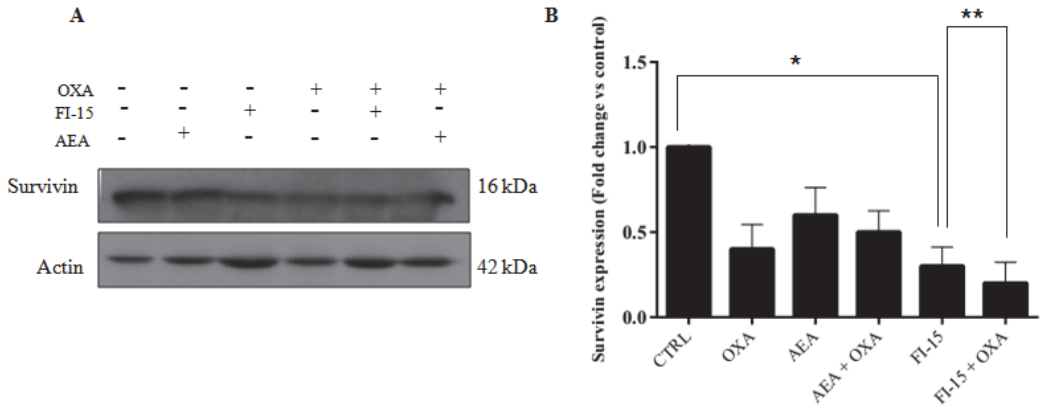


Figure 30. FI-15 in combination with oxaliplatin downregulated survivin expression.
(A) Lysates from DLD1 cells, left untreated (-) or treated with FI-15 (300 nM) and AEA (10 μM) alone or in combination with oxaliplatin (3 μM) for 72 hours were immunoblotted with anti-survivin antibody. To confirm equal loading, the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments. *(B)* Histograms show densitometric analysis of survivin expression, reported as fold increase *vs* control. Error bars depict means ± SD (n = 3) (ANOVA *vs* control, *p < .05, **p< .01).

It has been shown that p38 MAP kinase is involved in the down-regulation of survivin by baicalein treatment (Chao et al. 2007) and that phosphorylation of p38 MAP kinase mediates the activation of caspases (Hsiao et al. 2007). In addition, it has been reported that oxaliplatin-induced downregulation of survivin in human colon cancer cells occurs through p38 MAP kinase activation (Huei et al. 2010). To explore the pathway responsible for survivin downregulation, the amount of phosphorylated p38 was examined after combined treatment. An increase of p38

kinase phosphorylation amount was observed in combined treated cells after 72 hours, consistent with cleavage of caspase 3 and PARP activation (Figure 31).

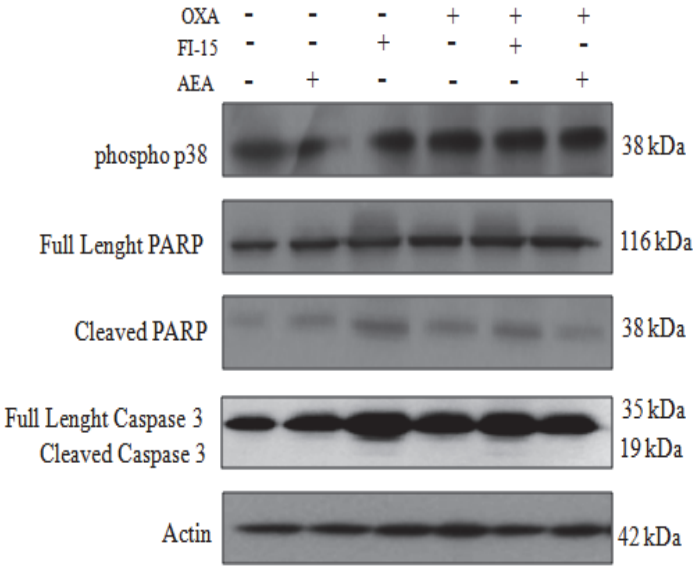


Figure 31. Combination of FI-15 with oxaliplatin activated the p38 MAP kinase phosphorylation and caspase-3.
Lysates from DLD1 cells, left untreated (-) or treated with FI-15 (300 nM) and AEA (10 μM) alone or in combination with oxaliplatin (3 μM) for 72 hours were immunoblotted with the indicated antibodies. To confirm equal loading, the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments.

Because the phosphorylation of survivin at Thr34 by cdc2 increases protein stability (O'Connor et al. 2002), survivin phosphorylation after co-administration of oxaliplatin with FI-15 was examined. As expected, oxaliplatin combination strongly reduced survivin phosphorylation in DLD1 cells more than alone (Figure 32A, B).

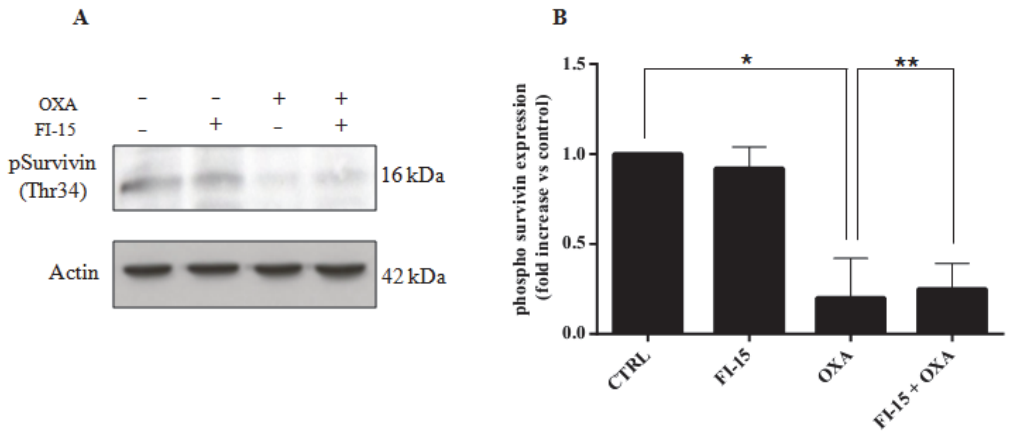


Figure 32. FI-15 in combination with oxaliplatin reduced survivin phosphorylation.
(A) Lysates from DLD1 cells, left untreated (-) or treated with FI-15 (300 nM) alone or in combination with oxaliplatin (3 μM) for 72 hours were immunoblotted with anti-survivin antibody. To confirm equal loading, the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments. *(B)* Histograms show densitometric analysis of survivin expression, reported as fold increase *vs* control. Error bars depict means ± SD (n = 3) (ANOVA *vs* control, *p < .05, **p< .01).

MG132, a specific proteasome inhibitor, was used in combination with oxaliplatin and FI-15 in examining the involvement of proteasome in survivin stability To this aim, DLD1 cells were pretreated for 1 hour with MG132 (10 μM) before exposure to oxaliplatin (3 μM) and FI-15 (300 nM) for 72 hours and survivin expression was valued through Western blot assay. Treatment with MG132 restored survivin protein levels in the oxaliplatin-FI-15-treated cells (Figure 33A, B). Taken together, these results suggest that protein down-expression of survivin is regulated by p38 MAP kinase and proteasomal pathway.

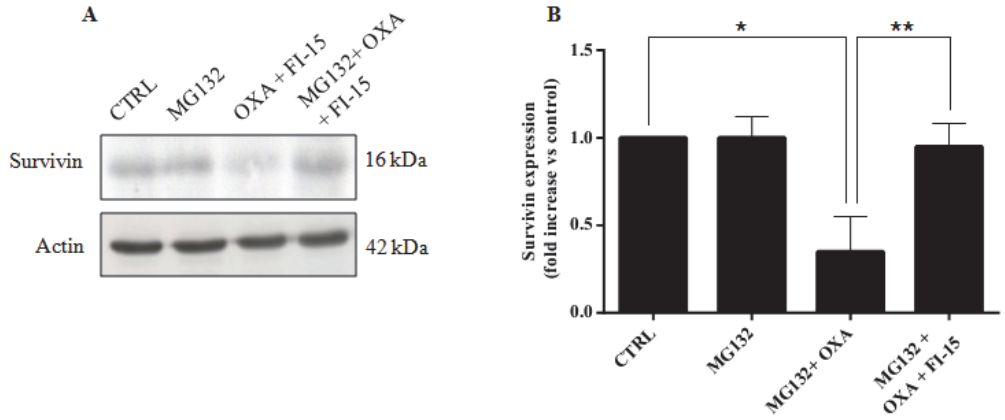


Figure 33. Blockage of proteasome pathway restored the combination-inhibited survivin protein expression.
(A) Lysates from DLD1 cells, left untreated (-) or pretreated with MG132 (10 μM) for 1 hour before exposure to FI-15 (300 nM) in combination with oxaliplatin (3 μM) for 72 hours were immunoblotted with anti-survivin antibody. To confirm equal loading, the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments. *(B)* Histograms show densitometric analysis of survivin expression, reported as fold increase *vs* control. Error bars depict means ± SD (n = 3) (ANOVA *vs* control, *p < .05, **p< .01).

Survivin downregulation through an oncolytic adenovirus (Shen et al. 2010) or shRNA (Shen et al. 2012) has also been reported to increase 5-fluouracil cytotoxicity, respectively in colon and gastric carcinoma. As a consequence, the hypotesis that the sinergic effect of FI-15 and 5-fluoruacil was due to FI-15-mediated downregulation of survivin expression was tested. Levels of expression of survivin were evaluated in DLD1 cells after exposure to FI-15 (300 nM) in combination with 5-FU (30 μM) for 72 hours. Compared to untreated control and to 5-FU alone, the co-treatment was able to strongly reduce survivin expression (Figure 34A, B).

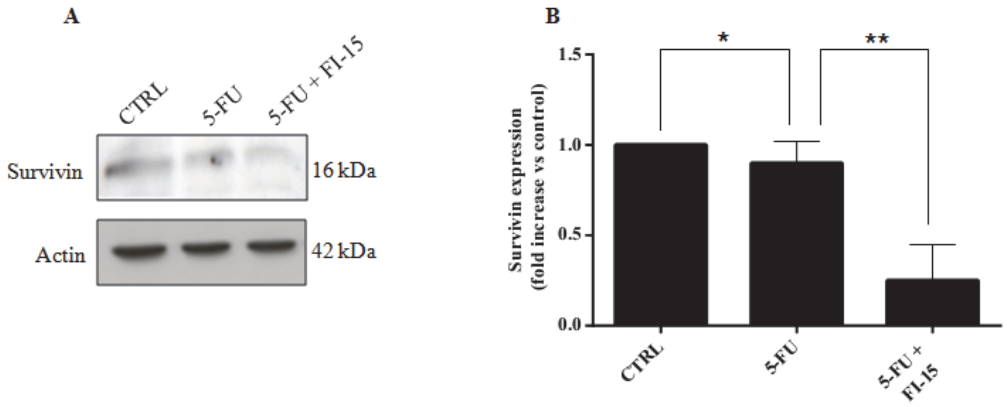


Figure 34. FI-15 in combination with 5-FU downregulated survivin expression.
(A) Lysates from DLD1 cells, left untreated (-) or treated with FI-15 (300 nM) alone or in combination with 5-fluorouracil (30 μM) for 72 hours were immunoblotted with anti-survivin antibody. To confirm equal loading, the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments. *(B)* Histograms show densitometric analysis of survivin expression, reported as fold increase *vs* control. Error bars depict means ± SD (n = 3) (ANOVA *vs* control, *p < .05, **p< .01).

5. DISCUSSION

Strong experimental evidence has suggested, *in vitro* and *in vivo*, the role of endocannabinoids in the control of CRC proliferation and in the modulation of the neo-angiogenic and metastatic processes underlying the progression of CRC cancer (Ligresti et al. 2003; Wang et al. 2008; Proto et al. 2012). However, the molecular mechanisms underlying these effects have not completely yet understood.

In this dissertation, the functional involvement of cannabinoid receptor 1 (CB1) signaling in CRC growth control has been explored through the use of a selective CB1 agonist, 2-Methyl-2-fluoro anandamide (Met-F-AEA), a stable analogue of the natural endocannabinoid anandamide. The exogenous administration of increasing doses of anandamide significantly inhibited CRC cellular proliferation in a dose- and time-dependent manner. This mechanism depends on basal levels of CB1 expression: CRC cells expressing high CB1 levels were more sensitive to anandamide-induced proliferation inhibition, instead cells expressing lower CB1 levels were more refractory to treatment, confirming what already observed in prostate (Sarfaraz et al. 2005), astrocitoma (Cudaback et al. 2010) and breast cancer (McKallip et al. 2005). Moreover, the pharmacological and genetic inactivation of CB1 receptor is capable of reversing the growth inhibition induced by the administration of anandamide, clearly suggesting that inhibition of CRC growth mediated by anandamide is CB1 receptor-dependent. Regional up-regulation of CB1 correlates with enhanced potency and efficacy of agonists at sites of disease in several animal models, including intestinal inflammation and hypertension (Batkai et al. 2004). Changes in cannabinoid receptor protein and mRNA levels were identified after anandamide exposure suggesting an anandamide-mediated CB1 up regulation in CRC cells. Despite the growing list of diseases that show cannabinoid receptor expression changes, relatively little is known about the mechanisms underlying this regulation. Since low basal expression levels of CB1 in human colorectal cancer cells has been attributed to methylation of the CB1 promoter and leads to enhanced tumor proliferation in animal models (Wang et al. 2008), the attention was focused on epigenetic role, which has emerged as a widespread regulation mechanism by which diseases cause long-lasting changes in gene expression by DNA and histone modifications (D'Addario et al. 2013). Histone methylation may be either activating or repressing gene expression, mainly depending on the sites of methylation (Martin and Zhang 2005). For example, K9 and K27 methylations of H3 are associated with transcriptional silencing, whereas methylations of K4, K36 and K79 of H3 have been linked to gene activation (Martin and Zhang 2005; Sims et al. 2003). Zhang et al. (2004) improved the knowledge of *CNR1* promoting regions and described three exons, localized at -212 to +140 bp from the transcriptional start

site, as candidate promoters able to confer reporter gene in cells expressing CB1 receptor. An increase in the amount of acetylated Histone H3 and H4 and a concomitant decrease in methylation at Lysine 9 and 27 of Histone H3 have been observed on exon 1 together with the activation of polymerase III and demonstrated a transcriptional regulation of CB1 through activation of its gene promoter in response to anandamide exposure. .

The promoter region of CB1 gene is known as the binding site of RARγ, responsible for the induction of CB1 expression induced by RA and 2-AG in mouse hepatocytes (Mukhopadhyay et al. 2010). In addition, the peroxisome proliferator-activated receptor gamma (PPARγ) dimerizes with retinoid receptors and binds to DNA sequences called PPARγ responsive elements, leading to the transcription activation of target genes after binding with the ligand (Ziouzenkova and Plutzky 2008). Anandamide is reported to bind directly PPARγ, activate its transcriptional activity and stimulate the differentiation of fibroblasts into adipocytes (Gasperi et al. 2007). Therefore, RXRα and PPARγ have been demonstrated as the transcription factors acting downstream anandamide induction and contributing to CB1 gene transactivation.

Each member of the endocannabinoid signaling system significantly contributes to the effectiveness of the endocannabinoid. A well-characterized enzyme, the fatty acid amide hydrolase (FAAH), has been described as responsible of anandamide degradation into arachidonic acid and ethanolamine (Giang and Cravatt 1997). A high FAAH basal expression has been identified in CRC cells, suggesting a role for the enzyme overexpression in the deregulation of the endocannabinoid metabolism in CRC tumors, confirming what already identified in prostate cancer (Endsley et al. 2008). Moreover, as a consequence of anandamide exposure, an accelerated metabolism of the endocannabinoid has been observed in CRC cells, partially depending on a concomitant increased FAAH expression. Since the protective mechanism of anandamide in CRC has demonstrated to be made incomplete through its FAAH-mediated rapid degradation, a potential approach to increase the local concentration of the endocannabinoid consists in delaying its inactivation (Di Marzo et al. 2004). Pharmacologically blocking FAAH enzyme through the selective inhibitor FI-15 has been represented as a convenient alternative to the CB1 and CB2 receptor agonists. As a consequence of increased anandamide availability, the FAAH inhibitor interfered with CRC cell proliferation in a dose- and time-dependent manner, inducing a significant cell cycle arrest in the G2/M phase. It has been reported that cell cycle progression from the G2 to M phase is regulated by activation of CDK1, whose activity is dependent upon coordination with cyclin B (Malumbres and Barbacid 2009; Lapenna and Giordano 2009). The activation of the CDK1/cyclin B complex (MPF, Maturation promoting factor) is maintained through phosphorylation at Thr161 and dephosphorylation at Thr14 and Tyr15 of CDK1 (Malumbres and

Barbacid 2009; Lapenna and Giordano 2009). Dephosphorylation of the Thr14 and Tyr15 residues in CDK1 is catalyzed by the phosphatase Cdc25C. It is thought as a rate-limiting step for G2 entry into mitosis (Malumbres and Barbacid 2009; FitzGerald et al. 2009). FI-15 has been demonstrated to induce a typical G2/M cell cycle arrest in CRC cells by regulating cyclin B1 and Cdc25C expression and CDK1 phosphorylation. Since the mitotic arrest has been reported to be induced through a G2/M checkpoint activation (Matsuoka et al. 2000), the attention has been focused on this important quality control measure that ensures the proper sequence of cell cycle events and allows cells to respond to DNA damage and to protect them from genomic instability (Bucher and Britten 2008). The G2/M DNA damage checkpoint involves the activation of ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR) proteins (Bucher and Britten 2008). Both ATM and ATR activate p53 by phosphorylation of Ser15 in response to DNA damage, which is required for inhibiting expression of the key regulators of the G2/M transition, CDK1 and cyclin B (Talos and Moll 2010). A DNA damage response signaling has been induced in CRC cells after 24- and 48 hours FAAH inhibitor exposure, as indicated through activation of ATR and its different substrates, such as Chk1. Reported evidence depict an interesting dichotomy of Chk1 functionality. On one hand, Chk1 activation by ATR results in nuclear export of Cdc25C and its subsequent cytoplasmic sequestration by 14-3-3 protein, which prevents the activation of the Cdc25C downstream target, the cyclin B/Cdc2 kinase, responsible for G2/M transition (Peng et al. 1997). On the other, Chk1 activation, together with BubR1 and Aurora B regulation, has also been required for spindle checkpoint function (Zachos et al. 2007) which monitors tension across attached kinetochores and initiates mitotic arrest in response to loss of microtubule tension (Elowe et al. 2007). A spindle checkpoint induction following FI-15 induced Chk1 activation has been demonstrated in CRC, as confirmed through BubR1 and Aurora B overexpression.

However, the sustained mitotic arrest has been demonstrated to be disrupted starting from 72 hours after FAAH inhibitor exposure, when cell cycle- and checkpoint-related factors involved were inactivated. The cell fate after mitotic arrest is controlled by two alternative pathways, one that promotes slippage out of mitosis and cellular survival from mitotic stress, the other that promotes cell death (Blagosklonny 2007). Mitotic slippage depends on cyclin B1 progressively proteolitic abilty during mitotic arrest by the same APC/C proteosome pathway that promotes normal mitotic exit when the SAC is satisfied (Brito and Rieder 2006), as well as on Bcl-x(L)/Bak ratio (Galán-Malo et al. 2012). Compared to the slippage pathway, the pathway that triggers cell death in mitotic arrest is unclear. It culminates in mitochondrial outer membrane permeabilization (MOMP) and activation of caspases (Shi et al. 2008). The two pathways are mechanistically independent and cell fate is determined by a stochastic kinetic competition

between them that results in cell-to-cell variation (Huang et al. 2010). Additionaly, Chk1 inhibition has been associated to the abolition of the spindle assembly checkpoint (SAC), premature entry in mitosis, catastrophic mitotic events, as well as induction of apoptosis (Parsels et al. 2011). Moreover, Chk1-mediated abrogation of cell-cycle checkpoint also results in potentiating the cytotoxicity of DNA-damaging drugs (Levesque et al. 2008; Tse et al. 2007; Xiao et al. 2005; Koniaras et al. 2001). In accordance with overall evidence, FI-15 has been argued to induce, after mitotic arrest, the activation of a late programmed cell death in CRC through an intrinsic apoptotic pathway.

Oxaliplatin used in combination with 5-fluorouracil (5-FU) and leucovorin (FOLFOX) for metastatic CRC therapy has led to response rates >50% with a median survival approaching 2 years (Cassidy et al. 2004) and has also been found to be very effective in the adjuvant setting (Maindrault-Goebel et al. 2004). Despite these impressive accomplishments, all metastatic CRC eventually become resistant to oxaliplatin, with a median time to progression of 8 months (Goldberg et al. 2004). Similar to oxaliplatin, 5-fluouracil resistance has also been observed in CRC patients (Zhang et al. 2008). Thus, new strategies to overcome chemotherapeutic resistance are under exploration, including combined therapies.

FAAH inhibitor, FI-15, in combination with oxaliplatin or 5-fluouracil has been argued to be effective in overcome chemoresistance in CRC. Since oxaliplatin resistance has been attributed, among others, to the overexpression of the anti-apoptotic (IAP) proteins (Arango et al. 2004), the attention was focused on the role of IAP protein survivin. Survivin have the unique properties to act both as a mitotic regulator, controlling cytokinesis, polyploidy, spindle assembly, chromatid separation and spindle-checkpoint activation on the mitotic apparatus and as a cell-death inhibitor of extrinsic/intrinsic apoptotic pathways, implicated in resistance to apoptosis (Altieri 2003). Survivin overexpression has been detected in tumors but rarely in normal adult tissues (Altieri 2003) and in particular has been associated with increased invasion and metastasis (Chu et al. 2012) and, as a consequence, with poor clinical outcome in patients with CRC (Kawasaki et al. 1998; Sarela et al. 2001). Moreover, Yie and his colleagues (2008) concluded that the detection of circulating cancer cells expressing survivin mRNA could be used to accurately identify gastric and colorectal cancer patients with high risks of relapse. Recently, it has been reported survivin as a key target for oxaliplatin (Baek 2010) since the chemotherapeutic drug induces downregulation of survivin in human colon cancer cells through p38 MAP kinase activation (Huei et al. 2010). In addition, survivin downregulation through an oncolytic adenovirus (Shen et al. 2010) or shRNA (Shen et al. 2012) has also been reported to increase 5-fluouracil cytotoxicity, respectively in colon and gastric carcinoma. Thus, downregulation of survivin has been proposed as the mechanism responsible of the synergic effect of FI-15 in combination with oxaliplatin and 5-fluorouracil.

Interestingly, the most studied potential therapeutic application of cannabinoids consists in the treatment of cancer chemotherapy side effects, including emesis, appetite inhibition, cachexia and pain (Bifulco et al. 2006). Indeed, a profound analgesic effects occurring through a direct activation of peripheral cannabinoid receptors has been recently described in experimental models of pain (Clapper et al. 2010) and a reduced neurotoxicity besides a reduced hyperalgesia induced after cisplatin treatment has been obtained through increasing AEA signaling at CB1 receptors (Khasabova et al. 2012). In addition, a peripheral endocannabinoid mechanism has also been explored: inhibiting FAAH increases the availability of endogenous AEA to activate CB1 and reduce hyperalgesia (Clapper et al. 2010) and includes a CB1-dependent neuroprotective effects such as attenuation of cytoskeletal damage (Karanian et al. 2005) and reduction of hippocampal neuronal activity in models of excitotoxicity (Coomber et al. 2008). Since, as other chemotherapeutics, oxaliplatin induces chronic painful peripheral neuropathy (Xiao et al. 2012), FI-15 could be a valid tool not only to overcome oxaliplatin resistance, but also to target oxaliplatin-induced side effects.

6. CONCLUSION

A deregulation of the endocannabinoid system occurs in colorectal cancer, leading to the need of a deeper characterization of the functional meaning of these changes. In this dissertation, I have provided strong evidence for a key tumor-suppressor action of the endocannabinoids and in particular of CB1 receptor signaling in the control of CRC growth. The ability of a selective CB1 agonist, 2-Methyl-2-fluoro anandamide (Met-F-AEA), a stable analogue of the natural endocannabinoid anandamide, to induce up regulation of CB1 receptor expression through an epigenetic mechanism acting on *CNR1* gene promoter and a significant anti-proliferative action in CRC cells, allows to explore the possibility of a direct targeting of endocannabinoid system for CRC treatment. Since this protective mechanism is made incomplete because of the rapid degradation of anandamide, an indirect approach to maintain a local endocannabinoid tone by targeting its inactivation through the degradative enzyme fatty acid amide hydrolase has proven effective, suggesting FAAH as a novel target for the development of anti-cancer drugs. Moreover, the FAAH inhibition associated with chemotherapeutics normally utilized in CRC treatment could represent a valid strategy to overcome drug resistance and side effects.

7. ACKNOWLEDGMENTS

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Attached manuscript II

Gangemi G *et al*.
Targeting the isoprenoid pathway in colorectal cancer:
a novel role for N6-isopentenyladenosine. *Manuscript in preparation*.

Targeting the isoprenoid pathway in colorectal cancer: a novel role for N6-isopentenyladenosine

Department of Pharmaceutical and Biomedical Sciences, University of Salerno, 84084 Fisciano (SA), Italy.

Department of Medicine and Surgery, University of Salerno, Via S. Allende, 1 - 84081 - Baronissi (SA), Italy.

ABSTRACT

Farnesyl diphosphate synthase (FDPS), a key enzyme of the mevalonate (MVA) pathway, has been demonstrated to promote colorectal cancer (CRC) development. FDPS regulation has been investigated in a CRC *in vitro* model through a N6-Isopentenyladenosine (i6A), a member of cytokinin family of hormones that regulate plant cell growth and differentiation. FDPS downregulation induced by i6A exposure has been associated to reduced CRC cell proliferation and deregulation of cell cycle. The molecular mechanisms underlying i6A-FDPS-regulation and i6A-direct interactions have been evaluated through a chemical proteomic approach. It has made possible to isolate and identify as a i6A direct partner H2B, whose post-translational modifications have been investigated. i6A increased the monoubiquitination of H2B, and, as a consequence, H3 methylation since a cross-talk responsible of chromatin boundary integrity maintenance between histones exist and suggest a transcriptional regulation acting on FDPS gene promoter. In addition, to characterize FDPS involvement in specific biological processes, i6A protein targets have been identified.

1. INTRODUCTION

N^6-Isopentenyladenosine (i^6A) is a member of cytokinin family of hormones that regulate plant cell growth and differentiation (Bifulco et al. 2008). i6A is present also in mammalian cells both as modified nucleoside (Faust et al. 1991) derived from isopentenylation of adenosine at position 37 (Persson et al. 1994) or selenocysteine (Sec) tRNA-bound, playing a major role in post-transcriptional processes. i6A have been demonstrated to exert potent *in vitro* anti-tumour activity on human epithelial cancer cell lines derived from different types of tumours (Spinola et al. 2007), including breast (Laezza et al. 2010) and Ehrlich carcinoma (Adair and Brennan, 1986) and leukemia (Ishii et al. 2002), in the latter case leading the way for a potential clinical application (Mittelman et al. 1975). Several i6A mechanisms of action have been hypothesized, including inhibition of both cell proliferation (Spinola et al. 2007) and protein prenylation (Laezza et al. 2006), induction of apoptosis (Ishii et al. 2002; Meisel et al. 1998), inhibition of DNA, RNA and/or protein synthesis (Burns et al. 1976; Gallo et al. 1969) and nucleoside transport (Hare and Hacker 1972; Hakala et al. 1975) through modulation of several transcription, cell cycle and apoptosis-related genes (Colombo et al. 2009).

Farnesyl diphosphate synthase (FPPS) was reported to catalyze the formation of the isoprenoid farnesyl pyrophosphate (FPP) from 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) during mevalonate metabolism (Szkopińska and Płochocka 2005). Increased FPPS activity and expression have been described in several tumors (Pilarsky et al. 2004), including colorectal cancer (Notarnicola et al. 2004). iPA anti-tumor activity identified in thyroid cells FTRL-5and in an *in vivo* model of athymic mice have been attributed to the enzyme farnesyl diphosphate synthase inhibition and then, as a consequence, to deregulation of the mevalonate pathway (Laezza et al. 2006).

Here, we evaluated the functional role of i6A in an *in vitro* model of CRC, focusing on FDPS involvement. Moreover, identification and validation of i6A-protein interactors and targets have been performed through a proteomic approach.

2. MATHERIALS AND METHODS

2.1 Chemicals

i6A (Sigma–Aldrich, Inc., St. Louis, MO) was dissolved in dimethylsulfoxide (DMSO) to a final concentration of 10 mM and stored at -20°C.

2.2 Cell Culture

Human colorectal cancer cell line DLD-1 (D.L. Dexter et al., 1979; Interlab Cell Line Collection ICLC no. HTL95011) derived from a colorectal adenocarcinoma of type C according to Dukes classification, was grown in RPMI 1640 medium (Lonza, Belgium) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (P/S; Euroclone) at 37°C in a humidified 5% CO_2 air atmosphere. Human colorectal cancer cell line SW620 (A. Leibovitz, et al., 1976; ATCC no. CCL-227™) derived from a lymph node metastatic site of a colorectal adenocarcinoma, was grown in Dulbecco's Modified Eagle's Medium (Lonza) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (P/S; Euroclone) at 37°C in a humidified 5% CO_2 air atmosphere. The colorectal cancer cell line HCT-116 (MG Brattain et al., 1982; ICLC no HTL95025) derived from a colorectal adenocarcinoma was cultured in McCoy's modified medium (Sigma Aldrich, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (Invitrogen,

Carlsbad, CA), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (P/S; Euroclone) at 37°C in a humidified 5% CO_2 air atmosphere.

2.3 Immunoblotting assay

For protein extraction, CRC cells were washed twice in ice-cold phospate-buffered saline (PBS) and lysed in buffer A (50 mM Tris-HCl pH 8.0 buffer containing 150 mM NaCl, 1% Nonidet P-40, 2 mg/ml aprotinin, 1 mg/ml pepstatin, 2 mg/ml leupeptin, 1 mM Na_3VO_4). Protein concentration was determined by the Bradford assay using bovine serum albumin as standard. Cell lysates were subjected to 10% SDS-PAGE. Gels were electroblotted into nitrocellulose membranes (Millipore Co., Bedford, MA) and filters were probed with the indicated primary antibodies: anti-H2Bub, anti-H2B, anti-di-methyl H3K4, anti-tri-methyl H3K4, anti-BMP4, anti-PMSC6, anti-phospho cofilin, anti-cofilin, anti-GNB1, all purchased by AbCam, anti-FDPS (Epitomics) and anti-β-actin (Sigma, St. Louis, MO). Proteins were visualized with peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence system (Amersham-Pharmacia Biosciences LTD, Uppsala, Sweden).

2.4 RNA extraction and Reverse-transcriptase polymerase chain reaction

Total RNA was extracted from cell lines by guanidinium thiocyanate isopropanol method (Chomczynski, 1987). To measure mRNA expression, reverse transcription (RT) was performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen, San Giuliano Milanese, Milan, Italy) and random oligonucleotide primers. The first-strand cDNA was amplified using the sense primer FDPS-F (5-TGCTGCGTTTTACTAAACGTG-3) and the anti-sense primer FDPS-R (5-CAATGGAAGGGCTGAAGTC-3). The primers used to amplify the

β2-microglobulin were the sense β2M1 (5-CCTGGATTGCTATGTGTCTGGGTTTCATCC-3) and the anti-sense β2M2 (5-GGAGCAACCTGCTCAGATAC-ATCAAACATG-3). The generated cDNAs were amplified by PCR, using the correspondent primers. After amplification, agarose gel electrophoresis was used to detect the expression of the genes.

2.5 Chromatin immunoprecipitation (ChIP)

For chromatin immunoprecipitation (ChIP), DLD1 cells were grown to 95% confluence in phenol red-free DMEM supplemented with 10% charcoal dextran-stripped fetal bovine serum for at least 3 days. ChIP assay was performed according to a modified version of the protocol previously reported by Villa et al. (2007). Briefly, following the addition of anadamide at various time points, cells were cross-linked with 0.8% of formaldehyde at room temperature for 6 minutes, and the reaction was stopped by the addition of glycine (0.125M). Cells were rinsed twice with cold PBS, resuspended in lysis buffer (1% SDS, 10 mM EDTA pH 8, 50 mM Tris HCl pH 8, PI) and sonicated. Lysates were diluted 10 times with the IP buffer (1%TritonX-100, 150 mM NaCl, 2 mM EDTA pH 8, 20 mM Tris HCl pH 8, PI) and then incubated overnight with 5 mg of each antibody. 40 microliters of protein A sepharose beads saturated with salmon sperm (Upstate, Billerica, MA) were added to the lysates for 2 h and then washed four times with Wash buffer 1 (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA pH 8, 20mM Tris HCl pH 8, PI) and once with Wash buffer 2 (1% Triton X-100, 500 mM NaCl, 2 mM EDTA pH 8, 20 mM Tris HCl pH 8, PI). The immunoprecipitated DNA was analyzed by PCR. We used the sense primer prFDPS-F: 5- TGCTGCGTTTTACTAAACGTG-3 and the anti-sense primer prFDPS-R: 5- CAATGGAAGGGCTGAAGTC -3. The specific antibodies anti-H3, anti-di-methyl-H3K4, anti-tri-methyl-H3K4 were purchased from Abcam.

2.6 Cell proliferation ELISA

Cell proliferation was evaluated, *in vitro*, by measuring BrdU incorporation during DNA synthesis through a colorimetric ELISA kit (Roche Diagnostics GmbH). In brief, DLD-1 cells were seeded in triplicate into 96-well plates. Cells (1×10^4/ 0.32 cm plate) were treated with the drugs and incubated for 24 or 48 hours. According to the protocol provided by the manufacturer, at the end of treatment, cells were first fixed with a denaturation solution for 30 minutes and then incubated with anti-BrdU antibody peroxidase conjugate solution (anti-BrdU-POD) for about 4 hours followed by incubation with substrate solution for 20 minutes. The colorimetric reaction was measured through a microplate reader (Molecular Devices) at 370 nm. The blank was performed in each experimental setup. The absorbance value of blank was subtracted from other experimental values and cell proliferation was expressed as the percentage of absorbance values of treated samples to untreated controls.

2.7 FACS analysis

To assess cell cycle progression, after treatment with vehicle or compound i6A for 24 hours, DLD1 cells (250 000 per 6 cm plate) were resuspended with Trypsin-EDTA, washed once in ice-cold phospate-buffered saline (PBS), fixed in 70% ethanol and resuspended in 1 ml of PBS containing 1mg/ml RNase (Roche) and 50 µg/ml propidium iodide (Sigma). After 4 hours of incubation at room temperature, propidium iodide incorporation in DLD1 cells was analyzed by a FACSCalibur flow cytometer (Becton Dickinson).

2.8 Statistical analysis

Statistical differences between the treatments and the control were evaluated by one-way analysis of variance (ANOVA). In the case of a significant result in the ANOVA, Student's t test was performed for all *in vitro* experiments. A p value less than 0.05 was considered statistically significant.

3. RESULTS

3.1 i6A-induced modulation of FDPS expression in CRC cells.

The levels of expression of FDPS after treatment of CRC cells with i6A were determined. To this aim, DLD1 cells were treated with i6A (10 μM) for increased times and FDPS expression levels were measured through Western blot analysis. As observed in Figure 1, FDPS protein expression was increased of about 40 % in i6A-treated CRC cells starting from 2 hours compared to control cells, treated only with vehicle. The increase was persistent until 24 hours of treatment when a marked downregulation has been observed (Figure 1).

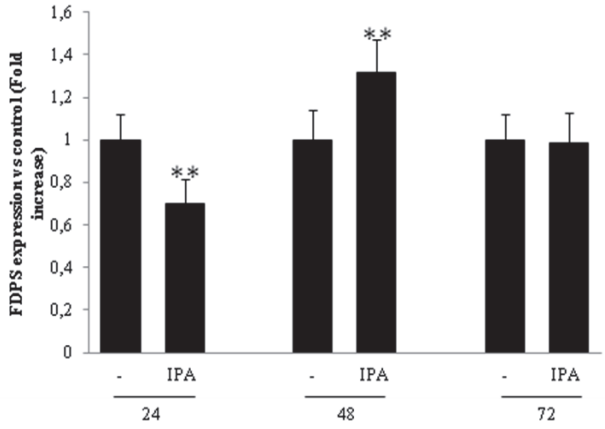

Figure 1. i6A-induced modulation of FDPS expression in CRC cells.
Histograms show densitometric analysis of FDPS protein expression, reported as fold increase *vs* control (mean± SD of 3 independent experiments) (ANOVA *vs* control, **p< .01).

3.2 Transcriptional mechanism is involved in FDPS modulation after i6A exposure

Incubation of CRC cells with i6A (10 μM) for different times resulted also in a drastic increase of messenger RNA (mRNA) for the FDPS gene after 2 hours, as evaluated by RT-PCR, suggesting that i6A-induced FDPS protein expression was consistent with increased levels of the corresponding FDPS mRNA (Figure 2). The increase is persistent until 12 hours of treatment when a marked downregulation has been observed. FDPS mRNA underwent a subsequent up regulation until 48 hours, when mRNA levels of treated cells were comparable to the control.

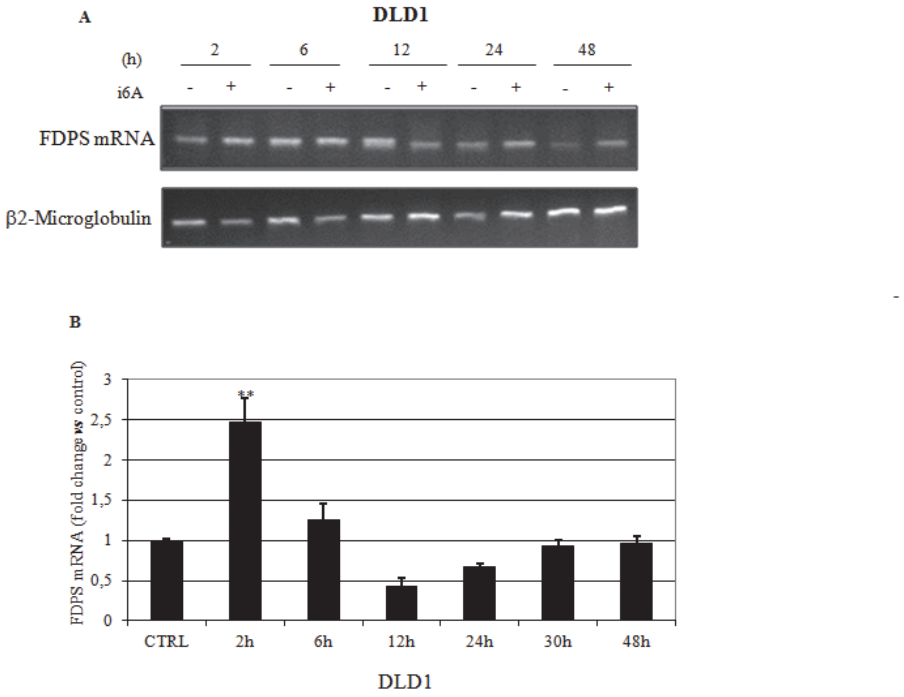

Figure 2. Transcriptional mechanism is involved in modulation of FDPS protein.
(A) cDNA from DLD1 cells, left untreated (-) or treated with i6A (10 μM) for the indicated incubation times, was analyzed for FDPS through RT-PCR. To confirm equal loading, mRNA expression level was normalized for the housekeeping gene β2-microglobulin. Results shown are representative of at least three independent experiments. *(B)* Histograms show densitometric analysis of FDPS mRNA expression, reported as percentage of control (mean± SD of 3 independent experiments) (ANOVA *vs* control, **p< .01).

3.3 FDPS downregulation affects CRC proliferation through cell cycle deregulation

In order to determine the role of FDPS into the control of CRC tumor growth, the ability of a iPA to induce apoptosis in human CRC cell lines was examined. As observed, the exogenous administration of increasing doses of the isoprenoid for 24 and 48 hours significantly inhibited DLD1 proliferation in a dose- and time-dependent manner, as evaluated by BrdU ELISA incorporation assay. In order to investigate whether i6A-mediated CRC inhibition may occur through cell cycle deregulation in CRC cells, flow cytometry was carried out. FACScan analysis of propidium iodide (PI)-stained DLD-1 cells revealed a strong accumulation of CRC population in a pre-G1 phase (Figure 3). This cellular accumulation occurred after 24 hours of treatment, reaching about 20% compared to the DMSO control. Accordingly, the percentage of cells in G0/G1 and S phase decreased of about 50%.

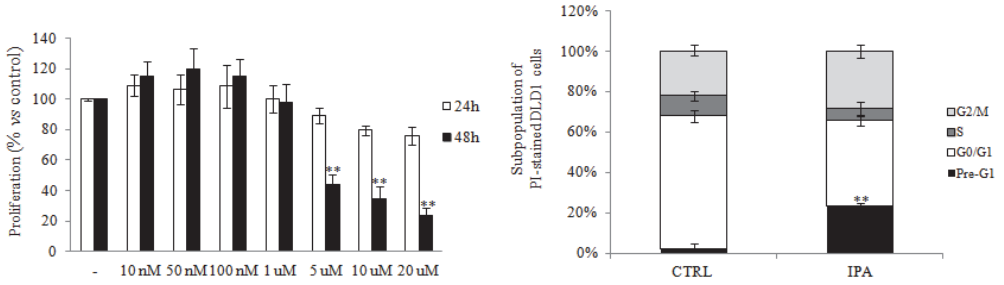

Figure 3. FDPS downregulation affects CRC proliferation through cell cycle dysregulation. *(A)* DLD1 cells (1 x 10^4/ 0.32 cm plate) were incubated with increasing doses of i6A for 24 and 48 hours. Cell proliferation was evaluated by measuring BrdU incorporation during DNA synthesis through a colorimetric ELISA assay. Results are shown as the mean ± SD from triplicate cultures. *(B)* Changes in the percentage of cells in G0/G1, S and G2/M phases after treatment of DLD1 cells with FI-15 (1 μM) for 24 and 48 hours are normalized to the cell cycle distribution of exponentially growing untreated control cells. Each value is the mean ± SD of 3 separate experiments performed in duplicates (**p<0.01).

3.4 Identification and validation of i6A interactors

Proteomic screening through fishing for partners gel free approach was used in order to identify potential molecular interactors of i6A, underlying cellular function of the isoprenoid in CRC. To this aim, DLD1 cells were exposed for 2 hours to the vehicle or to i6A (10 μM). Since previous reports suggested the existence of potential targets of the isoprenoid also in the nucleus (Mehdi et al. 2010), total protein extract obtained from CRC cells was fractionated and cytosolic and nuclear fractions were used for following experiments. As a result, by means of binding studies to iPA-biotinylated, purification of the interactors and subsequent sequence analysis of databases, some proteins potentially able to bind biotinylated IPA were identified and summarized in Table 1. The attention was focused on H2B protein, since it was the only protein identified both in cytosolic and nuclear extracts and that some of the other proteins identified (including nucleophosmin and histone H1.2) are direct partners of H2B.

A

Swiss Prot code	Identified protein	Peptides
H2B1B_HUMAN	Histone H2B type I-B	5
ACTA_HUMAN	Actin, aortic smooth muscle	2
ENOA_HUMAN	Alpha-enolase	3
G6PI_HUMAN	Glucose-6-phosphate isomerase	1

Swiss Prot code	Identified protein	Peptides
H2B1B_HUMAN	Histone H2B type I-B	6
H12_HUMAN	Histone H1.2	11
NPM_HUMAN	Nucleophosmin	2
THOC4_HUMAN	THO complex subunit 4	1
HNRPR_HUMAN	Heterogeneous nuclear ribonucleoprotein R	3

B

Table 1. *(A)* List of proteins identified in nuclear extracts of DLD1 treated cells. *(B)* List of proteins identified in cytosolic fraction of DLD1 treated cells.

3.5 Effect on H2B monoubiquitination

Since H2B is modified *in vivo* by the attachment of ubiquitin (West and Bonner 1980), H2Bub levels were determined through Western Blot analysis after i6A (10μM) exposure of DLD1 cells for increasing times from 0.5 to 5 hours. iPA increased the amount of mono-ubiquitinated histone H2B compared to total H2B expression from 0.5 hours (Figure 4A) until 24 hours when a drastic reduction occurred (Figure 5). The effect was independent from the human CRC cell line used since a similar effect was observed also in SW620 and HCT-116 cells (Figure 4B, 4C, 5B).

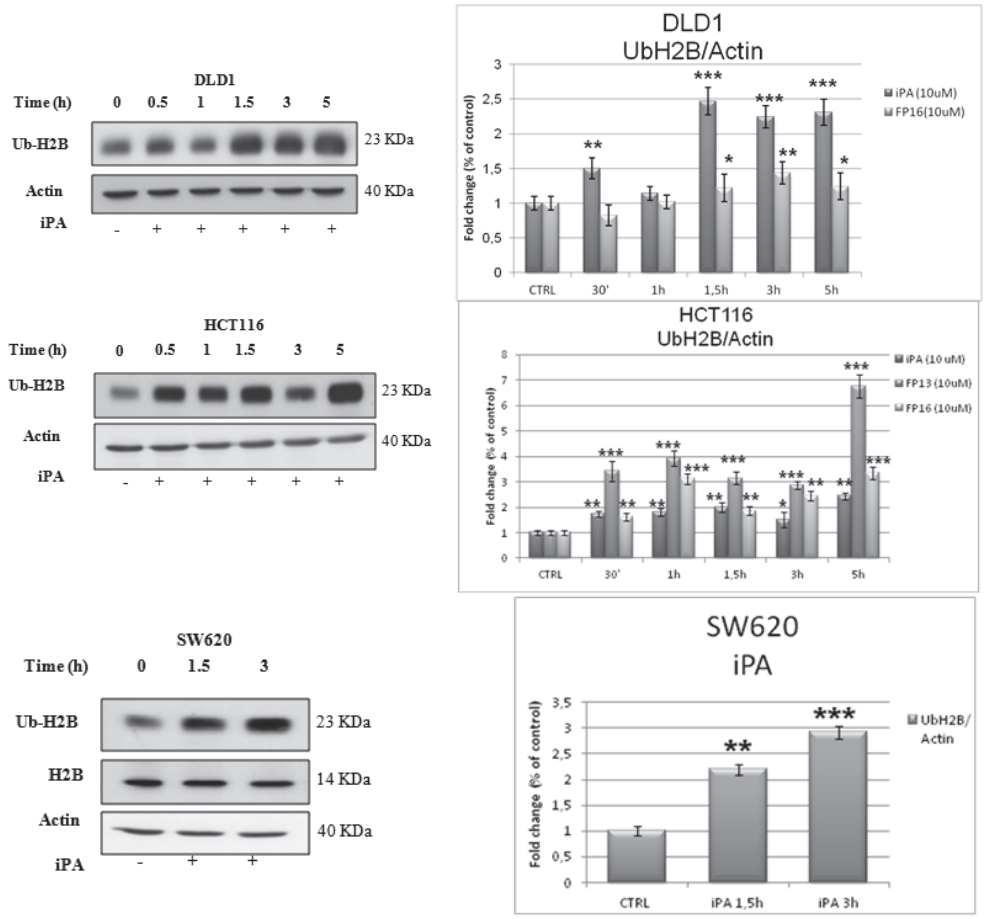

Figure 4. H2Bub protein upregulation induced by i6A in human CRC cells.
(A,B,C) Lysates from SW620, DLD1 and HCT-116 cells, left untreated (-) or treated with i6A (10 μM) for the indicated incubation times, were immunoblotted with anti-H2Bub antibody. To confirm equal loading, the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments. (*D, E, F)* Histograms show densitometric analysis of CB1 protein expression, reported as percentage of control (mean± SD of 3 independent experiments) (ANOVA *vs* control, **p< .01).

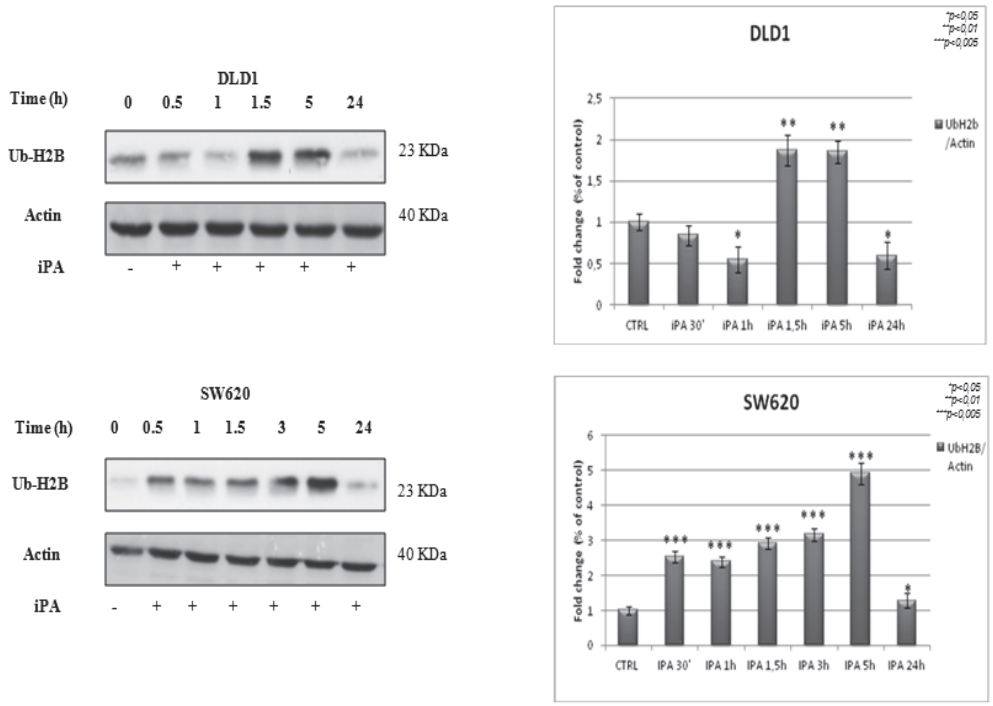

Figure 5. H2Bub protein downregulation induced by i6A in human CRC cells for prolonged times.
(A,B) Lysates from DLD1 and SW620 cells, left untreated (-) or treated with i6A (10 μM) for the indicated incubation times, were immunoblotted with anti-H2Bub antibody. To confirm equal loading, the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments. *(C,D)* Histograms show densitometric analysis of H2Bub protein expression, reported as percentage of control (mean± SD of 3 independent experiments) (ANOVA *vs* control, **p< .01).

3.6 H2B-H3 crosstalk on FDPS promoter gene

Several studies reported that H2B monoubiquitination is a prerequisite for Lys-4 H3 and Lys-79 H3 methylation (Weake and Workman 2008) on the transcribed region of highly expressed genes in human cells (Minsky et al. 2008). In order to evaluate whether the H2Bub observed after i6A-esposure was able to directly affect H3 methylation status on FDPS promoter gene, immunoprecipitation assays (ChIP) in DLD1 cells were performed. As shown in Figure 6, since after 1.5 and until 5.5 hours of treatment with i6A, a strong

increase in the amount of di-methyl K4 Histone H3 was observed, in conjunction with a little increase in tri-methyl K4 Histone H3 after 1.5 hours of i6A exposure.

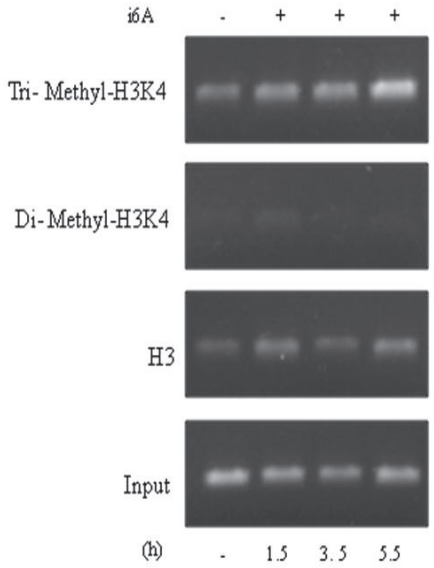

Figure 6. H2B-H3 crosstalk on FDPS promoter gene
Chromatin from DLD1 cells, left untreated (-) or treated with i6A (10 μM) for the indicated incubation times (1.5, 3.5, 5.5 hours), was analyzed through ChIP assays with the indicated specific monoclonal antibodies. The input confirms the comparable strength of the primer pairs specific for promoter region. As a positive control, non-precipitated input DNA, from both treated and untreated cells, was used as a template for PCR. As a negative control, IgG antibodies did not immunoprecipitate the *FDPS* promoter region and gave no PCR product. Results shown are representative of at least three independent experiments.

The i6A-induced modifications of H3 methylation status were consistent with histone H3 protein expression alterations in DLD1 cells exposed to i6A for increased times from 0.5 hours to 24 hours, as assessed through Western Blot analysis. The increment of both Di-methyl H3K4 and Tri-Methyl H3K4 was transiently, evident only at 0.5 and 1 hour. Consistent with a diminished H2Bub at 24 hours, H3 methylation tended to reduce for prolonged times (Figure 7).

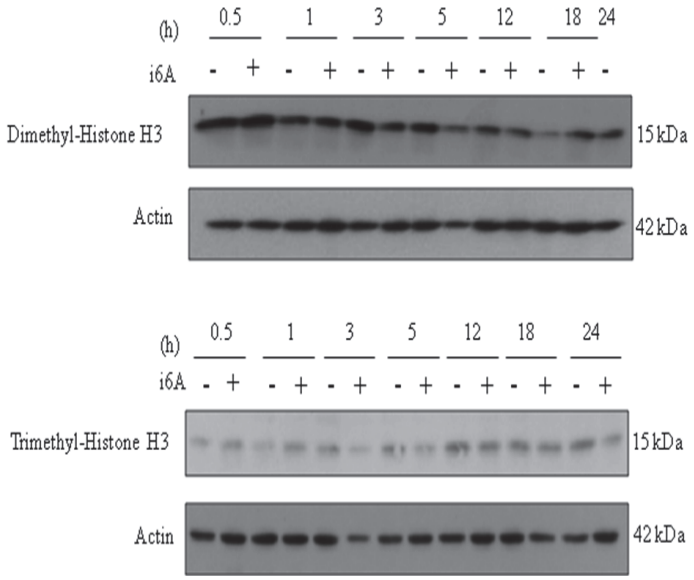

Figure 7. i6A-induced H3 modifications.
Lysates from DLD1 cells, left untreated (-) or treated with i6A (10 μM) for increasing times (0.5-24 hours) were immunoblotted with the indicated antibodies. To confirm equal loading, the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments.

3.7 Identification of i6A target genes

In order to identify i6A target genes and exploit FDPS involvement in CRC biological processes, DLD1 cells were treated for 2 hours with i6A and protein extract collected was subjected to a proteomic approach. Results summarized in Table 2 described proteins identified as proteic targets upregulated or downregulated by IPA and a fold change was obtained comparing i6A-treated cells *versus* control.

Protein	Fold Change (i6A/CTRL)
Thymidylate synthase	2.68
26S protease regulatory subunit 10B	2.68
Cofilin-1	2.34
Heterogeneous nuclear ribonucleoproteins C1/C2 O	1.99
Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	1.91
Poly(rC)-binding protein 1	1.85
Glyceraldehyde-3-phosphate dehydrogenase	1.75
Axin interactor, dorsalization-associated protein	1.74
DNA-directed RNA polymerases I, II, and III subunit RPABC1	1.64
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	-1.60
Bone morphogenetic protein 4	-2.24
Ankyrin repeat and SOCS box protein 2	-1.97
RNA-binding protein 4	-1.96
Follistatin-related protein 5	-1.95

Among these, we further selected 2 proteins upregulated and 2 downregulated for further validation. The proteins upregulated further studied were cofilin-1 and PMSC6 (26S protease regulatory subunit 10B); the downregulated BMP-4 (Bone morphogenetic protein 4) and GNB1 (Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1). The expression levels of these proteins were evaluated through Western Blot analysis in the same experimental conditions. As shown in Figure 8, previous data were confirmed.

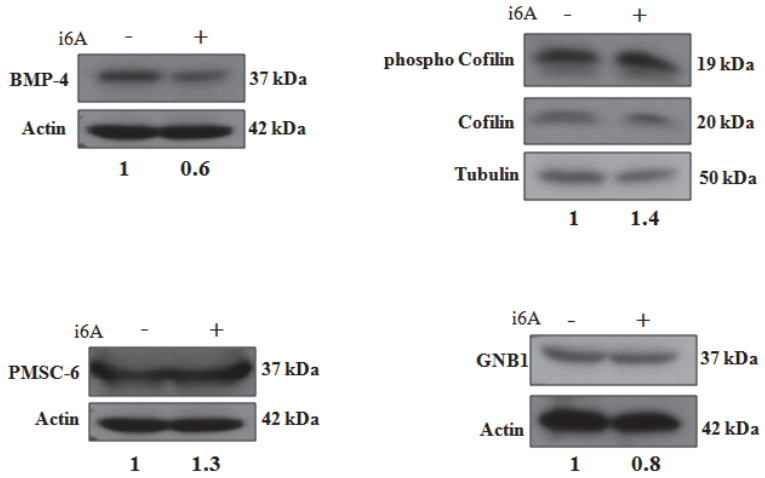

Figure 8. i6A target genes.
Lysates from DLD1 cells, left untreated (-) or treated with i6A (10 μM) for 2 hours were immunoblotted with the indicated antibodies. To confirm equal loading, the filters were stripped and reprobed with anti β-actin antibodies, as indicated. Blots shown are representative of at least three independent experiments.

4. DISCUSSION

The functional involvement of lipid metabolism in carcinogenesis has been widely explored (Zhang and Du, 2012). Deregulation of the mevalonate (MVA) pathway, paced by its rate-limiting enzyme, hydroxymethylglutaryl coenzyme A reductase (HMGCR), required for the generation of several fundamental end-products including cholesterol and isoprenoids, has been demonstrated to promote transformation (Clendening et al. 2010). Several enzymes of lipid metabolism are involved in colorectal cancer development (Notarnicola et al. 2012), including farnesyl diphosphate synthase (FDPS) (Dhar et al. 2012). A first evidence of FDPS activity was demonstrated in human CRC, with a higher FDPS activity and mRNA expression in cancer compared to normal mucosa (Notarnicola et al. 2004). In this study, the role of N^6-Isopentenyladenosine (i^6A), a member of cytokinin family of hormones that regulate plant cell growth and differentiation, in FDPS modulation has been investigated in a CRC *in vitro* model. I6A was able to regulate FDPS

expression both at protein and mRNA level. Moreover, FDPS downregulation observed after 24 hours of i6A exposure was associated to reduced CRC cell proliferation and deregulation of cell cycle. In order to dissect the molecular mechanisms underlying i6A-FDPS-regulation and to determine i6A-direct partners, studies of interaction of the molecule have been performed through a chemical proteomic approach. It has made possible to isolate and identify potential protein interaction targets of i6A from a total protein extract derived from treated cells. Among others, H2B has been isolated and identified as i6A direct partner. H2B is an essential component of the nucleosome, therefore compacting DNA together with the other histones, thus limiting their access to all those machines that require cellular DNA as template and therefore playing a central role in the regulation of transcription, DNA replication and repair (Chandrasekharan et al. 2010). Post-translational histone modifications are one of the mechanisms employed by cellular processes to remodel chromatin and gain access to the underlying DNA template (van Attikum et al. 2009). Histones undergo a wide variety of reversible covalent modifications, including arginine (R) methylation, serine/threonine phosphorylation and several types of modifications on their lysine (K) residues (acetylation, methylation, ubiquitination, sumoylation and ADP-ribosylation) (Campos et al. 2009). Histone 2B can be modified by the attachment of ubiquitin (West and Bonner 1980) through the H2B ligase RNF20 (Jääskeläinen et al. 2012). iPA increased the amount of mono-ubiquitinated histone H2B compared to total H2B expression until 5 hours when a drastic reduction occurred. The mono-ubiquitination of H2B is broadly recognized as a mark of transcriptional activity (Weake and Workman 2008). Indeed, H2B monoubiquitination (H2Bub1) has been demonstrated to cause an increase in H3K4 and -K79 methylation, through the activation of H3K4 methyltransferase activity of the SET1 complex (Wu et al. 2008), thus revealing the existence of a histone “cross-talk” responsible of chromatin boundary integrity maintenance.

According to these reports, the ability of i6A to affect H3 methylation was investigated. Consisting with increased H2Bub amount, di-methyl K4 Histone H3 and tri-methyl K4 Histone H3 levels increased for short times in i6A-treated cells compared to control. Since H2Bub1 is enriched in highly expressed genes throughout yeast and mammalian genomes, requiring many factors involved in the early steps of transcription elongation (Minsky et al. 2008), H3 methylation status of FDPS promoter was examined. As a consequence of H2Bub, di-methyl K4 Histone H3 and tri-methyl K4 Histone H3 were increased on FDPS promoter, suggesting a transcriptional regulation of the gene through histone modifications on its promoter. To better characterize the protein targets of i6A and, as a consequence, biological functions in which i6A was involved, a proteomich approach was performed. Several proteins have been reported upregulated or downregulated after i6A exposure and validation of selected ones have been carried out. These results overall underline a novel role for FDPS enzyme in CRC, suggesting isoprenoid derivative i6A as a potent tool to modulate its expression and its transcriptional regulation and to characterize its involvement in specific biological processes.

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Attached manuscript III

Malfitano AM, Ciaglia E, **Gangemi G**, Gazzerro P, Laezza C, Bifulco M.
Update on the endocannabinoid system as an anticancer target.
Expert Opin Ther Targets 2011;15(3):297-308.

Expert Opinion

Update on the endocannabinoid system as an anticancer target

Anna Maria Malfitano, Elena Ciaglia, Giuseppina Gangemi, Patrizia Gazzerro, Chiara Laezza & Maurizio Bifulco[†]
[†]*University of Salerno, Department of Pharmaceutical Sciences, Fisciano, Salerno, Italy*

Introduction: Recent studies have shown that the endocannabinoid system (ECS) could offer an attractive antitumor target. Numerous findings suggest the involvement of this system (constituted mainly by cannabinoid receptors, endogenous compounds and the enzymes for their synthesis and degradation) in cancer cell growth *in vitro* and *in vivo.*

Areas covered: This review covers literature from the past decade which highlights the potential of targeting the ECS for cancer treatment. In particular, the levels of endocannabinoids and the expression of their receptors in several types of cancer are discussed, along with the signaling pathways involved in the endocannabinoid antitumor effects. Furthermore, the beneficial and adverse effects of old and novel compounds in clinical use are discussed.

Expert opinion: One direction that should be pursued in antitumor therapy is to select compounds with reduced psychoactivity. This is known to be connected to the CB1 receptor; thus, targeting the CB2 receptor is a popular objective. CB1 receptors could be maintained as a target to design new compounds, and mixed CB1–CB2 ligands could be effective if they are able to not cross the BBB. Furthermore, targeting the ECS with agents that activate cannabinoid receptors or inhibitors of endogenous degrading systems such as fatty acid amide hydrolase inhibitors may have relevant therapeutic impact on tumor growth. Additional studies into the downstream consequences of endocannabinoid treatment are required and may illuminate other potential therapeutic targets.

Keywords: cancer, cannabinoid receptors, endocannabinoid system, endocannabinoids

Expert Opin. Ther. Targets [Early Online]

1. Introduction

The discovery of the endocannabinoid system (ECS) has initiated a novel field of pharmacological studies to reveal its therapeutic potential in several pathological conditions including cancer. The ECS consists of cannabinoid receptors, their endogenous ligands (endocannabinoids) and proteins for their synthesis and inactivation [1]. Two definitive cannabinoid receptors have been identified [1], CB1 mainly located in the CNS [2-4] and CB2 expressed by immune cells [5], although found also in rat [6] and human brain, where it was demonstrated to occur under pathological conditions [7,8]. More recently, the orphan receptor GPR55 was shown to function as a novel cannabinoid receptor [9] that might play a physiological role in lipid or vascular biology [10]. Cannabinoid receptors modulate ion channels [5] and several pathways involved in the control of cell proliferation and survival [11-13]. Endocannabinoids are released 'on demand' in response to diverse physiological and pathological stimuli [14]. The best known are anandamide (AEA) and 2-arachidonoylglycerol (2-AG). However, a group of *N*-acylethanolamides (oleylethanolamide, palmitoylethanolamide, homo-γ-linoleylethanolamide and docosatetraenylethanolamide) is also known. Some of these compounds behave as 'entourage compounds' regulating endocannabinoid functions through unknown

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mechanisms [15]. AEA has been implicated in several pathological processes by acting as a CB1/CB2 agonist and activating the GPR55 receptor [9]. Complex enzymatic cascades regulate endocannabinoid production and inactivation [1]; all the enzymes involved in these pathways are potential targets for pharmacological intervention in a wide range of diseases including cancer. Recent findings show that endocannabinoids affect cancer cell viability and invasiveness modulating the activity of proteins and nuclear factors involved in cell proliferation, differentiation and apoptosis [16,17]. Numerous plant-derived (tetrahydrocannabinol (THC), cannabidiol (CBD)), synthetic (WIN-55, 212 – 2, HU-210) and endogenous cannabinoids control cancer cell growth and death [18-21]. More importantly, cannabinoid administration to nude mice curbs the growth of various types of tumor xenografts, including lung carcinoma [22] glioma [23], thyroid epitelioma [24], lymphoma [25], skin carcinoma [26], pancreatic carcinoma [27] and melanoma [28]. Moreover, cannabinoids appear selective antitumor agents with good safety profile as suggested by a pilot clinical trial on patients with glioblastoma multiforme [29]. The aim of this review is to update the current knowledge of the ECS in cancer and highlight very recent findings on its modulation in several tumors, considered novel potential targets for drug development in cancer therapy.

2. Body

2.1 Endocannabinoid levels in cancer

The ECS is highly conserved among species and shows modulating activity on proteins and nuclear factors involved in cell proliferation, differentiation and survival. Endocannabinoid levels are finely modulated under physiological and pathological conditions. A transient increment appears to be an adaptive reaction to restore cell homeostasis when this is acutely perturbed. For instance, high AEA concentrations were found in placenta, umbilical vein and plasma from maternal circulation for pregnancy maintenance and parturition [30]. However, in some chronic conditions, the alteration of the ECS seems to contribute to the progress and symptoms of neurodegenerative diseases [31]. Elevated levels of endocannabinoids have been reported in several tumors compared to the respective healthy tissue, for example, in glioblastoma, meningioma [32], pituitary adenoma [33], prostate [34], colon carcinoma and endometrial sarcoma [35-37] (**Table 1**). Both AEA and 2-AG are increased in human colorectal adenomatous polyps and carcinomas compared to normal colorectal mucosa [35]. AEA levels were enhanced in glioblastomas while 2-AG levels were increased in meningiomas when compared with human non-tumor brain tissue [32]. However, the levels of endocannabinoids were found to differ depending on the grade of tumor [36]. Compared with normal tissues, AEA levels remained unchanged in pancreatic ductal carcinoma pancreas [38], ileum lymphoma and bladder carcinoma while it decreased in stomach carcinoma [36]. Mass spectrometry analysis of endometrial carcinoma extracts showed significant increase of 2-AG levels in comparison with samples from healthy patients [39].

2.2 Endocannabinoid degrading system and cancer

A local endocannabinoid tone is an important factor to control the malignancy of different cancer cells. Thus, modulation of the levels of endocannabinoids, by use of inhibitors of endocannabinoid synthesis or metabolism, results in a change in the invasive properties of cancer cells in a manner consistent with a protective effect of endocannabinoids.

After synthesis, AEA is rapidly hydrolyzed and degraded by the enzyme fatty acid amide hydrolase (FAAH) [40] while monoacylglyceride lipase (MAGL) is the hydrolytic enzyme which degrades the 2-AG (**Figure 1**). MAGL has been recently found highly expressed in aggressive human cancer cells and primary tumors, including melanoma, ovarian and breast cancer, where it regulates a lipid network enriched in protumorigenic signaling molecules [41]. A correlation between FAAH and cancer has been primarily investigated in prostate adenocarcinomas, where tumor epithelial FAAH–immunoreactivity has been recently associated with prostate cancer severity and outcome [42,43] and in pancreatic ductal adenocarcinomas where a correlation of high FAAH/MAGL levels and survival has been observed [38]. Therefore, blocking the degradation pathway and in particular the FAAH activity by specific inhibitors may enhance the anti-proliferative effects of AEA and have beneficial effects in cancer treatment. Indeed, treatment of human breast cancer cells *in vitro* with palmitoylethanolamide reduced up to 30 – 40% FAAH expression thereby allowing the accumulation of AEA and increasing its anti-proliferative effects [44].

In addition, treatment of athymic mice with thyroid tumor xenografts with VDM-11 (a selective inhibitor of endocannabinoid cellular reuptake) or arachidonyl–5-HT (a selective blocker of endocannabinoid hydrolysis) increased the intra-tumor levels of AEA and significantly decreased tumor volume [19]. Of note, FAAH pharmacological inhibition or siRNA knockdown decreases prostate cancer cell invasion [43]. Inhibitors of these enzymes have demonstrated therapeutic benefit in animal models of other several disorders too, including neuropathic pain, anxiety and inflammatory bowel diseases [45]. Of note, in a lipopolysaccharide (LPS) mouse model of inflammatory pain, FAAH(-/-) mice exhibit reduced edema and hyperalgesia. Through the same mechanism, the FAAH inhibitor URB597 attenuates the development of LPS-induced edema and hyperalgesia and more importantly reduces the production levels of IL-1β and TNF-α pro-inflammatory cytokines, offering a potentially powerful strategy to treat chronic inflammatory pain syndromes [46].

2.3 Cannabinoid receptors and cancer

Several studies suggested that the cannabinoid receptor system is altered during carcinogenesis. Cannabinoid

Table 1. Levels of endocannabinoids and CB receptors in malignant vs normal cells.

Levels of CB receptors	Tumor type	Levels of endocannabinoids	Ref.
↑ **CB1 and CB2 receptors**	Mantle cell lymphoma	↑ AEA	[47]
	Acute myeloid leukemia	↑ AEA	[48]
	Well-differentiated hepatocellular carcinoma	↑ AEA	[49]
	Prostate carcinoma	↑ 2-AG; ↓ AEA	[50]
	Malignant astrocytoma	↑ AEA	[51]
	Pancreatic carcinoma	= AEA and 2-AG	[27]
↓ **CB1 and CB2 receptors**	Poorly differentiated hepatocellular carcinoma	↑ AEA	[52]
CB1 and CB2 receptors similar to control levels	Kaposi's sarcoma		[53]
	Non-melanoma skin cancer		
	Astroglial carcinoma	↑ AEA	[34]
	Pituitary adenoma	↑ AEA and 2-AG	[54] [33]
↑ **CB2 receptors**	Glioblastoma	↑ AEA	[55]
	Meningioma	↑ 2-AG	[32]
	Estrogen receptor-negative primary breast carcinoma	↑ AEA	[56]
	Endometrial carcinoma	↑ 2-AG	[39]
	Colon carcinoma	↑ AEA and 2-AG	[35,59]
↑ **CB1 receptors**	Rhabdomyosarcoma		[58]
	Gastrocarcinoma	↓ AEA	[57]
↓ **CB1 receptors**	Primary breast carcinoma	↑ AEA	[25]
↑ **TRPV1 receptors**	Prostate carcinoma	↑ 2-AG; ↓ AEA	[50]
	Squamous cell carcinoma of the human tongue		[61]

2-AG: 2-Arachidonoylglycerol; AEA: Anandamide; CB: Cannabinoid.

receptors play a key role in endocannabinoid antitumor effects; however, the regulation of their expression in cancer tissues is a topic not sufficiently investigated. It seems that the levels of cannabinoid receptors are differently regulated in normal versus malignant cells (**Table 1**). This pattern of expression seems to be a common mechanism of protection of normal cells from pro-apoptotic and anti-proliferative effects of cannabinoid agonists [17]. Increased expression of CB1 and CB2 has been reported in mantle cell lymphoma [47], acute myeloid leukemia [48], hepatocellular carcinoma [49], prostate cancer cell lines [50], malignant astrocytomas [51] and human pancreatic cancer [27] compared to normal tissue (**Table 1**). Interestingly, high expression of CB1 and CB2 was detected by *in situ* hybridization in cirrhotic liver samples and in well-differentiated human hepatocellular carcinoma, while the expression in poorly differentiated hepatocellular carcinoma was low [52]. Instead, the levels of both receptors were similar to healthy tissue in Kaposi's sarcoma cells [53], non-melanoma skin cancer [34], pituitary adenomas [33] and astroglial tumors [54]. In several types of tumor, a remarkable increase of CB2 receptors levels has been detected, such as in endothelial cells of glioblastoma [55], in primary breast cancer compared with normal breast tissue [25], in estrogen receptor (ER)-negative breast tumors compared with ER-positive tumors [25] and in 91% of ErbB2-positive breast tumors (**Table 1**) [56]. Other types of tumors are instead accompanied by overexpression of the CB1 receptor, such as in the parietal cells of human gastric mucosa and in human gastric cancer cell HGC-27 [57]. Indeed, gene expression profiling revealed that the gene coding for CB1 is highly upregulated in rhabdomyosarcoma biopsies (**Table 1**) [58]. Significant levels of CB1 and CB2 receptors were found in both normal and colorectal cancer tissue. It was observed that AEA and 2AG antiproliferative effect in colon cancer cells was mediated by CB1 activation [35]; however, an anti-proliferative role associated with CB2 has also been proposed in the same study. A recent study [59] also confirmed that human colorectal cancer specimens and the corresponding normal colonic mucosa express CB1 and CB2 receptors at the mRNA and protein levels as previously reported (**Table 1**) [35]. Although the antitumor effects of (endo)cannabinoid can be mediated by cannabinoid receptors, the role of these receptors in cancer is still unclear; however, their levels of expression could have a prognostic value alone or in association with other recognized prognostic markers. CB2 receptor expression positively correlates with the histological grade of breast cancer [60]. In addition, high pancreatic cancer cell level of CB1 is associated with shorter survival, together with low FAAH and MGLL levels [38]. A role also for the transient vanilloid receptor TRPV1 has been suggested in cancer; increased expression of this receptor was found in human

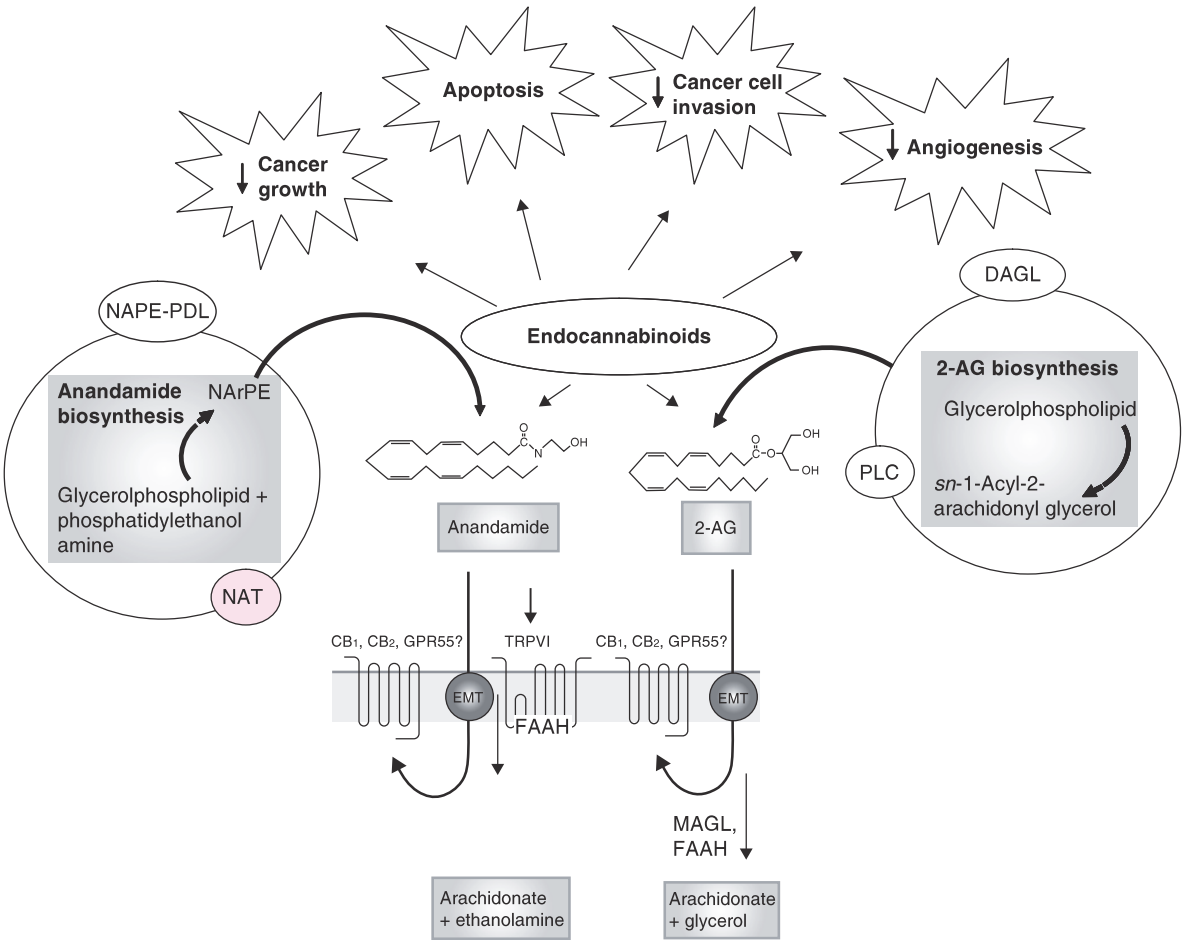

Figure 1. Anabolic and catabolic pathways of endocannabinoids and effects on cancer growth. The anandamide biosynthesis occurs through enzymatic hydrolysis of the membrane lipid precursor, NArPE. The anandamide biosynthetic enzymes NAT and NAPE-PLD and the inactivating enzyme FAAH are all located on intracellular membranes. The enzymes for 2-AG biosynthesis are the PLC and the sn-1-selective DAGLs mostly localized on the plasma membrane. After biosynthesis, endocannabinoids can activate cannabinoids receptors, either after previous release into the extracellular space or directly moving within the cell membrane. EMT seems to facilitate both endocannabinoid release and re-uptake. The enzyme FAAH hydrolyzes anandamide and related compounds in arachidonate and ethanolamine and MAGL hydrolyzes 2-AG in arachidonate and glycerol. Endocannabinoids might directly inhibit tumor growth by mechanisms involving induction of apoptosis, inhibition of cancer cell invasion and inhibition of neo-angiogenesis.

2-AG: 2-Arachidonoylglycerol; DAGL: Diacylglycerol lipase; EMT: Endocannabinoid membrane transporter; FAAH: Fatty acid amide hydrolase; MAGL: Monoacylglyceride lipase; NAPE-PLD: *N*-acylphosphatidyl-ethanolamine-specific phospholipase D; NArPE: *N*-arachidonoyl phosphatidylethanolamine; NAT: *N*-acyltransferase; PLC: Phospholipase C.

prostate carcinoma [50] and in squamous cell carcinoma (SCC) of the human tongue compared to normal tongue. However, statistical analysis revealed that the marked overexpression of TRPV1 found in all grades of SCC showed no correlation with the degree of malignancy of the tumors [61].

At present, little is known about the regulation of cannabinoid receptor expression in tumor tissue; the different response of normal and malignant cells to cannabinoids and the abnormal expression of cannabinoid receptors in cancer call for further research on the regulation of cannabinoid receptors.

3. Antitumor mechanisms of endocannabinoids and their derivates

The activation of cannabinoid receptors on tumor cells modulates signaling pathways involved in cell proliferation and survival, and although the downstream events are not completely unraveled, there is substantial evidence for the involvement of at least four mechanisms: antiproliferative action through the suppression of mitogenic signal, induction of apoptosis, inhibition of tumor cell invasion and neo-angiogenesis.

3.1 Anti-proliferative effects

Numerous pharmacological studies have demonstrated the anti-proliferative action of endocannabinoids and their derivates in several tumor models through cannabinoid receptor-dependent or -independent mechanisms that may involve cytotoxic or cytostatic effects, apoptosis induction and anti-metastatic effects. AEA exerts anti-proliferative effects on cholangiocarcinoma [62], human thyroid carcinoma [63], hepatocellular carcinoma [49], COX-2 overexpressing non-melanoma skin cancer [64] and breast cancer proliferation; this effect on mammary tumors is accompanied by reduced levels of the long form of the prolactin receptor [65] and trk nerve growth factor (NGF) receptor [66]. These effects are due to cell cycle arrest with activation of Chk1 and suppression of Cdk2 activity [67]. Furthermore, the AEA analog R(+)-methanandamide (MET-A) induces growth inhibition and apoptosis in prostate cancer PC-3 cells through CB2-mediated signaling [68] and decreases the EGFR expression [69]. The CB1–CB2 agonist, WIN-55,212 – 2, induces gastric cancer cell apoptosis [70], WIN-55,212 – 2 and the CB2 synthetic agonist JWH-133 inhibit human breast cancer cell proliferation [25]. Indeed, *in vivo* studies reported that administration of endocannabinoids induces the regression of a broad array of cancer types, such as thyroid epitheliomas through CB1 receptors [24], lymphomas [71], gliomas [15], skin carcinomas [26] and pancreatic cancer [27] through CB2 receptors. AEA suppresses rat C6 glioma cell growth; this effect was partially blocked by the CB1, CB2, and TRPV1 antagonists, but was completely attenuated when these antagonists were added in combination [72]. Of note, on human glioma cells, CBD exerts anti-proliferative effects correlated to induction of apoptosis, not reverted by cannabinoid and vanilloid receptor antagonists [73].

3.2 Pro-apoptotic effects

Apoptosis induced by cannabinoids can involve caspase-dependent and -independent pathways. For instance, the CB1 receptor antagonist, rimonabant (SR141716), inhibits leukemia cell growth promoting apoptosome-complex formation and caspase pathway activation. [74]. Moreover, several studies confirmed pro-apoptotic and antitumor effects of cannabinoids via mechanisms involving *de novo* synthesis of ceramide [23]. For instance, in colorectal cancer cells, CB1 activation mediated induction of apoptosis, through inhibition of RAS-MAPK and PI3K-AKT pathways, downregulation of anti-apoptotic factors and activation of ceramide [75]. CB1 and CB2 receptor activation stimulates ceramide *de novo* synthesis in different human tumors such as glioma, pancreatic cells, leukemia and mantle cell lymphoma. AEA can induce growth suppressive/pro-apoptotic effects in cholangiocarcinoma cells via stabilization of the lipid raft structures within the plasma membrane, increased production of ceramide and subsequent recruitment of death receptor complex components into the lipid raft structures through activation of the non-canonical Wnt signaling pathway [62].

3.3 Effects on tumor invasion

Modulation of cancer cell invasion has recently emerged as a topic of increasing interest. MMPs are emerging as a family of enzymes that exert important functions during tumor invasion. Tissue inhibitors of MMPs (TIMPs), and in particular TIMP-1, have been shown to inhibit the proteolytic activity of MMPs and suppress vascular tumor growth and angiogenesis in xenograft animal models [76]. For instance, the effects of AEA on MMP and TIMP expression were evaluated in various cancer cells. Using a cervical cancer cell line, Met-AEA as well as Δ9-THC and CBD inhibited the invasive properties of these cells via increased expression of TIMP-1 [77,78]. Inhibitory properties of endocannabinoids in tumor migration have also been described. AEA inhibited migration of colon carcinoma cells [48]; in an *in vivo* model of metastatic spreading using breast cancer cells, Met-AEA significantly reduced the number and dimension of metastatic nodes, an effect inhibited by specific CB1 receptor antagonists [18]. Indeed, there was a marked decrease in the phosphorylation of focal adhesion kinase and src, both of which are involved in the metastatic formation and development.

3.4 Effects on angiogenesis

Several studies suggest that angiogenesis is also regulated by cannabinoids. The AEA analog 2-methylarachidonyl-2′-fluoro-ethylamide (Met-F-AEA) has been shown to reduce sprout number and length of endothelial cell spheroids, inhibit capillary-like tube formation and suppress angiogenesis in the *in vivo* chick chorioallantoic membrane model [79]. Other studies indicate anti-angiogenic effects of cannabinoids related to the expression of VEGF. Met-F-AEA was found to decrease VEGF and VEGFR-1 levels in K-ras-transformed thyroid cells and experimental tumors of nude xenograft mice [80].

The effects described on endocannabinoid involvement in different stages of tumor progression support the emerging notion that targeting the ECS through drugs aimed at regulating this system may represent useful therapeutic tools for cancer treatment.

4. Signaling pathways involved in endocannabinoid anticancer effects

Anticancer effects elicited by (endo)cannabinoids can be mediated by CB1, CB2 or transient vanilloid TRPV1 receptors, but in some cases other mechanisms can be involved (e.g., COX, lipid rafts, PPAR-γ) [81]. AEA has been shown to inhibit breast cancer cell proliferation by acting via the CB1 receptor to activate the cAMP/PKA/MAPK pathway [80]. These effects ultimately result in decreased expression of prolactin receptor [65] and trk NGF receptor [66], with blocking of cell cycle progression by activation of checkpoint kinase Chk1 or cyclin-dependent kinase inhibitor p27/kip1 [80] and suppression of Cdk2 activity [67]. Furthermore, AEA through a CB1-dependent mechanism reduces the expression of

EGFR in several human prostatic cancer cell lines, a phenomenon that has been associated with G1 arrest and cell death [69] possibly by an increase of ceramide production. Similarly, the stimulation of CB1 receptor by Met-F-AEA inhibits the growth of xenograft tumors of thyroid origin by affecting the expression of VEGF tyrosine kinase receptor by blocking the activity of p21ras [80]. AEA may activate either CB2 or TRPV1 to elicit a similar response, although the mechanism by which this occurs is not clear. Recently, HMG-CoA reductase has been suggested as a new target of antitumor effect of AEA. In particular, the decreased activity of this enzyme is responsible for the inhibition of farnesylation of Ras oncogenic protein involved in cell proliferation of human mammary carcinoma cell lines [82]. Cannabinoids can also induce apoptosis via pathways not mediated by cannabinoid- or vanilloid receptor activation. In particular, in human H4 neuroglioma cells the endocannabinoid analog R(+)-methanandamide (R(+)-MA) has been shown to induce apoptosis through a pathway linked to lipid raft microdomains involving increased synthesis of pro-apoptotic sphingolipid ceramide. These effects involve COX-2 expression and subsequent formation of prostaglandin E2 via activation of p38 and p42/44 MAPKs [83,84]. Interestingly, increased Cox-2 expression is fundamental to sensitize the apoptosis-resistant colon cancer cells to AEA-induced cell death [85]. In the same way, AEA induces cell death in Cox-2 overexpressing squamous carcinoma cells but not in keratinocytes, which express low basal level of Cox-2. Resistance to AEA-mediated cell death in these cells was then reversed by overexpression of Cox-2 [86]. Alternatively, AEA-induced ceramide production can facilitate the Fas/FasL death receptor complex within the lipid raft structure, resulting in increased apoptosis of cholangiocarcinoma cells [87]. A change in cytosolic free $Ca2^{+}$ levels is a pivotal signal for various cellular responses. Recent studies suggested that AEA induced apoptosis in human osteosarcoma cells by causing $Ca2^{+}$ influx and release that converge on p38MAPK phosphorylation and subsequent activation of caspase-3 leading to apoptosis [88].

The pro-apoptotic effects of (endo)cannabinoid converge on ceramide accumulation and activation of (ER) stress-related pathway. The stress-regulated protein p8 plays a key role in this effect controlling the expression of ATF-4, CHOP and TRB3 factors [27]. This cascade of events triggers the activation of mitochondrial intrinsic apoptotic pathway through mechanisms not unraveled yet. However, it has been suggested that the pro-apoptotic activity of (endo)cannabinoids may involve the JNK activation [68] as well as the inhibition of both RAS–MAPK/ERK and PI3K–AKT survival signaling cascades, accompanied by the activation of the pro-apoptotic BCL-2 family member Bad. Very recently, it was demonstrated that non-psychoactive CB2-selective agonists such as JWH-133 were efficient to treat highly aggressive and low responsive tumors of MMTV-neu mice, a clinically relevant animal model of ErbB2-positive breast cancer [56]. This antitumor action relies, at least partially, on the inhibition of the pro-tumorigenic PI3K–Akt pathway, whose importance in breast cancer is corroborated by clinical studies showing in most ErbB2-overexpressing tumor activation of Akt. A recent publication furthermore demonstrated the induction of autophagy by cannabinoids. In human glioma cells, THC induces ceramide accumulation and eukaryotic translation initiation factor 2α phosphorylation; thereby, activated ER stress response promoted autophagy via TRB3-dependent inhibition of the Akt–mammalian target of rapamycin complex 1 axis [89]. Interestingly, the effects of cannabinoids on the Notch transmembrane receptor family that play a pivotal role in cellular differentiation, proliferation and apoptosis have been recently demonstrated. In particular, AEA and 2-AG have opposite effects on cholangiocarcinoma growth, depending on the differential activation of Notch signaling that requires the γ-secretase complex for activation. Anti-proliferative effects of AEA are associated with increased Notch 1 mRNA, presenilin 1-dependent proteolytic cleavage and activation, and nuclear translocation while the growth promoting effects of 2-AG are associated with presenilin 2-dependent activation of Notch 2 signaling [90]. Furthermore, AEA inhibits angiogenesis, tumor cell migration and invasion. Met-AEA inhibits angiogenesis via decreased VEGF expression and inhibition of the basic fibroblast growth factor-stimulated endothelial cell proliferation [79]. Of note, in thyroid tumors the same compound inhibits the angiogenetic process via downregulation of the pro-angiogenic growth factor VEGF and its receptor Flt-1 expression, thereby counteracting the cancer growth *in vivo* [80]. Regarding the AEA-induced inhibition of migration, it is probably due to decreased activation in breast cancer cells of focal adhesion kinases and src kinase [18]. Met-AEA also inhibits human cervical cancer (HeLa) cell invasion by decreasing the expression of proteins responsible for breaking down the extracellular matrix of the target organ, such as MMPs and increasing the expression of TIMPs. These effects were reversed by specific inhibitors of p38 and p42/44 MAPKs [77] suggesting involvement of this signaling pathway. Finally, in androgen-independent prostate cancer cells, endogenous 2-AG and CB1 agonists reduced invasion through CB1-dependent inhibition of adenylyl cyclase, decreasing phospho-kinase A activity [34].

5. Preclinical/clinical use of endocannabinoid-derived drugs: beneficial and undesired effects

The ECS modulation is involved in various pathophysiological conditions in both central and peripheral tissues and is implicated in the hormonal regulation of food intake and energy metabolism, cardiovascular, gastrointestinal, immune, behavioral, anti-proliferative and mammalian reproduction functions. Intense research efforts yielded numerous drugs interacting with the main elements of the ECS, cannabinoid receptor agonists and antagonists, inhibitors of FAAH and

MAGL and diacylglycerol lipases. Therapeutically relevant ECS modulators include THC in combination with CBD (sativex), its synthetic forms (marinol), and its closely related compounds (cesamet). These compounds remain the only clinically applied cannabinoid receptor agonists. The principal pharmacological effects of these compounds include analgesia, muscle relaxation, anti-emesis, appetite stimulation and psychoactivity [91]. Rimonabant is the first CB1 blocker launched to treat cardiometabolic risk factors in obese and overweight patients. Phase III clinical trials showed the drug's ability to regulate intra-abdominal fat tissue levels, lipidemic, glycemic and inflammatory parameters [92]. However, safety concerns led to its withdrawal, as probably interfering with the 'autoprotective' role of the ECS it induced ansiogenic and pro-depressant effects. Considering that many side effects rely on CNS action, it would be adequate for the development of peripherally-restricted antagonists to be unable to cross the BBB or to use partial CB1 agonists in order to limit adverse effects. A different approach could be the use of phytocannabinoids such as CBD [93] with very weak or no psychotropic effects. Cannabinoid receptor ligands are potential therapeutic agents in neurodegenerative diseases such as Parkinson and Alzheimer and AIDS-related neurodegeneration [94]. Cannabinoids represent also a novel class of anti-inflammatory drugs due to their ability to negatively impact the release of inflammatory mediators and the induction of pro-inflammatory transcriptional programs. Of note, the activation of CB2 receptor is a key factor of the cannabinoid suppressive effects, while apparently pro-inflammatory effects seem to be attributable to the CB1 receptor expression [95]. Given the growing evidence of the expression of the CB1 receptor on numerous immunocytes, it is likely that the clinical promise of cannabinoids as anti-inflammatory drugs may rely on the development of highly selective CB2 agonists. In terms of clinical application, the inhaled or ingested THC can acutely suppress ongoing airway or gastrointestinal inflammation, leading to particular interest in the development of cannabinoid therapeutics for treatment of asthma, chronic obstructive pulmonary disease and Crohn's disease/IBD.

An emerging research area is represented by the role of ECS elements in mammalian reproduction given their implication in oogenesis, embryo oviductal transport, blastocyst implantation, placental development and pregnancy outcomes, and sperm survival, motility, capacitation and acrosome reaction [96].

5.1 Preclinical evidence of cannabinoid efficacy in cancer

Cannabinoid-related drugs are emerging as valuable adjunctive agents for the management of multiple symptoms of cancer and therapy-induced side effects. Available data support a broad spectrum of palliative properties, including appetite stimulation, inhibition of nausea and emesis associated with chemotherapy or radiotherapy, pain relief, mood amelioration and relief from insomnia [81]. Marinol, cesamet and sativex have been already approved by the FDA for these indications. Recently, results of multi-center, double-blind, randomized, placebo-controlled, parallel-group study showed the efficacy, safety and tolerability of THC:CBD extract and THC extract in 177 patients with intractable cancer-related pain. This study shows that THC:CBD extract is a more promising efficacious treatment than THC extract alone for relief of advanced cancer pain in patients not achieving an adequate analgesic response to opioids [97]. However, side effects of cannabinoids are known: euphoric mood alteration, sedation and alleviating inhibition but also disphoric mood alteration including anxiety, panic and psychosis. Until now, the use of cannabinoid-derived drugs has shown a fair safety profile with respect to current chemotherapeutics. However, only a single pilot Phase I–II clinical trial, approved by the Spanish Ministry of Health in 2002, has been performed so far and the results have been recently disclosed [98]. This study evaluated the safety profile of THC intracranial administration and its antitumor efficacy in a cohort of nine terminal patients affected by recurrent glioblastoma multiforme, an aggressive primary brain tumor with poor prognosis and no efficacious treatment. Cannabinoid delivery resulted as safe without psychoactive effects and median survival was prolonged by 24 weeks. Indeed, THC decreased tumor cell proliferation and increased apoptosis when administered to two patients. Although this pioneer study suffers from several limitations, good safety profile of THC was demonstrated. To optimize the results, the protocol should also involve a larger cohort of patients and combinatorial studies with commonly used chemotherapeutics. Finally, a non-invasive, less traumatic administration route would be more desirable for clinical practice.

6. Conclusion

Findings widely reported in literature support the regulatory action elicited in health and pathological conditions by the ECS. The modulation of both endocannabinoid levels and their receptors in cancer has prompted the development of numerous (endo)cannabinoid derived agents aimed to target cannabinoid receptors or modulate the enzymes involved in endocannabinoid production or degradation. Thus, myriad effects on tumor models both *in vitro* and *in vivo* have been reported suggesting antitumor properties such as inhibition of cancer cell growth, induction of apoptosis and block of processes involved in tumor progression, such as angiogenesis, and cell migration. These effects might involve several signaling pathways being both cannabinoid receptor-dependent or -independent. Overall research has led to the use of some of these compounds in clinical trials, and although only few data are available, these studies strongly suggest the safety profile of cannabinoid derivatives. The proposed antitumor efficacy of (endo)cannabinoid-related drugs alone or in combination with other currently used chemotherapeutics is not completely investigated and needs a deeper research at both

preclinical and clinical level in order to allow a safe translation into the clinical setting.

7. Expert opinion

Key findings from current literature show that the study of the ECS is emerging as a relevant topic to be targeted in cancer. However, research performed up to date is still at basic or preclinical levels but we believe in the high potential of multiple studies.

One of the issues that should be pursued is to better select compounds in order to reduce psychoactivity known to be connected to the CB1 receptor; thus, for example, focusing on CB2 receptor as a primary target is becoming the objective of ongoing studies that could probably lead to new highly selective compounds. The design of novel CB2 ligands with high affinity at the CB2 receptor could reduce side effects due to high doses; thus, design studies could identify promising candidates to test *in vitro* and *in vivo* models and in clinical trials in the near future. Furthermore, CB1 receptors could be maintained as a target to design new compounds and or mixed CB1–CB2 ligands only if these substances are able to not cross the BBB. In addition, phytocannabinoids could be considered as potential candidates to pursue studies on clinical trials; these substances are able to maintain cannabinoid anticancer effects and indeed present very low psychoactivity, and their efficacy is supported by the applications of CBD in several clinically applied drugs. Research in this field requires further studies to better characterize the molecular mechanisms on which anticancer effects of (endo)cannabinoids are based, exhaustive clinical trials to establish the real advantage with respect to currently used chemotherapeutic or combinatory studies in order to reduce undesired side effects. Another relevant issue that probably will be the objective of studies is the regulation of cannabinoid receptor in carcinogenesis and the possibility of being considered as a potential marker of tumor progression. The aim of these studies should be the evaluation of cannabinoid receptor expression in tumor versus normal tissues in order to achieve significant antitumor efficacy without immunosuppression. Furthermore, targeting the ECS with agents that activate cannabinoid receptors or inhibitors of endogenous degrading systems such as FAAH inhibitors may be of relevant therapeutic impact on tumor growth and spreading; indeed, the increase of the endogenous levels of endocannabinoids may prove to be beneficial in the treatment of various cancers. Further studies into the downstream consequences of endocannabinoid treatment are required and may illuminate other potential therapeutic targets. The potential related to the study of the ECS is high considering also that preclinical study and clinical trials revealed the safety profile of cannabinoid derived drugs; thus, improving the studies on this field could show not only the efficacy of such compounds as palliative and antinociceptive drugs but also potential application as therapeutic agents, probably taking to new advances in cancer research.

Declaration of interest

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•• **Of considerable importance, this work is the only pilot Phase I – II clinical trial performed so far on cannabinoids.**

Affiliation

Anna Maria Malfitano[1], Elena Ciaglia[1], Giuseppina Gangemi[1], Patrizia Gazzerro[1], Chiara Laezza[2] & Maurizio Bifulco[†3]
[†]Author for correspondence
[1]University of Salerno, Department of Pharmaceutical Sciences, Fisciano, Salerno, Italy
[2]Istituto di Endocrinologia e Oncologia Sperimentale del CNR, IEOS, Napoli, Italy
[3]Proffesor, University of Salerno, Department of Pharmaceutical Sciences, Via Ponte don Melillo, 84084 Fisciano, Salerno, Italy
Tel: +39 089 969742; Fax: +39 089 969602;
E-mail: mbifulco@unisa.it

Attached manuscript IV

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Pharmacological Actions of Statins: A Critical Appraisal in the Management of Cancer

Patrizia Gazzerro, Maria Chiara Proto, Giuseppina Gangemi, Anna Maria Malfitano, Elena Ciaglia, Simona Pisanti, Antonietta Santoro, Chiara Laezza, and Maurizio Bifulco

Department of Pharmaceutical and Biomedical Sciences, University of Salerno, Fisciano, Italy (P.G., M.C.P., G.G., A.M.M., E.C., S.P., A.S., M.B.); and Istituto di Endocrinologia e Oncologia Sperimentale, Consiglio Nazionale delle Ricerche, Napoli, Italy (C.L.)

Address correspondence to: Prof. Maurizio Bifulco, Department of Pharmaceutical and Biomedical Sciences, University of Salerno, Via Ponte Don Melillo, 84084 Fisciano (Salerno), Italy. E-mail: maubiful@unisa.it
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Abstract*——Statins, among the most commonly prescribed drugs worldwide, are cholesterol-lowering agents used to manage and prevent cardiovascular and coronary heart diseases. Recently, a multifaceted action in different physiological and pathological conditions has been also proposed for statins, beyond anti-inflammation and neuroprotection. Statins have been shown to act through cholesterol-dependent and -independent mechanisms and are able to affect several tissue functions and modulate specific signal transduction pathways that could account for statin pleiotropic effects. Typically, statins are prescribed in middle-aged or elderly patients in a therapeutic regimen covering a long life span during which metabolic processes, aging, and concomitant novel diseases, including cancer, could occur. In this context, safety, toxicity, interaction with other drugs, and the state of health have to be taken into account in subjects treated with statins. Some evidence has shown a dichotomous effect of statins with either cancer-inhibiting or -promoting effects. To date, clinical trials failed to demonstrate a reduced cancer occurrence in statin users and no sufficient data are available to define the long-term effects of statin use over a period of 10 years. Moreover, results from clinical trials performed to evaluate the therapeutic efficacy of statins in cancer did not suggest statin use as chemotherapeutic or adjuvant agents. Here, we reviewed the pharmacology of the statins, providing a comprehensive update of the current knowledge of their effects on tissues, biological processes, and pathological conditions, and we dissected the disappointing evidence on the possible future use of statin-based drugs in cancer therapy.

I. Introduction

Many studies have highlighted the fact that statins, besides their application in cardiovascular and coronary heart diseases as cholesterol-lowering agents, exhibit a wide range of pleiotropic effects that may significantly contribute to the treatment of conditions other than cardiac diseases, such as inflammatory and neurological pathologic conditions and even tumors. The commonly known pharmacological activity of statins relies on a potent inhibition of the endogenous mevalonate pathway, which leads directly to the biosynthesis of cholesterol and isoprenoids. Statins bind to mammalian HMG-CoA reductase at nanomolar concentrations, leading to an effective displacement of the natural substrate HMG-CoA, which binds instead at micromolar concentrations (Moghadasian, 1999). The interactions between statins and HMG-CoA reductase prevent the conversion of HMG-CoA to L-mevalonate resulting in the inhibition of the downstream cholesterol biosynthesis and numerous isoprenoid metabolites such as geranylgeranyl pyrophosphate (GGPP[1]) and farnesyl pyrophosphate (FPP) (Fig. 1). GGPP and FPP are lipid attachments that constitute key intermediates for post-translational events of several cell signaling proteins, including the small GTPase family members Ras, Rac, and Rho (Chow, 2009). The attachment of these lipids also known as isoprenylation is fundamental for the activation and intracellular transport of these proteins that act as molecular switches controlling multiple pathways and cell functions such as maintenance of cell shape, motility, factor secretion, differentiation, and proliferation. Considering that the key role of these prenylated proteins is an obvious expectance that statin effects may extend beyond their cholesterol-lowering actions. These cholesterol-

[1]Abbreviations: 5-FU, 5-fluorouracil; Aβ, amyloid β; AD, Alzheimer disease; Akt, protein kinase B; AML, acute myeloblastic leukemia; APP, amyloid precursor protein; ARF, ADP-ribosylation factor; ATRA, all *trans*-retinoic acid; AUC, area under the concentration versus time curve; BMD, bone mineral density; CAD, coronary artery disease; Cav1, caveolin-1; CI, confidence interval; CIITA, MHC-II transactivator; COX, cyclooxygenase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; eGFR, estimated glomerular filtration rate; eNOS, endothelial nitric-oxide synthase; EPC, endothelial progenitor cells; ER, endoplasmic reticulum; ERK, extracellular signal-regulated protein kinase; FOLFIRI, folinic acid (leucovorin)/5-FU/irinotecan; FPG, fasting plasma glucose; FPP, farnesyl pyrophosphate; GGPP, geranyl-geranyl pyrophosphate; HCC, hepatocellular carcinoma; HDL-C, high-density lipoprotein cholesterol; ICAM, intercellular adhesion molecule; IFN, interferon; IL, interleukin; JNK, c-Jun NH_2-terminal kinase; LDL-C, low-density lipoprotein-cholesterol; LFA-1, lymphocyte function-associated antigen-1; MAPK, mitogen-activated protein kinase; MHC, major histocompatibility complex; MS, multiple sclerosis; NF-κB, nuclear factor-κB; NSAID, nonsteroidal anti-inflammatory drug; OATP, organic anion transporting polypeptide; P450, cytochrome P450; P-gp, P-glycoprotein; PI3K, phosphatidylinositol 3-kinase; RAD-001, everolimus; RR, relative risk; SCC, squamous cell carcinoma; SMC, smooth muscle cell; STAT, signal transducer and activator of transcription; TACE, transarterial chemoembolization; TGF-β, transforming growth factor β; UGT, UDP-glucuronosyl transferase; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

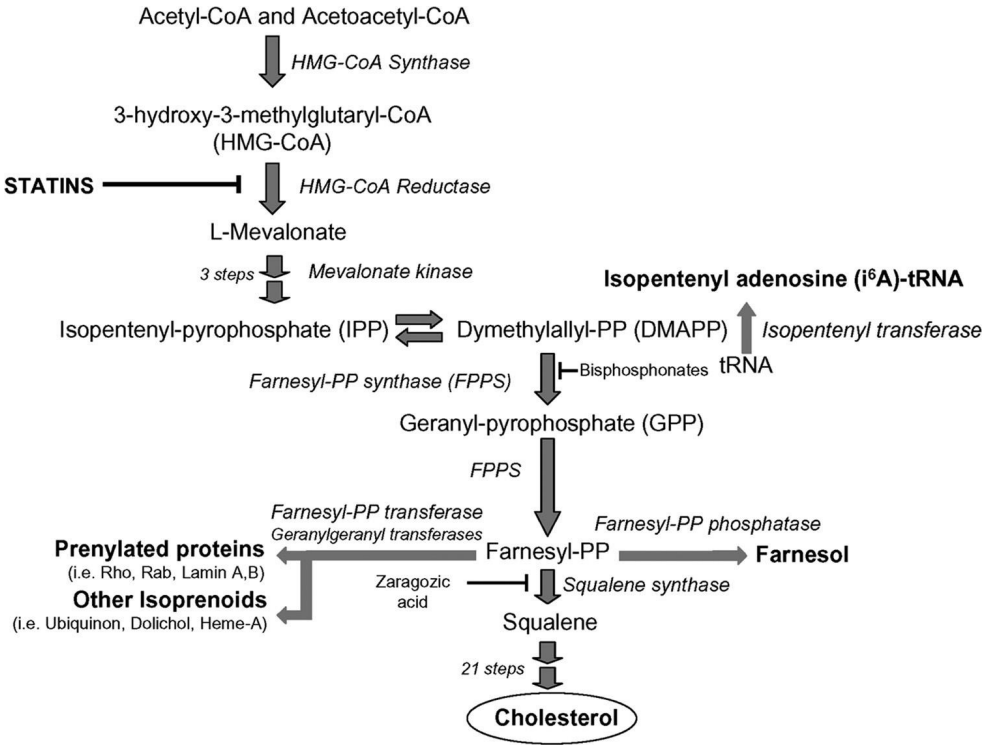


FIG. 1. The mevalonate pathway. Statins act by inhibiting HMG-CoA reductase, the key enzyme of the mevalonate pathway. Statins could have pleiotropic effects possibly through other products of the mevalonate pathway (e.g., i^6A tRNA, prenylated proteins, and other isoprenoids) that play central roles in cell signaling, protein synthesis, and cytoskeletal organization.

independent effects are known as pleiotropic effects and include, among others, improvement of endothelial function, inhibition of vascular inflammation and oxidation, and stabilizing of atherosclerotic plaques (Zhou and Liao, 2010).

In the present study, the pharmacology, mechanism of action, and metabolism of statins are reviewed, as well as their effects on tissues and numerous biological processes, such as those involved in the immune system, endothelia, smooth muscle, platelet function, in the metabolism, in the bone, and in the nervous system. Furthermore, a careful analysis is undertaken to provide a comprehensive view of pros and cons of the statin effects in cancer, including their cancer risk and prevention, their potential application as chemopreventive agents, and their use in combination with currently adopted chemotherapeutics.

II. The Isoprenylated Proteins

In the 1980s, studies on cholesterol biosynthesis led to the discovery that a compound derived from mevalonic acid, other than cholesterol, is incorporated into a specific set of protein-containing cysteine linked to a 15-carbon farnesyl or a 20-carbon geranylgeranyl group (Glomset et al., 1990). The synthesis of FPP and GGPP is catalyzed by FPP synthase and GGP synthase, respectively. FPP and GGPP are substrates of isopentenyl transferase involved in post-translational prenylation of a variety of proteins (Casey and Seabra, 1996). Three distinct heterodimeric protein isoprenyl transferases have been described in metazoans, protozoans, fungi, and plants. Protein farnesyltransferase transfers a farnesyl group from farnesyl diphosphate to the cysteine residue of a carboxyl terminal CaaX motif (where "C" is cysteine, "a" is an aliphatic amino acid, and "X" is usually methionine, glutamine, serine, alanine, or cysteine) (Yokoyama et al., 1992). Protein geranylgeranyltransferase type I usually transfers a geranylgeranyl group from geranylgeranyl diphosphate (GGPP) to the cysteine residue of a similar CaaX motif (where "X" is leucine or isoleucine) (Taylor et al., 2003). Protein geranylgeranyltransferase type II (also called Rab geranylgeranyltransferase) transfers two geranylgeranyl groups from GGPP to the cysteine residues of XCCXX, XXCXC, XXCCX, XXXCC, XCXXX, or CCXXX motifs at the carboxyl terminus of Rab proteins bound to the Rab escort protein (Leung et al., 2006). After the attachment of the isoprenoids, proteins undergo two additional post-translational modifications, collectively referred to as CaaX processing. The diphosphate is cleaved off by the Ras-converting enzyme, and the Ste24p endogenous proteases remove the terminal three amino acids (-aaX). Upon cleavage of the terminal tripeptide, the remaining prenylated cysteine residue undergoes carboxymethylation by a methyl group, delivered from *S*-adenosylmethionine. This conversion is catalyzed by isoprenylcysteine carboxyl methyltransferase, which is located in the Golgi apparatus, ER, and nuclear membranes. Under

physiological conditions, the carboxymethylation is reversible. It is assumed that the intermediates during these subsequent enzymatic reactions exist only transiently and are rapidly converted into the mature prenylated proteins (Winter-Vann and Casey, 2005). Overall, prenylation enhances lipophilicity and favors lipid-lipid interactions of these proteins with cellular membranes, although, in many cases, the modified C terminus is important in protein-protein interactions as well (Zhang and Casey, 1996). Proteins containing a carboxyl-terminal CAAX motif are small GTPases proteins that play a fundamental role in a multitude of intracellular signal transduction pathways involving vesicle trafficking, cell growth, differentiation, and cytoskeletal function (Konstantinopoulos et al., 2007). RAS proteins containing the CAAX motif are members of this family and are particularly interesting because of their well established role in oncogenesis. H-Ras, K-Ras, and N-Ras are the most renowned members of this family and they are constantly activated because of the mutation in the proto-oncogen (Downward, 2003). Furthermore, the majority of the Ras subfamily members are known to be farnesylated and, interestingly, K-Ras and N-Ras but not H-Ras can be geranylgeranylated when physiological farnesylation is inhibited (Brunner et al., 2003; Downward, 2003). Several other CAAX proteins are involved in the initiation and progression of cancer, such as the RHO family of GTPases, which includes RAC and cell division cycle 42, which is implicated in both oncogenesis and metastasis (Ridley, 2001). Increased signaling by yet another GTPase, RAP1A, has been associated with myeloproliferation (Ishida et al., 2003). The 60 Ras-like proteins in the brain (Rab) represent the largest group within the superfamily of small GTPases (Pereira-Leal et al., 2001) and are mainly involved in intracellular vesicular transport (Zerial and McBride, 2001; Kimura et al., 2008; Bergbrede et al., 2009; Zhu et al., 2009).

Aberrant expression of RAB has also been documented in a variety of cancers. RNA microarray analyses demonstrated that approximately 50% of the RAB genes are overexpressed in ovarian cancer. RAB25 is also up-regulated in prostate cancer and transitional-cell bladder cancer (Cheng et al., 2004). Overexpression of RAB5A and RAB7 has been documented in thyroid adenomas, and RAB1B, RAB4B, RAB10, RAB22A, RAB24, and RAB25 are up-regulated in hepatocellular carcinomas and cholangiohepatomas (He et al., 2002; Croizet-Bergeret al., 2002). The ADP-ribosylation factor (ARF) and secretion-associated and Ras-related proteins are mainly involved in vesicle formation and intracellular trafficking (Takai et al., 2001; Memon, 2004). From the ARF family, ARL5, SARA1 (also known as SAR1A), and SARA2 have been shown to be overexpressed in hepatocellular carcinoma, whereas the levels of ARF6 correlate with breast-cancer-cell invasiveness (He et al., 2002; Hashimoto et al., 2004). ARF-like tumor suppressor protein 1, another member of the ARF family (also known as ARL11), functions as a tumor suppressor gene in humans, and a nonsense ARF-like tumor suppressor protein 1 polymorphism predisposes patients to familiar cancer (Calin et al., 2005). Constitutive activation of G-protein-coupled receptor pathways can also contribute to transformation (Schwindinger and Robishaw, 2001; Daaka, 2004) and the γ-subunits of heterotrimeric G proteins are all CAAX proteins (Schwindinger and Robishaw, 2001). CAAX proteins also include many phosphatases and kinases and their mutations are associated with cancer (Cates et al., 1996; Collins et al., 2000). In both normal and transformed cells, CAAX proteins, including the nuclear lamins A and B, and the centromeric proteins CENP-E and CENP-F, are involved in processes that are important for cell division and nuclear-envelope assembly/disassembly (Ashar et al., 2000; Hutchison, 2002). In particular, three mammalian nuclear lamin proteins, lamin B1, lamin B2, and the lamin A precursor, prelamin A, undergo canonical farnesylation and processing at CAAX motifs. In the case of prelamin A, there is an additional farnesylation-dependent endoproteolysis, which is defective in two congenital diseases: Hutchinson-Gilford progeria and restrictive dermopathy (Young at al., 2006). Finally, one of the earliest myelin-related proteins expressed when OLGs differentiate, 2′,3′-cyclic-nucleotide-3′phosphodiesterase is farnesylated and palmitoylated and is involved in the regulation of cytoarchitecture through its interaction with microtubules and microfilaments (Braun et al., 1991; Laezza et al., 1997; Bifulco et al., 2002).

III. The Pharmacology of Statins

A. Chemical Structure and Pharmacological Activity

The structural design of the statins has been modeled to achieve different functionalities tightly related to each particular component of the molecule. The chemical structure of the statins is constituted by two components, the pharmacophore, which is a dihydroxyheptanoic acid segment, and its moiety composed of a ring system with different substituents. The function of the pharmacophore relies on the inhibition of the HMG-CoA reductase enzyme in a competitive, dose-dependent, and reversible manner. The stereoselectivity of the HMG-CoA reductase enzyme dictates the stereochemistry of the statins, which present two chiral carbon atoms, C3 and C5, on their pharmacophore. The moiety of the pharmacophore, according to the chemical modified ring systems and the nature of the substituents, generates the different structures of the statins. The ring system is a complex hydrophobic structure, covalently linked to the pharmacophore, that is involved in the binding interactions to the HMG-CoA reductase. The binding interactions of the ring are able to reduce the competition for the binding site between the statin and the endoge-

nous HMG-CoA substrate because keeping the statin closed to the enzyme precludes the possibility of statin displacement by the endogenous substrate. The structure of the ring can be a partially reduced naphthalene (lovastatin, simvastatin, pravastatin), a pyrrole (atorvastatin), an indole (fluvastatin), a pyrimidine (rosuvastatin), a pyridine (cerivastatin), or a quinoline (pitavastatin). The substituents on the rings define the solubility of the statins along with many of their pharmacological properties. Different substituents on the ring generate different structures. For instance, on the partially reduced naphthalene ring, as substituent, can be located a CH_3 group and a 2-methylbutyrate ester (lovastatin), or a 2,2- methylbutyrate ester (simvastatin), which substantially increases the potency of the drug; on nitrogen-containing rings isopropyl and *p*-fluorophenyl substituents (atorvastatin and fluvastatin) can be attached. The statins are commonly grouped in two types; type 1, natural or fungal-derived statins (lovastatin, simvastatin, pravastatin), exhibit close structural homology and differ from the type 2 constituted by the synthetic statins (Schachter, 2005). Type 1 statins were originally identified as secondary metabolites of fungi (Alberts, 1988). Mevastatin, one of the first identified, was isolated from *Penicillium citrinum* by Endo et al. (1976) and, in its active form, resembles the cholesterol precursor HMG-CoA. Subsequently, a more active fungal metabolite, mevinolin or lovastatin, was isolated from *Aspergillus terreus* by Alberts et al. (1980). The functional difference between natural and synthetic statins relies on their ability to interact and inhibit the HMG-CoA reductase and on their lipophilicity. Type 2 statins are known to form more interactions with HMG-CoA reductase because of their structural characteristics; for instance, atorvastatin and rosuvastatin have additional hydrogen binding interactions. Indeed, rosuvastatin also exhibits a polar interaction between the methane sulfonamide group and the HMG-CoA reductase enzyme. These structural properties render this statin the most efficient in terms of dose able to reduce HMG-CoA reductase activity by 50% (Davidson, 2002). Among the statins mentioned, lovastatin, simvastatin, atorvastatin, and fluvastatin are lipophilic, whereas pravastatin and rosuvastatin are more hydrophilic. The lipophilic properties of the statins are accompanied, except for pitavastatin, by low systemic bioavailability because of an extensive first-pass effect at the hepatic level (García et al., 2003). Although this effect can be desirable, because, as site of cholesterol biosynthesis, the liver is the target organ, the statins' lipophilicity enables them to passively penetrate the cells of extrahepatic tissues, possibly leading to side effects that in some cases can be undesirable. On the other hand, hydrophilicity depends on an active transport process to enter the hepatocyte; thus, hydrophilic statins are more hepatoselective, because they are excluded by other tissues. However, the balance between desired and undesired effects of lipophilic and hydrophilic statins remains not clearly established. In summary, the different chemical structures, the lipophilicity/hydrophilicity rate, and as reviewed in section III.B, the kinetic profile, the rate of metabolism, and the formation of active and inactive metabolites govern the variability of the statin pharmacological activity, nonetheless contributing to their pleiotropic actions.

B. Pharmacokinetic Properties of Statins

The pharmacokinetic properties of the statins are orchestrated by several factors, including their active or lactone form, their lipophilic/hydrophilic rate, and their absorption and metabolism. Statins are administrated orally as active hydroxy acids, except for lovastatin and simvastatin, which are administrated as lactone prodrugs and then hydrolyzed to hydroxy acid form (Corsini et al., 1995). The statin pharmacological properties, referred to as doses administered as open acid and lactone forms, are shown in Table 1.

The percentage of absorption is between 30 and 98% and the time to reach peak plasma concentration (T_{max}) is within 4 h after administration (Pan et al., 1990; Tse et al., 1992; Cilla et al., 1996; Mück et al., 1997). The daily absorption may vary according to the time of administration (Cilla et al., 1996) and food intake (Garnett, 1995); for instance, changes in lipid and apolipoprotein values were similar after morning and evening administration of atorvastatin. Rate and extent of equivalent absorption of atorvastatin were lower during evening than morning administration (Cilla et al., 1996). When consumed with food, lovastatin is more efficiently absorbed (Garnett, 1995) with respect to fluvastatin (Smith et al., 1993), atorvastatin (Radulovic et al., 1995), and pravastatin (Pan et al., 1993a), which have a reduced absorption, whereas rosuvastatin (Davidson, 2002), simvastatin (Garnett, 1995), and cerivastatin (Mück et al., 1997) absorption is not affected by food consumption.

Because the liver is the target organ of statins, an efficient first-pass uptake may be more important than high bioavailability to achieve the statin effect. An extensive first-pass extraction implies a low systemic bioavailability; indeed, bioavailability of cerivastatin is approximately 60% (Mück et al., 1997) and that of pitavastatin is 80% (Kajinami et al., 2000), whereas fluvastatin bioavailability ranges from 19 to 29% (Tse et al., 1992). Furthermore, increased doses of fluvastatin enhance the drug circulating levels without time-related changes of its pharmacokinetic profile, thus suggesting a saturable first-pass effect of fluvastatin (Tse et al., 1992; Dain et al., 1993).

Pravastatin is the only statin not bound to plasma proteins; thus, as result of a systemic exposure to unbound drug, the pharmacologically active drug is relatively low (Corsini et al., 1999), and its circulating level is high compared with other statins (Hamelin and Turgeon, 1998).

TABLE 1
Pharmacokinetics of the statins

	Atorvastatin	Cerivastatin	Fluvastatin	Lovastatin	Pitavastatin	Pravastatin	Rosuvastatin	Simvastatin
Dose, mg	40[a]	0.3[a]	20–40[a]	40[a]	2[a]	40[a]	20–40–80[a]	40–60[a]
Dose form	Open acid[a,b]	Open acid[a,b]	Open acid[a,b]	Lactone[a,b]	Open acid[a,c]	Open acid[a,b]	Open acid[a]	Lactone[a,b]
Optimal time of dosing	Any time of day[d]	Evening[d]	Bedtime[d]	With meals morning and evening[d]	Any time of day[c]	Bedtime[d]	Any time of day[d]	Evening[d]
Absorption, %	30[b,c]	98[b,c]	98[b,c]	31[b,c]	80[c]	37[b,c]	50[c]	65–85[b,c]
t_{max}, h	2–4[a,b,c]	2.5–3[a,b,c]	0.5–1.5[a,b,c]	2–4[a,b,c]	1–1.8 [a,c]	0.9–1.6[a,b,c]	3–4[a,c]	1.3–2.4[a,b,c]
Bioavaibility, % [a,b,c,d]	12[a,b,c,d]	60[a,b,c,d]	10–35[a,b,c,d]	<5[a,b,c,d]	>60[a,c,d]	18[a,b,c,d]	20[a,c,d]	<5[a,b,c,d]
Solubility	Lipophilic[d]	Lipophilic[d]	Lipophilic[d]	Lipophilic[d]	Lipophilic[d]	Hydrophilic[d]	Hydrophilic[d]	Lipophilic[d]
Effect of food on bioavailability	↓ 13[b,c,d,e]	No[b,d] ↑ 23[c]	↓ 15–25[b,c,d,e]	↑ 50[b,c,d,e]	NO[d,e]	↓ 30[b,c,d,e]	NO[c,d,e]	NO[b,c,d,e]
Protein binding	>98[c,d,e]	>99.5[b,c,d]	>98[b,c,d,e]	96–98.5[b,c,d,e]	96[c,d,e]	43–54[c,d,e]	88[c,d,e]	>95[b,c,d,e]
logP (*N*-octanol/water partition coefficient)	1.11[a,c]	1.69[a,c]	1.27[a,c]	1.70[a,c]	1.49[a,c]	−0.84[a,c]	−0.33[a,c]	1.60[a,c]
Primary metabolic pathway	CYP3A4[a,b,c,d,e]	CYP3A4 CYP2C8[a,b,c,d,e]	CYP2C9[a,b,c,d,e]	CYP3A4[a,b,c,d,e]	CYP2C9 Minimally[a,c,d,e]	CYP3A4 Minimally[a,b,c,d,e]	CYP2C9 Minimally[a,c,d,e]	CYP3A4[a,b,c,d,e]
Lipid-lowering metabolites	Active[c,e]	Active[c,e]	Mainly inactive[c,e]	Active[c,e]	No[c,e]	Mainly inactive[c,e]	No[c,e]	Active[c,e]
IC_{50}, nM	15.2[c,e]	13.1[c,e]	17.9[c,e]	2.7–11.1[c,e]	6.8[c,e]	55.1[c,e]	12[c,e]	18.1[c,e]
Hepatic excretion, %	>70[b,c,e]	NA	>68[b,c,e]	>70[b,c,e]	NA	46–66[b,c,e]	90[c,e]	78–97[b,c,e]
Renal excretion, %	2[c,d,e]	<30[c,d,e]	6[c,d,e]	30[c,d,e]	<2[c,d,e]	60[c,d,e]	10[c,d,e]	13[c,d,e]
Clearance, $l \cdot h^{-1} \cdot kg^{-1}$	0.25[b]	0.20[b]	0.97[b]	0.26–1.1[b]		0.81[b]		0.45[b]
$t_{1/2}$, h	11–30[b,c,e]	2–3[b,c,e]	0.5–2.3[b,c,e]	2.5–3[b,c,e]	11[b,c,e]	0.8–3[b,c,e]	20[b,c,e]	1.9–3[b,c,e]

[a] Shitara and Sugiyama (2006).
[b] Corsini et al. (1999).
[c] Mukhtar et al. (2005).
[d] Schachter (2005).
[e] Saito (2009).

The solubility profile is a fundamental characteristic that governs the hepatoselectivity of the statins and their inhibitory effect on HMG-CoA reductase. Lipophilic statins enter the hepatocytes by passive diffusion, whereas hydrophilic statin uptake is carrier-mediated (Hamelin and Turgeon, 1998; Nezasa et al., 2003). Lipophilic statins show an efficient activity at both hepatic and extrahepatic sites, whereas hydrophilic statins are more hepatoselective (Hamelin and Turgeon, 1998). The human transporters involved in the hepatic uptake of statins are located either at the basolateral or apical membrane in polarized cells and may be classified as influx (uptake into cells) and efflux (out of cells) transporters. The sequential crossing of the basolateral and apical membranes may require interplay of influx and efflux transporters together with phase I and II metabolism. Indeed, in the liver, organic anion transporting polypeptides (OATP) may transport drug substrates from the portal blood into hepatocytes. In particular, pravastatin, cerivastatin, pitavastatin, rosuvastatin, and atorvastatin are substrates of human OATP1B1, a member of the OATP family (Sirtori, 1993; Hsiang et al., 1999; Shitara and Sugiyama, 2006). In the hepatocytes, other drug transporters, such as multidrug resistance protein, breast cancer resistance protein, and bile salt export pump, may be involved in the metabolite efflux (Ho and Kim, 2005). These mechanisms of transport may represent a crucial step for the statin metabolism and elimination (Niemi, 2007).

C. Metabolism of the Statins in Health and Disease

1. Cytochrome P450-Mediated Metabolism of Statins. In the liver, statin lactones are hydrolyzed to their open acid forms chemically or enzymatically by esterases or paraoxonases (PONs) (Duggan and Vickers, 1990). The open acid form is converted to its corresponding lactone via a CoA-dependent pathway and via glucuronidation by UDP-glucuronosyl transferase (UGT). Both acyl glucuronide and acyl CoA derivatives may return to statin acids by hydrolysis. In addition, whereas statin open acids are irreversibly cleared by β-oxidation and glucuronidation processes, statins as lactone forms rapidly undergo oxidation through the microsomal cytochrome P450 (P450) family of enzymes (Bottorff and Hansten 2000). The CYP3A4 isoenzyme is the major microsomal enzyme that metabolizes many statins, including lovastatin, simvastatin, atorvastatin, and cerivastatin, into active derivates responsible for HMG-CoA reductase inhibition (Lennernäs, 2003). In particular, the major active metabolites of simvastatin are the β-hydroxy acid and its 6′-hydroxy, 6′-hydroxymethyl, and 6′-exomethylene derivatives (Prueksaritanont et al., 2003), whereas for atorvastatin, 2-hydroxy- and 4-hydroxy-atorvastatin acid are reported (Jacobsen et al., 2000). The formation of these active metabolites in *Bacillus megaterium* has been reported to occur through an enzymatic reaction catalyzed by another isoenzyme of cytochrome P450 BM3, CYP102A1 (Kim et al., 2011).

On the other hand, the metabolism of pravastatin in the liver cytosol and in the gastric tract (Quion and Jones, 1994) and of fluvastatin, predominantly occurring through the isoenzyme CYP2C9 (50–80%) and also through CYP3A4 and CYP2C8 (Fischer et al., 1999), produces several inactive metabolites. Likewise, cerivastatin can also be biotransformed by CYP2C8 (Mück, 1998).

Pitavastatin (NK-104), a non–P450-metabolizable statin, is rapidly glucuronized by UGT1A3 and UGT2B7 and then converted to pitavastatin lactone, its major inactive metabolite, by the glucuronic acid elimination reaction (Fujino et al., 2003). Unlike other statins, the cyclopropyl group diverts the drug away from metabolism by CYP3A4 and allows only a small amount of CYP2C9-mediated metabolism (Catapano, 2010).

2. Statin Excretion. Liver and kidney are involved in the elimination of statins from the systemic circulation via the bile into the feces. The hepatic elimination of the statins is limited by their uptake and controlled by the transporters on the basolateral membrane of the liver. Canalicular efflux transporters P-glycoprotein (P-gp) and multidrug resistance-associated protein 2 are two of the major ATP-dependent efflux pumps for statin excretion into the bile. For example, the biliary efflux of rosuvastatin is mediated by multiple transporters multidrug resistance-associated protein 2, multidrug resistance protein 1, and breast cancer resistance protein (Kitamura et al., 2008).

On the other hand, the urinary excretion of statins, except for pravastatin, is quite low. Unlike other statins, up to 60% of intravenously administered pravastatin is excreted in the urine in humans (Hatanaka, 2000). Tubular secretion is the main mechanism involved in the renal excretion of pravastatin and is primarily mediated by the OAT3 transporter. However, when renal elimination is low, the exposure of statins in the liver depends only on the sequestration clearance and is independent of the uptake activity. Instead, when statins, such as pravastatin, undergo significant renal elimination, the increase in the AUC of the plasma concentration does not compensate the reduced hepatic uptake activity, resulting in a weaker pharmacological effect. The half-life elimination of all statins, except atorvastatin and pitavastatin, is very short (0.5–3 h), and drugs do not accumulate in plasma after repeated administrations (Table 1).

3. Factors That May Affect Statin Metabolism. Other factors or their concomitant occurrence may influence the statin metabolism. These factors including race or ethnicity, food intake, age and sex, and concomitant diseases may affect the pharmacokinetic and pharmacodynamic profile of the statins.

a. Race or ethnicity. There is no evidence of clinically relevant interethnic differences in cerivastatin pharmacokinetics in white, black, and Japanese patients after oral therapeutic doses (Mück et al., 1998).

b. Food intake. Concomitant administration of statins with food may alter their pharmacokinetic and pharmacodynamic profile. It has been reported that consumption of pectin or oat bran soluble fiber together with lovastatin reduces its absorption (Metzger et al., 2009), whereas alcohol intake does not affect the efficacy and safety of fluvastatin treatment (Smit et al., 1995). On the other hand, fluvastatin treatment in rats on high-fat and high-sucrose diet was lethal, suggesting that both altered statin metabolism and elimination increase plasma levels of aspartate aminotransferase and creatine kinase, resulting in skeletal muscle toxicity (Sugatani et al., 2010). Moreover, olive oil, consumed in a Mediterranean-style diet, can increase the cholesterol-lowering effect of simvastatin compared with sunflower oil. In contrast, the consumption of polyunsaturated rich oils, through the cytochrome P450 activation, could decrease the half-life of some statins and therefore their cholesterol-lowering effects (Vaquero et al., 2010).

c. Age and sex. The influence of differences in age and sex on pharmacokinetic properties of statins has also been reported. The administration of separate dosage regimens of lovastatin and simvastatin in patients with hypercholesterolemia increases the plasma concentrations of active and total statins only in elderly persons (aged 70–78 years) and in women. However, these age- and sex-related differences do not require modification of dosage regimens, because statin plasma concentrations are not necessarily related to their efficacy and the therapeutic window of lovastatin and simvastatin is quite wide (Cheng et al., 1992).

Likewise, age- and sex-related differences have been reported in the equivalent maximum concentration (C_{max}), in the AUC_{∞}, and in the half-life after the administration of a single dose of atorvastatin (Gibson et al., 1996). In contrast, the pharmacokinetic profiles of pravastatin are not affected by age and sex. Indeed, although the mean AUC of pravastatin is higher in the elderly women, C_{max} and β $t_{1/2}$ values are similar in young and elderly volunteers (Pan et al., 1993b).

Finally, several studies demonstrated that pharmacogenetic variants in HMG-CoA reductase influence the degree of lipid reduction during statin therapies. In particular, patients carrying HMG-CoA reductase single-nucleotide polymorphisms experienced reduced statin sensitivity and smaller reductions in cholesterol, apolipoprotein B, and triglyceride (Chasman et al., 2004; Medina et al., 2008).

d. Concomitant diseases. Statin treatment is required in patients affected by renal and hepatic diseases (Yoshida et al., 2009). However, in pathological conditions of severe renal dysfunction, the elimination kinetic of statins seems to be altered: indeed, plasma levels of total and active lovastatin are increased in affected compared with healthy subjects (Quérin et al., 1991). In contrast, in patients with hyperlipidemia and chronic renal failure subjected to hemodialysis, there was no

evidence of increased accumulation of atorvastatin or its major active metabolite upon multiple dosing, compared with healthy volunteers (Lins et al., 2003). Similar evidence has been also reported for fluvastatin administration (Ichimaru et al., 2004).

In patients receiving long-term dialysis, plasma concentrations of cerivastatin and its metabolites are higher (up to 50%) than in healthy subjects. The half-lives of both parent drug and metabolites remain unaffected without accumulation under repeated dosage. In addition, cerivastatin clearance is not increased by concurrent dialysis as would be predicted from the high plasma protein-binding without significant difference in cerivastatin exposure between the dialysis and the dialysis-free profile days (Mück et al., 2001). Moreover, in patients with end-stage kidney disease undergoing continuous ambulatory peritoneal dialysis, the pharmacokinetic profile of rosuvastatin is very similar to that observed in healthy volunteers; therefore, a lower dose of rosuvastatin may be administered (Bologa et al., 2009).

With regard to hepatic diseases, the steady-state pharmacokinetics of rosuvastatin and its lactone, after the administration of a single dose, are very similar in male patients with liver cirrhosis and male volunteers without liver disease. In contrast, these patients showed increased pitavastatin plasma concentration after administration (Hui et al., 2005).

It is noteworthy that, according to available data, genetic variations in the P450 family of enzymes alter the in vivo availability of many commonly used statins. For instance, gain or loss of catalytic function in the *CYP2C8* gene causes an alteration of cerivastatin metabolic clearance of up to six-fold compared with the wild-type enzyme, altering cerivastatin pharmacokinetics and influencing, at least in part, the susceptibility to the development of myotoxicity (Kaspera et al., 2010). Conversely, a recently discovered polymorphism of CYP3A5 gene seems not to be an important factor in the modification of atorvastatin disposition and pharmacodynamics in humans (Park et al., 2008).

4. Clinically Relevant Drug-Drug Interactions with HMG-CoA Reductase Inhibitors. Statins are commonly well tolerated. The most frequent adverse effects are mild (such as gastrointestinal upset or discolored urine). The major clinical trouble associated with statin therapy is the hepatotoxicity characterized by an increase of hepatic aminotransferases, hepatocellular and cholestatic injury, autoimmune-type reactions, and fulminant liver failure (Liu et al., 2010). In addition, myotoxicity (myalgia, myopathy) occurs in approximately 10% of statin-treated patients, and it may progress to rhabdomyolysis, commonly characterized by massive muscle necrosis, myoglobinuria, and acute renal failure (Williams and Feely, 2002). The rank order of myotoxicity was cerivastatin > simvastatin acid > fluvastatin > atorvastatin > lovastatin acid > pitavastatin ≫ rosuvastatin = pravastatin, without a correlation with their cholesterol-lowering effects (Kobayashi et al., 2008). The adverse effects are generally due to excessive statin dosing or drug-drug interactions that inhibit statin metabolism.

Drug interactions involving statins have been studied since 2001, when the first case of fatal rhabdomyolysis after cerivastatin and gemfibrozil coadministration was reported (Pasternak et al., 2002). The inhibition or induction of P450 isoenzymes, involved in the metabolism of more than 50% of the drugs currently available in clinical practice, is the mechanism responsible for many drug-drug interactions (Bertz and Granneman, 1997).

a. Statins and CYP3A4 inhibitors. Most of the drug interactions with statins result from the inhibition of CYP3A4 enzyme. Indeed, statin binding and thereby its metabolism could be blocked by drugs with a higher affinity for CYP3A4 enzyme. Consequently, the coadministration of these drugs with a CYP3A4-dependent statin leads to an increase of its plasma levels and bioavailability of the statin and of the risk of statin-related side events. Among statins, simvastatin and lovastatin have the highest potential for clinically relevant interactions, followed by atorvastatin (Jacobson, 2004). The coadministration of the CYP3A4 inhibitor itraconazole with simvastatin and lovastatin increases their mean peak concentration and the AUC, causing rhabdomyolysis (Tiessen et al., 2010); this effect is lower on atorvastatin metabolism (Dong et al., 2008).

On the other hand, itraconazole does not interact with statins that are not substrates of CYP3A4 (Cooper et al., 2003) and with cerivastatin, although it is metabolized by CYP3A4 (Kantola et al., 1999) because of the greater contribution of CYP2C8 compared with CYP3A4 on its metabolism (Shitara et al., 2004). Several studies have reported that many substrates of CYP3A4 in the intestinal wall are also substrates of P-gps (Bertz and Granneman, 1997) and significantly contribute to drug interactions with statins (Benet et al., 2003).

b. Statins and calcium antagonists. The effect of calcium channel antagonists on the pharmacokinetics of statins, by inhibition of CYP3A4 and/or P-gp, has been widely reported (Wang et al., 2001). The coadministration of verapamil, a calcium blocker, substrate of both P-gp and CYP3A4 (Döppenschmitt et al., 1999), with lovastatin or simvastatin (Jacobson, 2004) as well as atorvastatin (Hong et al., 2009) increased their plasma concentrations. These interactions are probably caused by the inhibition of CYP3A-mediated metabolism in small intestine or in the liver and P-gp efflux pump in the small intestine (Choi et al., 2009).

Likewise, diltiazem, another calcium channel-antagonist, in combination with simvastatin, lovastatin and pravastatin (Azie et al., 1998), fluvastatin (Choi et al., 2006) and atorvastatin therapy (Hong et al., 2007), increases plasma levels of the statins and the risk of associated rhabdomyolysis and hepatitis (Kanathur et al.,

2001). A novel mechanism of simvastatin interaction with diltiazem, not based on CYP3A4 inhibition, has been proposed. In cardiac and skeletal muscle of rabbits, several biochemical changes, including an increase of serum creatine kinase MB and of troponin I levels (Jasińska et al., 2006) have been described. The massive creatine kinase MB production increases ATP release by depletion of ATP stores, resulting in a secondary insult to the initial muscle damage.

c. Statins and macrolides/ketolide antibiotics. Several macrolides/ketolide antibiotics, including erythromycin, clarithromycin, and azithromycin, are potent inhibitors of CYP3A4 isoenzymes and consequently can increase the plasma concentrations of coadministered CYP3A4-dependent statins (Niemi et al., 2001). Indeed, coadministration of erythromycin with simvastatin, lovastatin, and atorvastatin induces higher plasma concentrations resulting in rhabdomyolysis (Kahri et al., 2004). Unlike erythromycin and clarithromycin, azithromycin does not increase the plasma concentration of atorvastatin (Chiu et al., 2002); indeed, its inhibitory effect is lower (Ito et al., 2003). Moreover, Burtenshaw et al. (2008) outline a case of rhabdomyolysis, probably as a result of interaction of fusidic acid, a bacteriostatic antibiotic, with simvastatin.

d. Statins and protease inhibitors. Statins are used for the treatment of hypercholesterolemia in patients with HIV subjected to a long-term antiretroviral therapy with HIV protease inhibitors (such as indinavir, nelfinavir, ritonavir and saquinavir) (Calza et al., 2008). Several interactions of statins with the protease inhibitors have been described. As an example, coadministration of nelfinavir increases the concentration of simvastatin by more than 500% and consequently the associated risk of skeletal muscle damage. On the contrary, the effect of nelfinavir is moderate on atorvastatin concentrations that are instead increased by a combined therapy with ritonavir and saquinavir (Hsyu et al., 2001). On the other hand, the combination therapy with ritonavir or saquinavir and pravastatin, by inhibition of OATP1A2, reduced the plasma concentration of pravastatin (Cvetkovic et al., 1999) that is instead not affected by the coadministration of raltegravir (van Luin et al., 2010).

e. Statins and organic anion-transporting polypeptide 1B1 inhibitors. Uptake transporters of the OATP (SLCO) family are new additional regulators of drug disposition (König et al., 2000), including fexofenadine, digoxin, rifampicin, methotrexate, nonsteroidal anti-inflammatory drugs (NSAIDs), and HMG-CoA reductase inhibitors. In particular, pravastatin (Hsiang et al., 1999) and cerivastatin are substrates of OATP1B1 (SLCO21A6), a liver-specific uptake transporter.

HMG-CoA reductase inhibitors are used for the management of dyslipidemia in transplant recipient patients subjected to a post-transplantation immunosuppressive therapy with cyclosporine A. Shitara et al. (2003) examined the relative contributions of metabolism versus transport in the clinically observed interaction between cyclosporin A and cerivastatin. The increase of cerivastatin systemic concentrations with cyclosporin A occurs through the inhibition of the hepatic uptake transporter OATP1B1 rather than inhibition of CYP3A4- or CYP2C8-mediated metabolism. In contrast, cyclosporin A increases through OATP1B1 the plasma levels also of non-P450-mediated type of statins such as pravastatin, pitavastatin and rosuvastatin in the clinical situation (Launay-Vacher et al., 2005). Consequently, the statin therapy in cyclosporine A-treated transplant recipients should be initiated at the lower end of the dosage range. In contrast, fluvastatin has a low interaction with cyclosporine A because it is mainly metabolized by CYP2C9 (Holdaas et al., 2006).

A similar mechanism of statin interaction occurs with some oral antidiabetic drugs and has been reported to be responsible for diabetes-related cardiovascular disease. In particular, repaglinide, rosiglitazone, and metformin influence the transport of pravastatin by inhibition of OATP1B1 (Bachmakov et al., 2008). On the contrary, after coadministration of vildagliptin, another oral antidiabetic drug, with simvastatin, no interaction was observed in healthy subjects (Ayalasomayajula et al., 2007).

It is noteworthy that, the pharmacokinetic of nateglinide was investigated in rabbits in the presence of HMG-CoA reductase inhibitors (fluvastatin, lovastatin) and calcium channel blockers (verapamil, nifedipine). Fluvastatin and nifedipine increase the systemic exposure of nateglinide, probably through the inhibition of the metabolism of nateglinide by CYP2C5 (human CYP2C9) (Kim et al., 2010).

f. Other interactions. Interactions between statins and coumarin anticoagulants such as warfarin, fluindione, phenprocoumon, and acenocoumarol have been reported. The enantiomers of warfarin are metabolized by different P450 isoenzymes in the liver: metabolism of (*R*)-warfarin is primarily catalyzed by CYP3A4 and CYP1A2, whereas (*S*)-warfarin is primarily metabolized by CYP2C9. Reduced clearance of both warfarin enantiomers (10–20%) and reduced levels of the 10-hydroxy metabolite (60%) after coadministration of simvastatin or lovastatin have been reported (Hickmott et al., 2003), through CYP3A4 oxidation. Likewise, potential interaction between fluvastatin and warfarin has also been reported in some patients, unlike pravastatin, cerivastatin, and atorvastatin.

In vitro studies have demonstrated that fibric acid compounds (fibrates) such as gemfibrozil interact with the same family of glucuronidation enzymes involved in statin metabolism (Prueksaritanont et al., 2005). As a result of statin glucuronidation inhibition, the coadministration of gemfibrozil with statins generally increases the statin AUC, with the exception of simvastatin, pravastatin, atorvastatin, and rosuvastatin.

The administration of ezetimibe in combination with simvastatin improves the pro-atherogenic lipoprotein profile in subjects with type 2 diabetes (Ruggenenti et al., 2010), in patients receiving continuous ambulatory peritoneal dialysis (Suzuki et al., 2010), and in patients with coronary heart disease who fail to reach recommended lipid targets with statin therapy alone (Rotella et al., 2010). Likewise, coadministration of ezetimibe with rosuvastatin is well tolerated in patients with hypercholesterolemia (Kosoglou et al., 2004). In contrast, no interactions of dalcetrapib, an inhibitor of cholesteryl ester transfer protein, with pravastatin, rosuvastatin, or simvastatin were found in healthy men (Derks et al., 2010).

It is noteworthy that grapefruit juice intake has been described to inhibit simvastatin metabolism. Indeed, its active ingredient, bergamottin, has been shown to increase serum concentrations of lovastatin and its active metabolite (Kantola et al., 1998), as well as that of simvastatin and its active metabolite simvastatin acid (Le Goff-Klein et al., 2003), by inhibition of CYP3A4 in the small intestine. Consequently, bergamottin could be used as a marker to adjust posology in food-drug interaction studies. Moreover, the effect on simvastatin concentration is lower when simvastatin is taken 24 h after ingestion of high amounts of grapefruit juice, compared with concomitant intake of grapefruit juice and simvastatin. This effect dissipates within 3 to 7 days after ingestion of the last dose of grapefruit juice (Lilja et al., 2000). Although grapefruit juice also increases the AUC of atorvastatin, the actual increase in activity is low, probably because of a simultaneous effect of decreasing the AUC of active metabolites of atorvastatin (Saito et al., 2005). On the other hand, no interactions of pravastatin, fluvastatin, and rosuvastatin with grapefruit juice have been reported.

In addition, histopathological studies revealed that ginger reduces liver lesions induced by atorvastatin. Therefore, a combination of ginger with low dose of statins could be useful for the treatment of patients with hypercholesterolemia who are susceptible to liver function abnormalities (Heeba and Abd-Elghany, 2010).

g. Statin interactions with cytochrome P450 inducers. Statin-drug interactions associated with enzyme induction have also been described. Coadministration of drugs that are enzyme inducers with statins reduced statin plasma concentrations and therefore decreased their cholesterol-lowering effects.

As an example, when coadministered with rifampicin or with carbamazepine, the plasma AUC of simvastatin and its metabolite are reduced, through the induction of CYP3A4 (Niemi et al., 2003; Ucar et al., 2004). In addition, rifampicin reduces the AUC of fluvastatin and pravastatin although they are not metabolized by P450, probably by a mechanism that involves the induction of drug transporters.

IV. Effects of the Statins on Tissues and Biological Processes

A. Statins and Immune System

Numerous findings suggest that statins display immunomodulatory effects mainly triggering the major histocompatibility complex (MHC), the costimulatory molecules, the leukocyte migration, and the cytokine network.

1. Statin Effects on the Major Histocompatibility Complex. Statins interfere with the interaction between MHC (class I/class II) and CD8/CD4 required to achieve efficient T-cell activation. Initially, their immunomodulatory action was ascribable to the inhibition of MHC-II molecule; however, a recent clinical trial showed block of T-cell activation markers by atorvastatin (Ganesan et al., 2011). All the statins are able to block interferon-γ (IFN-γ)-induced MHC-II expression on endothelial cells, macrophages, and microglia by a mechanism involving block of the IFN-γ inducible expression of MHC-II transactivator (CIITA) promoter pIV that regulates the MHC-II expression. Another IFN-γ inducible CIITA promoter, promoter I, has also been found to be inhibited by statins (Kwak et al., 2000; Sadeghi et al., 2001; Youssef et al., 2002; Lee et al., 2008); however, simvastatin does not down-regulate CIITA mRNA or activity of the CIITA-PIII or CIITA-PIV promoters in several cells (Kuipers and van den Elsen, 2005a), suggesting that these drugs could regulate multiple promoters. Conflicting data have been reported on the regulation of MHC-I, possibly ascribed to different types of statins, natural or synthetic, and/or the different rate of lipophilicity. For instance, atorvastatin does not affect MHC-I expression on endothelial cells, whereas simvastatin inhibits both IFN-γ-induced MHC-I and also constitutively MHC-I expressed in several cells (Kuipers et al., 2005b). Thus, besides the direct immunosuppressive action, the reduced MHC-II availability might be related to potential therapeutic strategies to promote immune tolerance and decrease the rejection of transplanted organs. Nonetheless statins might find applications in disorders related to aberrant expression of MHC-II (type I diabetes, multiple sclerosis, rheumatoid arthritis) and chronic inflammatory pathologic conditions.

2. Statin Effects on Costimulation. An effective T-cell response requires the assistance of costimulatory molecules interacting with their ligands, such as CD80/CD86, CD28/CTLA4 and CD40/CD154. Statins inhibit constitutive as well as IFN-γ induced up-regulation of costimulatory molecules, CD80, CD86, CD40 on lymphocytes, macrophages, microglia and endothelial cells (Kuipers et al., 2005b, 2006). Indeed, statins suppress the cytokine-induced maturation of dendritic cells, which consequently fail to express these costimulatory molecules and to induce T-cell response (Yilmaz et al., 2004). Statins can elicit their immunosuppressive effects at various stages; however, it remains unknown

whether these effects actually take to immunosuppression in humans.

3. Statin Effects on Adhesion Molecules. Another component of the immunological synapse selectively blocked by the statins is the lymphocyte function-associated antigen-1 (LFA-1) (Weitz-Schmidt, 2003), an α/β heterodimeric receptor belonging to the β2 integrin subfamily that plays a central role in lymphocyte homing and leukocyte trafficking. Initially, lovastatin was shown to block LFA-1 by binding to the allosteric site of the extracellular I domain on the α_L chain (therefore known as the lovastatin site). However, subsequent studies showed that lovastatin derivates inhibited LFA-1 more potently without effect on the HMG-CoA reductase (Welzenbach et al., 2002). The interaction between activated LFA-1 and the intracellular adhesion molecule-1 (ICAM-1) providing signals for both leukocyte migration and costimulation is also blocked by statins. Other adhesion molecules in monocytes and T cells have been shown to be inhibited by statins, ICAM-1, CD11b, CD18, and CD49 (Weitz-Schmidt, 2003). A recent study in patients with acute coronary syndrome confirmed the reduced levels of adhesion molecules ICAM-1 and vascular cell adhesion molecule-1 after short-term atorvastatin preload (Patti et al., 2010). These effects might result in reduced migration and infiltration of the leukocytes along with strongly reduced T-cell activation.

4. Statin Effects on Inflammatory Mediators. Numerous studies suggest inhibitory effects of statins on proinflammatory cytokine production, such as IFN-γ, tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 in several cells, including microglia, astrocytes, and mononuclear cells. These studies also propose a switch from Th1 to Th2 response by statins. However, whether this switch really occurs remains controversial, because several in vitro and in vivo models suggest a statin induction of Th2 cytokines, IL-4, IL-5, IL-10, and transforming growth factor (TGF-β) (Youssef et al., 2002; Zeiser et al., 2007), whereas, in a murine model of inflammatory arthritis, simvastatin suppresses the Th1 response without enhancement of the Th2 response (Leung et al., 2003). Moreover, in experimental autoimmune uveitis, lovastatin suppressed the disease without induction of Th2 (Gegg et al., 2005), whereas in a model of allergic asthma, simvastatin reduced Th2 production in the lung (McKay et al., 2004). Statin also affects the expression of chemokines and their receptors; macrophage inflammatory protein-1α and IL-8 are reduced in peripheral blood mononuclear cells by atorvastatin in patients with coronary artery disease as well as the mRNA expression of the macrophage inflammatory protein-1α receptors CCR1 and CCR2 (Waehre et al., 2003). In normal subjects, a recent study by DNA microarray analysis on human peripheral blood lymphocytes showed that atorvastatin significantly decreased the expression of six cytokines [IL-6, IL-8, IL-1, plasminogen activator inhibitor type, PAI-1, TGF-β1, TGF-β] and five chemokines (CCL2, CCL7, CCL13, CCL18, CXCL1) and affected the expression of many inflammatory genes (Wang et al., 2011). Indeed, other inflammatory mediators are reduced by statins, such as matrix metalloproteinases (Hillyard et al., 2004) and nitric oxide in microglia and monocytes (Cordle and Landreth, 2005). The suppression of the immune response by statins is mainly ascribed to impaired cell activation, adhesion, cross-talk, and trafficking.

5. Molecular Mechanisms of Statin Immunoregulation. The molecular mechanisms of statin immunomodulation often involve multiple pathways along with the regulation of genes encoding key molecules of the antigen presentation and immune regulation. STAT family members represent a statin target. Lovastatin suppression of IFN-γ-induced CD40 expression in microglia is mediated by inhibition of STAT activation (Townsend et al., 2004); atorvastatin decreased the phosphorylation of STAT-4 and induced STAT-6, required for Th1 and Th2 commitment, respectively (Youssef et al., 2002). Another mechanism involves the down-regulation of the nuclear factor-κB (NF-κB) encoding the transcription of many immune genes such as MHC-1, chemokines, interferon-inducible protein-10, monocyte chemoattractant protein 1 (MCP-1), and COX-2. It was suggested that atorvastatin reduces these chemokines by inhibition of NF-κB activation (Martín-Ventura et al., 2005; Li et al., 2010). Statins are also able to disrupt lipid raft structures whose main component is cholesterol. This finding showed the relevance of rafts in the immune cell signaling, because several surface molecules are found in lipid rafts, and their association increases their local concentration at the level of the immunological synapse (He et al., 2005). Another mechanism of immunomodulation is the regulation of isoprenylated proteins such as Rho and Rac and their function (Greenwood et al., 2006). Simvastatin suppresses T-cell activation and proliferation by selectively impairing the Ras/MAPK pathway (Ghittoni et al., 2005). Several mechanisms can contribute to the immunomodulatory effects of statins; however, the precise mode of action is still an open issue.

B. Statins and Endothelial Function

1. Statins and Angiogenesis. Improvement of endothelial function and vasculoprotective action are well recognized statin pleiotropic effects. Statins have been reported to protect the brain from ischemic strokes and ischemia-reperfusion injury of the heart in animal models (Endres et al., 1998) and to increase blood flow, ameliorating vasomotor response in patients (Dupuis et al., 1999). Simvastatin administration induced neovascularization both in vitro and in the ischemic limbs of normocholesterolemic rabbits, through increased endothelial nitric-oxide synthase (eNOS) activity mediated by Akt pathway (Kureishi et al., 2000). The induction of

the angiogenic response is a protective physiological mechanism against ischemia and hence is considered a therapeutic strategy for coronary artery and peripheral vascular diseases. On the other hand, pathological angiogenesis is involved in the pathogenesis of cancer, atherosclerosis, diabetic retinopathy, rheumatoid arthritis, and other diseases. Statins were able to inhibit tumor-induced angiogenesis in mice and neovascular growth both in vitro and in vivo, through RhoA-dependent inhibition of vascular endothelial growth factor receptor (VEGFR), Akt, and focal adhesion kinases (Feleszko et al., 1999; Park et al., 2002). Actually, a dual effect of statins on angiogenesis is reported and explained by a dose-dependent biphasic effect: low doses (between 0.005 and 0.05 μM) are proangiogenic and induce the PI3K/Akt pathway, leading to eNOS activation, and high doses (>0.05 μM) are antiangiogenic and induce apoptosis and VEGF down-regulation. In murine models, low-dose statin therapy (0.5 mg/kg/dose) induced angiogenesis, whereas high concentrations of cerivastatin or atorvastatin (2.5 mg/kg/dose) were inhibitory (Weis et al., 2002). Because the serum levels reached by statins in patients range from 0.002 to 0.1 μM (Desager and Horsmans, 1996), a standard statin therapy might induce rather than inhibit neovascularization. Some exceptions to the biphasic theory have been reported; for instance, in swine, the same dose of simvastatin was proangiogenic in the ischemic kidney and antiangiogenic in early coronary atherosclerosis (Wilson et al., 2002; Chade et al., 2006). In the same animal and at the same dose, statins inhibited atherosclerosis progression by block of atheroma neovascularization and stimulated angiogenesis in the ischemic hind limb, meanwhile being effective to inhibit xenograft tumor growth (Sata et al., 2004). Moreover, cerivastatin was able to stimulate collateral vessel development after ischemia, even at a dose 1000-fold higher than those reported for serum statin levels in patients. On the other hand, the pro- and antiangiogenic effects might be related to the specific angiogenic stimulus, the mechanism of angiogenesis (physiological, pathological, inflammatory), and the local microenvironment (Sata et al., 2004). Low doses of simvastatin stimulated angiogenesis triggered by hypoxia, whereas inhibited tumor necrosis factor α-induced inflammatory angiogenesis. It is noteworthy that high doses of simvastatin (10 μM) inhibited angiogenesis under both conditions, probably as a result of cytotoxic effects. Inflammatory angiogenesis was inhibited by atorvastatin at both low and high doses (Araújo et al., 2010). The inhibitory effect of statins has been reported only when angiogenesis is stimulated by specific proangiogenic or inflammatory mediators (Vincent et al., 2002). On the contrary, statins may act in synergism with proangiogenic stimuli, such as hepatocyte growth factor and endothelial progenitor cells, stimulating angiogenesis (Uruno et al., 2008). Statin ability to inhibit angiogenesis in pathological setting could be a useful tool to contrast atherosclerosis as a result of plaque stabilization, cancer progression, and retinal angiogenesis. In this frame, statins could be able to promote collateral vessel growth in ischemic tissues, without proangiogenic effects or even being antiangiogenic in the atherosclerotic plaque (Sata et al., 2004). Fluvastatin has been reported to prevent retinal neovascularization through down-regulation of STAT3 and hypoxia-inducible factor-1α and VEGF signaling (Bartoli et al., 2009). Statins may also exert beneficial effects on endometriosis, because inhibiting the proliferation of endometrial stroma affects both the angiogenic and inflammatory processes (Bruner-Tran et al., 2009).

2. Statins and Endothelial Dysfunction. Endothelial dysfunction has been recognized as an independent predictor of cardiovascular disease risk. All statins significantly ameliorate endothelial dysfunction in patients with coronary artery disease (CAD) (Järvisalo et al., 1999) through low-density lipoprotein cholesterol (LDL-C)-lowering effect and pleiotropic actions such as eNOS up-regulation and nitric oxide (NO) production; through Akt activation; and through inhibition of Rho prenylation, antioxidant, and anti-inflammatory effects. Atorvastatin increased NO availability, prevented the production of oxygen free radicals, and down-regulated the expression of COX-2 and the production of the contracting prostanoid 8-isoprostane (Virdis et al., 2009). Long-term pravastatin treatment in spontaneously hypertensive rats improved blood pressure, restored endothelial function, and decreased oxidative stress (Kassan et al., 2009). Pitavastatin treatment in long-term smokers was associated to reduced LDL-C oxidation and protection of endothelium from oxidative stress (Yoshida et al., 2010). In patients with stable CAD, pitavastatin ameliorated postprandial endothelium-dependent vasodilation, inhibiting oxidative stress (Arao et al., 2009). Moreover, pravastatin and fluvastatin had a direct scavenging radical activity (Yamamoto et al., 1998; Kassan et al., 2010). Pravastatin was also reported to inhibit the stimulatory activity of angiotensin II on NADPH oxidase, thereby contrasting the production of superoxide radicals (Alvarez et al., 2010).

Endothelial apoptosis is associated with endothelial dysfunction and is involved in the pathophysiology of atherosclerosis, leading to plaque erosion and thrombosis (Bombeli et al., 1997). Short-term atorvastatin treatment in patients with CAD was reported to be regenerative on the endothelium, through the inhibition of endothelial apoptosis (Schmidt-Lucke et al., 2010), even induced by hyperhomocysteinemia (Bao et al., 2009). On the other hand, high micromolar concentrations of statins, 100- to 200-fold higher than serum statin levels in patients, have been reported to induce apoptosis (Katsiki et al., 2010). Moreover, the inhibition of ubiquinone synthesis by statins, which is essential for a proper mitochondrial function, might be responsible for mitochondrial dysfunction, which has been proposed as a

possible cause of statin-induced myopathy, suggesting the possibility of contrasting such detrimental effect with supplements of ubiquinone (Dai et al., 2010).

Several pharmacological agents, called preconditioning agents, are able to protect the endothelium from the damage triggered by ischemia-reperfusion. The preconditioning potential of statins is multifactorial, because they up-regulate several enzymes, including ecto-5′-nucleotidase, eNOS and COX-2 (Liuni et al., 2010). Statins are also able to induce a postconditioning effect; that is, the protection of a tissue that suffered an intense ischemic episode. Post-treatment with simvastatin or atorvastatin protected from oxygen and glucose deprivation, stimulating reperfusion in endothelial cells (Wu et al., 2010). Endothelial cells under a disturbed proatherogenic blood flow show increased apoptosis and oxidative stress, eNOS inhibition, altered leukocyte adhesion, and LDL-C permeability (Berk, 2008). Atorvastatin induced the vasculoprotective heme oxygenase-1 expression through the Akt pathway, mainly at sites of laminar stress (Ali et al., 2009a). Endothelial response to statins could be therefore affected by wall shear stress, because it has been recently observed that the protective action of simvastatin depends on the hemodynamic forces, being compromised by low shear stress with reversing flow (Rossi et al., 2011).

3. Statins and Endothelial Progenitor Cell Biology. Bone marrow-derived endothelial progenitor cells (EPC) in peripheral blood express CD34, CD133, and VEGFR2 markers, possess a regenerative potential, and are able to differentiate into mature endothelial cells (Asahara et al., 1997). Neither ischemia- nor cytokine-induced mobilization of EPC, as well as ex vivo expansion and reinfusion in animal models, has been shown to promote new blood vessel formation in the injured areas, enhancing perfusion, and leading to recovery of ischemic tissue (Takahashi et al., 1999). Statins promote the mobilization of hematopoietic progenitor cells from the bone marrow and increase EPC proliferation, survival, and functional activity (Dimmeler et al., 2001; Llevadot et al., 2001). Statins increased EPC levels with a peak at 3 to 4 weeks of treatment (Vasa et al., 2001), whereas a treatment >4 weeks augmented the late EPC population, which displays higher proliferative potential than early EPC subset (Deschaseaux et al., 2007). Intensive statin treatments (80 versus 20 mg of atorvastatin) have been associated with higher EPC numbers (Leone et al., 2008), whereas longer standard therapeutic regimens (>8 weeks) have been associated with a reduction in EPC count in the peripheral blood (Hristov et al., 2007), probably because of the increased incorporation of the mobilized EPC into injury sites. The effects of statins on EPC could be due to their pleiotropic activity, because, at least in animal models, no significant changes of serum cholesterol levels were reported. However, a modified diet and lifestyle leading to cholesterol reduction also enhance EPC number (Umemura and Higashi, 2008). Potential molecular mechanism of statin action on EPC might involve the PI3K/Akt pathway (Dimmeler et al., 2001) and the inhibition of apoptosis (Urbich et al., 2005). The essential role of eNOS for mobilization of bone marrow–derived stem and progenitor cells has been ascertained; indeed the beneficial effects of atorvastatin on EPC were abolished in eNOS(−/−) mice (Landmesser et al., 2004). Moreover, the adverse effects of oxidized LDL-C, a known risk factor for CAD, on the functionality of EPC is reverted by statin treatment through the Akt/eNOS pathway (Ma et al., 2009). A limit in EPC cell therapy in humans is their rapid senescence during ex vivo expansion procedures as a result of low telomerase activity. An advantage of statins is their ability to prevent senescence, through a mechanism dependent on protein prenylation (Assmus et al., 2003) and the induction of telomere repeat-binding factor 2 (Spyridopoulos et al., 2004). Compared with cytokines or chemokines able to regulate EPC number, such as granulocyte-colony stimulating factor, statins improve re-endothelialization after balloon injury or carotid artery injury, also inhibiting neointimal thickening (Walter et al., 2002; Werner et al., 2002) and avoiding restenosis (Kang et al., 2004). The positive effect on re-endothelialization induced by fluvastatin treatment after implantation of sirolimus-eluting stents, is due in part to the increased mobilization of EPC (Fukuda et al., 2009). An innovative stent technology designed to trap CD34+ cells has been recently introduced into the clinic (Klomp et al., 2011), and the therapy with high doses of atorvastatin (80 mg) before stent implantation was reported to enhance the number of trapped EPC (Hibbert et al., 2011).

The therapeutic potential of a pharmacological strategy aimed to enhance EPC number and functions may extend also to other pathological conditions, such as systemic sclerosis, characterized by low EPC levels and inadequate recruitment to sites of vascular injury (Mok et al., 2010). It is noteworthy that statin treatment was reported to transiently increase the EPC pool in patients affected by systemic sclerosis (Kuwana et al., 2009).

C. Statins and Vascular Smooth Muscle Cell Function

The phenotypic switching of vascular smooth muscle cells (SMCs) from contractile to synthetic state is critical for vascular repair but is also involved in vascular proliferative diseases (Owens et al., 2004). Statins have been reported to inhibit SMC proliferation, migration, and invasion in a way prevented by the recovery of the isoprenoid pathway intermediates and not by cholesterol (Corsini et al., 1993; Erl, 2005). In particular, the inhibition of Rho prenylation seems a predominant mechanism by which statins affect SMC functions (Laufs et al., 1999). Lipophilic statins have been shown to induce apoptosis directly or to sensitize SMC to apoptotic inducers. Hydrophilic statins seem to protect from apoptosis. However, the apoptotic effect is present at

doses higher than those administered in the clinical practice (Katsiki et al., 2010) and has been observed exclusively in cell culture studies, because in the spontaneously hypertensive rat, atorvastatin was unable to induce aortic SMC apoptosis (Doyon et al., 2011). Low doses of fluvastatin exerted a cytoprotective effect against oxidative stress, whereas higher doses were pro-apoptotic, suggesting a potential biphasic effect (Makabe et al., 2010).

Injury-induced SMC proliferation and migration in the arterial wall is a principal feature of restenosis after angioplasty and stent coronary implantation. The drug-eluting stents, coated with the antimitotic paclitaxel or the immunosuppressive agent sirolimus, reduced the rate of restenosis and improved patient outcome (Inoue and Node, 2009). However, a major issue about the efficacy and safety of this approach is the negative impact of these compounds on endothelial proliferation, which could result in late thrombotic events. Because statins improve endothelial function and re-endothelialization through EPC mobilization and display direct inhibitory effects on SMC, they could be the "gold standard" for the new generation of drug-eluting stents. Indeed, beyond the efficacy of statins to inhibit neointimal thickening in experimental models of angioplasty (Preusch et al., 2010), observational studies in large cohorts of patients have shown that both pre- and postoperative statin treatment decreases neointimal thickening and restenosis after successful stent implantation (Corriere et al., 2009; Takamiya et al., 2009). It is noteworthy that a synergistic antiproliferative effect of fluvastatin and everolimus on SMC has been demonstrated in vitro (Ferri et al., 2008). Moreover, atorvastatin inhibited the PDGF-induced expression of Nur-77, a nuclear orphan receptor overexpressed by neointimal SMC after angioplasty (Wang et al., 2010b), which indeed could be a new putative target of statins. However, an oral statin therapy has been reported to not so efficiently inhibit in-stent restenosis (Verzini et al., 2011), probably as a result of insufficient local concentrations at the injury site. Cerivastatin-eluting stents display a safe profile and better efficacy in animal models (Jaschke et al., 2005; Miyauchi et al., 2008). A polymer-free cerivastatin drug-eluting stent based on the new technique of bioabsorbable "sol-gel" has been shown to inhibit neointimal thickening more efficaciously than the routinely used a polymer-based paclitaxel-eluting stent (Pendyala et al., 2010).

Hypertension alters the vascular structure through imbalance of SMC proliferation and apoptosis that is normalized by antihypertensive drugs (Deblois et al., 2005). In animal models of hypertension, long-term statin administration improved blood pressure and contributed to the normalization of vessel wall (Doyon et al., 2011). It is noteworthy that a synergism between statins and antihypertensive drugs has been observed in several clinical trials. Atorvastatin reduced primary events of CAD by 35% versus placebo group; this effect was augmented up to 53% in combination with the calcium channel blocker amlodipine (Clunn et al., 2010). In spontaneously hypertensive rats, quinapril administered in combination with atorvastatin lowered blood pressure, ameliorating cardiac and vessel function and hypertrophy, through increased rates of SMC apoptosis (Yang et al., 2005). Moreover, statins, promoting the dedifferentiation of SMC, could up-regulate the expression of calcium channels, thereby reverting the loose of efficacy of calcium channel blockers that occurs with disease progression (Clunn et al., 2010). Simvastatin per se has been reported to block calcium entry through the inhibition of Rho/Rho kinase (Pérez-Guerrero et al., 2005). Statins have been also reported to protect from pulmonary arterial hypertension, reducing neointimal thickening and improving endothelial dysfunction and inflammation, in hypoxic, high pulmonary blood flow and embolism conditions (Nishimura et al., 2003; Girgis et al., 2007). Simvastatin inhibited platelet-derived growth factor-induced proliferation and migration of SMCs isolated from the lungs of patients undergoing lung transplant as a result of idiopathic pulmonary arterial hypertension (Ikeda et al., 2010).

An intensive field of research is represented by the possibility to target airway SMCs for asthma treatment. Indeed, asthma is characterized by hyperplasia and hypertrophy of airway SMCs, which may exacerbate airway narrowing and contribute to airway remodeling and inflammation (Camoretti-Mercado, 2009). With the exception of bronchial thermoplasty, which partially removes airway muscle mass, there are no therapeutic approaches targeting airway SMCs in asthma. Statins inhibited the proliferation of airway SMCs though RhoA (Takeda et al., 2006). In murine models of allergic airway inflammation and asthma, lovastatin administration decreased the magnitude of inflammatory cell infiltrate (McKay et al., 2004) and improved airway SMC hyper-reactivity through RhoA inhibition (Chiba et al., 2008).

D. Statins and Platelet Function

Some of the statin effects in reducing cardiovascular events can be ascribed to their ability to prevent thrombus formation by exerting modulatory effects on blood coagulation cascades, profibrinolytic mechanisms and platelet functions. One of the first effects reported is the reduction of the cholesterol content of the platelet membrane, which results in low cytosolic Ca^{2+} levels (Le Quan Sang et al., 1995) and intraplatelet pH modifications (Puccetti et al., 2002), as well as in decreased biosynthesis of thromboxane A_2 (Kaczmarek et al., 1993; Notarbartolo et al., 1995). The reduced platelet activity under statin treatment might also be due to its inhibitory effect on Rho-GTPase family such as Rap-1b members (Kaneider et al., 2002; Rikitake and Liao, 2005;) and on the activity of other important signaling mole-

cules, such as Erk2, NF-κB, and Akt, which have the capacity to affect platelet function (Mitsios et al., 2010).

Statins can also decrease platelet activation by modulating the NO bioavailability in platelets (Laufs et al., 2000; Haramaki et al., 2007; Lee et al., 2010) and rapidly reducing the CD36 and lectin-like ox-LDL receptor-1 (Mehta et al., 2001; Puccetti et al., 2005), specific receptors for ox-LDL that are considered potent platelets agonists. Furthermore, statins inhibit the platelet-induced tissue factor expression by monocytes and macrophages (Puccetti et al., 2000), counteracting the prothrombotic complications of atherosclerosis (Aikawa et al., 2001). In this context, statins, such as agonists of PPAR-α and -γ are also highly effective in reducing the platelet-mediated foam-cell generation via inhibition of matrix metalloproteinase 9 secretion (Daub et al., 2007). Moreover, statins inhibit collagen-induced platelet CD40 ligand (CD154) expression and release (Sanguigni et al., 2005; Pignatelli et al., 2007), whose high levels have been found in atherothrombosis and in the major adverse cardiovascular events (Aukrust et al., 1999; Garlichs et al., 2001; Cipollone et al., 2002; Heeschen et al., 2003; Semb et al., 2003; Varo et al., 2003). Just through this molecule, platelets can interact with endothelium and, at the same time, quickly activate CD40-bearing immune cells and platelets themselves (Henn et al., 1998; Prasad et al., 2003; Zhang et al., 2011). Statins and fibrates, by activating the PPAR system in platelets (Ali et al., 2009b), may dampen the release of proinflammatory/prothrombotic mediators and aggregation (i.e., CD40L, thromboxane A_2, IL-1β) (Phipps and Blumberg 2009; Marx et al., 2003). The protease-activated receptor-1 inhibition by statins study (Serebruany et al., 2006) has suggested for the first time that statins can also specifically target platelet thrombin protease-activated receptor-1, thereby modulating antiplatelet and antithrombotic properties. Finally, several statins exhibited an in vitro and in vivo inhibitory effect of the platelet-activating factor (Tsantila et al., 2011) and, more importantly, can also exert their antiplatelet effects by reducing platelet adhesion to the vessel wall or the endocardium (Tailor et al., 2004; Schäfer et al., 2005; Chello et al., 2008; Molins et al., 2010). Beyond platelets, statins may inhibit plasmatic pathways of thrombus formation (Undas et al., 2005) and may affect fibrinolytic pathways (Bourcier and Libby, 2000).

The first strong evidence of potential association between statin administration and reduced risk of thromboembolism has come from a case control study in postmenopausal women (Doggen et al., 2004) in which statin administration was associated with a slightly lower risk of venous thrombosis. Other case control studies (Lacut et al., 2004; Ramcharan et al., 2009; Sørensen et al., 2009) have also shown reduction in the risk of venous thrombosis ranging from 26 to 58%. On the other hand, two additional observational studies showed no association between the use of statins and the risk of venous thrombosis (Yang et al., 2002; Smeeth et al., 2009). However, the recent randomized double blind Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) study showed that rosuvastatin significantly reduced the occurrence of symptomatic venous thromboembolism in apparently healthy subjects with no significant differences between treatment groups in the rates of bleeding episodes (Glynn et al., 2009). This finding is in contrast with the previously registered protective effect of long-term statin use against the risk of bleeding in warfarin users (Atar et al., 2006; Douketis et al., 2007). Given the success of statins in preventing cardiovascular events and their promising antiplatelet and antithrombotic action, especially in CAD progression and regression (Heart Protection Study Collaborative Group, 2002; Nissen et al., 2004; Walter et al., 2010), they have been tested or are still under evaluation for efficacy outside the cardiovascular system in some related conditions characterized by increased platelet activation and risk of thrombotic events, such as in diabetes (Watala et al., 2007) and in subjects with hypercholesterolemia (Davì et al., 1992; Opper et al., 1995). Diabetes has a major impact on morbidity and mortality because of cardiovascular atherothrombotic events (Tschoepe et al., 1997; Resnick et al., 2000). Rosuvastatin treatment has been demonstrated to normalize endothelial function and reduce platelet activation in diabetic rats, which may account for the reduction of cardiovascular events by statins in patients with diabetes (Schäfer et al., 2007). In fact, in the recently completed Collaborative Atorvastatin Diabetes Study (CARDS), atorvastatin treatment resulted in 48% reduced relative risk of stroke in patients with diabetes without history of coronary artery disease (Colhoun et al., 2004). It is noteworthy that the effect of atorvastatin in patients with type 1 diabetes characterized by high levels of procoagulant platelet-derived microparticles (Mobarrez et al., 2010) resulted in efficient reduction by statin therapy (Tehrani et al., 2010). Multiple effects of statin treatment have been also described in hypercholesterolemia, including reversal of hypercholesterolemia-associated platelet activation and reduction of platelet reactivity, thromboxane biosynthesis, thrombin generation and aggregation, and thrombogenic potential (Takemoto and Liao, 2001; Thompson et al., 2002). Statins might have beneficial effects also in reducing arterial thrombosis and cardiovascular risk in postmenopausal women subjected to hormone therapy (Peverill et al., 2006; Canonico et al., 2008).

In conclusion, these data on statins are quite promising; however, it remains to be determined to what extent these pleiotropic effects account for a potentially beneficial statin therapy in the clinical setting. It is noteworthy that a large population-based cohort study, examining a range of clinical outcomes found to be positively or negatively associated with statins, failed to confirm a

protective effect of statins on the risk of venous thromboembolism (Hippisley-Cox and Coupland, 2010). However, in our opinion, this prospective study was characterized by more potential confounders, and a different cut-off of statical significance used for the analysis ($p < 0.01$) might have underestimated the potential positive secondary effects of statins.

E. Statins and Metabolism

Recent randomized controlled trials and meta-analyses focused on the effects of different regimens of statin therapy in patients with coronary artery disease or at risk for cardiovascular events (Josan et al., 2008). The Cholesterol Treatment Trialists collaboration (Baigent et al., 2005, 2010) reported data from two cycles of meta-analyses, the first on 14 randomized clinical trials and the second on a total of 26 clinical trials including 170,000 participants. They found that for every 1 mM reduction of serum LDL-C achieved during standard statin therapy (e.g., 20–40 mg/day simvastatin), there was a proportional reduction of approximately 20% in the 5-year incidence of major coronary events (Baigent et al., 2005). More intensive statin treatments or the use of more potent and newer statins (40–80 mg/day atorvastatin or 10–20 mg/day rosuvastatin) resulted in a further reduction of approximately 15 percentage points in cardiovascular events (Baigent et al., 2010). Authors did not report evidence of any significant increase of adverse effects in statin-intensive trials compared with standard therapy. These findings, together with the observations by Josan et al. (2008) that the effects of a more intensive statin therapy (80 mg/day atorvastatin alone or in combination with antioxidant vitamins) is more efficacious than standard therapies (e.g., 40, 20, or 10 mg/day atorvastatin) in decreasing LDL-C levels, strongly suggest that targeting LDL-C is essential to reduce cardiovascular morbidity and mortality. Low HDL-C and elevated triglyceride content are a common pattern in patients with type 2 diabetes, metabolic syndrome, or obesity and account for the prevalence of cardiovascular events in these pathologic conditions (Bell et al., 2011). Some trials, analyzing the effects of statins in patients with diabetes, showed a significant decrease in cardiovascular events (Cziraky et al., 2008). The Collaborative Atorvastatin in Diabetes Study (CARDS) reported that 10 mg/day atorvastatin reduced cardiovascular disease outcomes by 37% in patients with type 2 diabetes without previous history of cardiovascular disease, with a mean decrease in LDL-C levels of 46 mg/dl and a mean triglyceride level decrease of 35 mg/dl (Colhoun et al., 2004). In contrast, other reports suggested that some statins such as lovastatin are almost ineffective in reducing tryglicerides, lipoprotein(a), or enhancing HDL-C plasma levels, although statin treatment was still efficacious in reducing cardiovascular events (Cziraky et al., 2008; Jialal and Bajaj, 2009). This provides a rationale to use combined therapy with fibrate or niacin to achieve either LDL-C- and triglyceride-lowering or HDL-C-enhancing goals in the management of diabetic dyslipidemia and metabolic syndrome (Cziraky et al., 2008; Jacobson, 2011). Few but rising studies explored the effects of statins on diabetic kidney disease. Data from trials involving patients with severe kidney disease showed modest beneficial effect either of atorvastatin or rosuvastatin therapy on cardiovascular events (Wanner et al., 2005; Fellström et al., 2009). The Collaborative Atorvastatin in Diabetes Study (CARDS) study group (Colhoun et al., 2009) analyzed the effects of atorvastatin on estimated glomerular filtration rate (eGFR) and albumin excretion rate in patients with diabetes. A moderate beneficial effect of statin therapy on eGFR was observed with an improvement of 0.18 ml/min per 1.73 m^2 in the annual rate of change. The improvement in eGFR rate reached 0.38 ml/min per 1.73 m^2 in the subjects with albuminuria. Nevertheless, independent of kidney disease stage, atorvastatin reduced cardiovascular disease endpoints (coronary events, revascularizations, and stroke) in these patients (Colhoun et al., 2009). The Long-term Intervention with Pravastatin in Ischemic Disease (LIPID) trials also reported a slight efficacy of pravastatin on eGFR (Tonelli et al., 2005). It has been hypothesized that the modest or absent effect of statins might be due to high rate of angiotensin-converting inhibitors used in patients with diabetes (Colhoun et al., 2009). It is noteworthy that data from the JUPITER trial (Ridker et al., 2008) indicated a significant decrease of eGFR after statin therapy at 1 year. However, it remained to be established whether changes in eGFR observed in the trials are the consequence of a permanent effect on kidney function or reflect transient effect on plasma creatinine levels (Colhoun et al., 2009). An analysis on 3 years of follow-up from large Veterans Integrated Service Network database (VISN 16) estimated that patients under statin therapy had 13% decrease in the odds of developing kidney disease (Sukhija et al., 2008). A recent study from the same group reported that statin use is associated with an increase of fasting plasma glucose (FPG) in patients with and without diabetes. In particular, among patients with diabetes, FPG increased with statin use from 102 to 141 mg/dl and among nonusers from 100 to 129 mg/dl. This relationship between statin use and FPG seems to be independent of age and use of aspirin, β-blockers, and angiotensin-converting enzyme inhibitors (Sukhija et al., 2009). More in vitro and in vivo studies and further meta-analyses are required to ascertain a possible positive/negative effect of statins on glucose metabolism. However, the results from the major clinical trials suggest that statin mono- or combined therapy might be useful not only to reduce LDL-C levels but also to improve several dyslipidemia and diabetic endpoints delaying renal dysfunction.

F. Statins and Bone

The ability of statins to influence bone metabolism was first reported by Mundy et al. (1999), who screened a library of more than 30,000 natural compounds for osteoinductive substances. Only lovastatin was found to have this effect, with the consequent ability to stimulate new bone formation both in vitro, as observed in cultures of neonatal murine calvaria, and in vivo in animal models of postmenopausal osteoporosis. Similar effects were found with the lipophilic statins (simvastatin, mevastatin, and atorvastatin) (Sugiyama et al., 2000) that also now seem to be more effective than the hydrophilic statins (rosuvastatin and pravastatin) in protecting bone (Uzzan et al., 2007). In a bisphosphonate-like manner, statins can also inhibit osteoclasts activation by preventing mevalonate production, which leads to the loss of prenylation of small GTPases and, consequently, disruption of downstream intracellular signaling pathways in osteoclasts (Dunford et al., 2006; Hughes et al., 2007). Moreover, statins can finely modulate the osteoprotegerin/receptor activator of NF-κB/receptor activator of NF-κB ligand system that is a critical determinant for maintenance of skeletal integrity (Kaji et al., 2005; Ahn et al., 2008a). The bone anabolic action of statins also involves an increased expression and synthesis of osteocalcin by reducing the inhibitory effect of Rho-associated kinase in human osteoblasts (Ohnaka et al., 2001). Statins are also able to partially suppress osteoblast apoptosis through a TGF-β–Smad3 pathway (Kaji et al., 2008) and regulation of estrogen receptor α expression (Park et al., 2011). Moreover, the proliferation and recruitment of osteoprogenitor cells, critical steps in the early stages of bone healing, were enhanced by simvastatin-stimulated TGF-β1 and bone morphogenetic protein-2 (Nyan et al., 2010). In addition to direct effects on bone, statins may increase bone formation by other indirect actions. Vascular invasion is a prerequisite for calcification during endochondral bone formation (Gerber et al., 1999); thus, the well established proangiogenic effect of statins might increase bone formation. Statins may also affect bone formation indirectly by inhibiting inflammation that is responsible for an imbalance in bone metabolism by favoring bone resorption (Tikiz et al., 2004; Tanaka et al., 2005).). It is noteworthy that Yavuz et al. (2009) have described an interesting relationship between statins and the vitamin D physiology that might represent a new pleiotropic effect of this class of drugs with great bone anabolic potential.

Of course, the next major question that arises is whether statins really would have beneficial effects on human bone by increasing bone mineral density (BMD) and consequently reducing fracture risk. Edwards et al. (2000)) published the first study in postmenopausal women to indicate a significant increase in BMD associated with statin administration. Next, statins have also been shown to exhibit a protective effect against nonpathological fractures among older women (Chan et al., 2000; Chung et al., 2000; Meier et al., 2000; Wang et al., 2000c). With regard to the effects of statins on BMD, more recent evidence came from results of the studies on this endpoint in patients in treatment with statins for hypercholesterolemia. Overall, patients taking statins have a higher femoral bone mass density (by a mean ± 0.2 S.D.) (Safaei et al., 2007; Uysal et al., 2007; Uzzan et al., 2007; Pérez-Castrillón et al., 2008; Tang et al., 2008). However, these studies have been conducted on small case series, so differences identified are minimal and fail to reach statistical significance (Luisetto and Camozzi, 2009). A recent large, randomized, placebo-controlled trial of atorvastatin showed instead a negative effect on bone mineral density and bone markers in dyslipidemic postmenopausal women (Bone et al., 2007), confirming data obtained in other past studies (Bjarnason et al., 2001; Stein et al., 2001; Braatvedt et al., 2004). A systematic review by Yue et al. (2010) of all randomized controlled trials involving postmenopausal women (3022 subjects) found that statin use does not prevent fractures or increase bone density in these subjects. At the same time, a recent prospective randomized control trial study enrolling 212 patients with hyperlipidemia and osteopenia has received particular attention in view of the positive effect of simvastatin to significantly increase bone mineral density and bone markers (serum c-telopeptide of type 1 collagen and N-terminal propeptide of procollagen type 1) even though, like many others, this study also suffers from some limitations and confounders that do not clarify whether statins are beneficial in either preventing and/or slowing bone loss in the aging osteoporotic population (Chuengsamarn et al., 2010). No clinical trials focusing on the statin effects on the reduction of fracture risk have been reported. In 2000, a first observational study found an inverse association between hip fractures and statin use (Wang et al., 2000c). After that, small retrospective studies (Chung et al., 2000; Meier et al., 2001; Pasco et al., 2002; Scranton et al., 2005) and a meta-analysis (Bauer et al., 2004) showed a lower risk of fractures. At the same time, a randomized trial (Bone et al., 2007) and three large population-based studies (van Staa et al., 2001; LaCroix et al., 2003, 2008) together with two previous cardiovascular prevention trials (Pedersen and Kjekshus, 2000; Reid et al., 2001), analyzed a posteriori, showed no benefits. These negative findings were recently confirmed by a very large population-based cohort study conducted to assess the effect of statins on a range of health outcomes (Smeeth et al., 2009). Likewise, in the last few months, another large population-based cohort study failed to confirm a protective effect of statins on the risk of osteoporotic fractures (Hippisley-Cox and Coupland, 2010). These disparate results can be explained by different possible reasons: differences in trial design; insufficiently large control group; patient identification methods; statin use definitions; insufficient dose to affect

bone; insufficient treatment duration; inclusion and exclusion criteria; and confounding factors controlled for obesity, physical activity, use of other drugs and comorbidities, diagnostic methods used, lack of objective assessment of fracture, and so called "publication bias."

Overall, the beneficial effects are largely reported from studies with weaker study design, such as case-control trials. These observations suggest that there is clearly a need for properly conducted, adequately powered, randomized controlled clinical trials to assess conclusively whether statins could potentially reduce fracture rates. Until that moment, patients at high risk of fractures should be treated with currently approved medications.

G. Statins and Nervous System

Hypercholesterolemia is associated with vascular diseases that may increase the risk of cognitive dysfunction from mild deficits to vascular dementia and Alzheimer disease (AD) (Sparks et al., 1994; Hofman et al., 1997; Notkola et al., 1998; Moroney et al., 1999; Nash and Fillit, 2006). Cholesterol and LDL levels are independent determinants for developing dementia (Kalmijn et al., 1996; Moroney et al., 1999) and correlate with total Alzheimer amyloid (Aβ) peptide, by shifting the cleavage of amyloid precursor protein (APP) from α to β product (Sparks et al., 1994; Racchi et al., 1997; Refolo et al., 2000). Observational studies showed that the prevalence of AD in statin users was 60% lower than in the total population and 73% lower than patients taking other cardiovascular medications (Wolozin et al., 2000). Yaffe et al. (2002) performed an observational study on 1037 postmenopausal women with coronary heart disease and showed that higher serum levels of total and LDL cholesterol were associated with worse cognitive scores and greater probability of cognitive impairment. They also observed a positive trend for better cognitive performance in statin users that seemed to be independent of total cholesterol levels. Cramer et al. (2008) analyzed the association between the use of statins and the incidence of combined dementia and cognitive impairment without dementia over 5 years of follow-up. Unadjusted analyses and two models of analyses adjusted for baseline covariates such as diabetes, stroke, smoking status, presence of any apolipoprotein E ε4 allele and Modified Mini-Mental State Examination (3MSE) score, showed that statin use was associated with a ~40% lower rate of dementia/cognitive impairment without dementia. Observational reports corroborated this finding in elderly patients suggesting that statin use could be associated with a lower risk of dementia and AD (Jick et al., 2000; Rockwood et al., 2002). Two other major studies reported no positive effects of statins in reducing the risk of dementia or AD (Shepherd et al., 2002; Zandi et al., 2005) and indicated that the use of statins such as pravastatin (PROSPER study) (Shepherd et al., 2002) or both water-soluble or lipophilic statins in the Cache County Study (Zandi et al., 2005) had no effect on cognitive outcomes. The discrepancy of the results could be due to the analytical method adopted (i.e., cross-sectional or prospective analysis) (Miida et al., 2007). Moreover, these studies were performed on elderly cohorts of men and women with different mean range of age and with already established cognitive impairment or AD. A recent observational study carried out on people who participated in the Ginko Evaluation of Memory Study (GEMS) showed that the use of statins was significantly associated with a reduced risk of dementia and AD among participants without mild cognitive impairment at baseline. On the contrary, statins did not seem to exert protective cognitive effect when treatment started in the presence of baseline mild cognitive impairment and after (cerebro)vascular disease has developed (Bettermann et al., 2011). Results obtained in clinical studies do not answer the question whether statins could be useful in the prevention of dementia and AD. First, most of the studies were not designed primarily to analyze the effects of statins on cognitive functions and enrolled patients with advanced vascular diseases. Second, only a few recent clinical trials analyzed the effect of a single statin, whereas a number of studies were carried out in patients who received different kinds of statins with different bioavailability profiles and solubility. Lipophilic statins, which are able to cross the blood-brain barrier, might be more efficacious than soluble statins in preventing cognitive impairment and AD (Haag et al., 2009; Bettermann et al., 2011). Finally, it remains unclear whether the protective effects of statins are related to lipoprotein levels, to their pleiotropic effects (Vaughan, 2003; Miida et al., 2007), or to a direct effect on protein prenylation within the central nervous system. Increasing evidence in animal models indicate that statins exhibit a neuroprotective effect on AD onset and progression. In cultured hippocampal neurons, the formation of Aβ is abolished after reducing cholesterol levels with lovastatin (Simons et al., 1998), and simvastatin is able to reduce levels of Aβ 42 and Aβ 40 in vitro and in vivo (Fassbender et al., 2001). In a recent study on a mouse model of neuroinflammation induced by intracerebroventricular injection of $A\beta_{1-40}$ peptide that mimics the early phase of AD, atorvastatin reduced neuroinflammation and oxidative stress response improving spatial learning and memory deficits (Piermartiri et al., 2010). Atorvastatin seems to reduce inflammation and synaptic loss by inhibiting the expression of glutamatergic transporter and COX-2 in the brain. Moreover, in APP transgenic (Tg) mice showing typical pathological hallmarks of AD, a 3 months' treatment with simvastatin improved memory (Li et al., 2006), decreased glial activation, cortical soluble Aβ levels, and the number of Aβ plaque-associated dystrophic neurites (Tong et al., 2009). Kurata et al. (2011) analyzed the effects of pitavastatin and atorvastatin in Tg mice and correlated serum lipid profiles with cognitive dysfunction, senile plaque, and phosphorylated τ-positive dystrophic neu-

rites. They demonstrated that statins prevented cognitive decline, but neither atorvastatin nor pitavastatin influenced serum triglycerides or HDL-C levels compared with control group. Indeed, statins down-regulate the isoprenoid pathway and its intermediate products, which are responsible for normal function of cellular isoprenylated proteins. In vitro experiments have demonstrated that statins at physiological concentrations are able to inhibit Rab family protein prenylation whose function is associated with Aβ production and APP trafficking (Ostrowski et al., 2007). Statins also reduce Rho GTPase protein expression in mouse microglial and neuronal cells reducing Aβ-induced inflammation and inhibiting Aβ secretion (Cordle et al., 2005; Ostrowski et al., 2007). It is noteworthy that the beneficial effects of statins on neuroinflammation and neurodegeneration have been reported also in non-AD animal models. Simvastatin has been shown to attenuate learning and memory impairment in both Tg and normal non-Tg mice without affecting Aβ levels in the brain (Li et al., 2006). These findings address a protective role of statins in preventing cognitive decline in non–AD-related dementia and suggest potential therapeutic applications in other chronic inflammatory disorders of the central nervous system such as multiple sclerosis (MS). Some studies in experimental allergic encephalomyelitis, the animal model of MS, indicated that lovastatin-treatment attenuates MS progression and reduces immune cell infiltration in the central nervous system (Stanislaus et al., 2001; Ifergan et al., 2006). The immunomodulatory effects of statins may exert protective effects in MS by down-regulation of proinflammatory Th1 cytokines or by promoting Th2 bias (Youssef et al., 2002; Peng et al., 2006). These observations provide a rationale to evaluate the efficacy of statins administered alone or combined with approved treatments for MS. At present only a few studies, enrolling a limited number of participants, showed that lovastatin or simvastatin decreased the relapses and the number and volume of gadolinium-enhanced lesions in relapsing-remitting MS (Sena et al., 2003; Vollmer et al., 2004). More recent studies provide contrasting results about reduction or progression of relapses in patients with relapsing-remitting MS (Birnbaum et al., 2008; Rudick et al., 2009). Aimed to investigate the effects of statin-treatment combined to IFNβ-1a in MS, at least six clinical trials are still ongoing (Kamm et al., 2009; Wang et al., 2010a), but results are incomplete; therefore, no sufficient information support mono or combination therapy with statins in MS.

V. Statins and Cancer

A. Effects of Statins in Cancer

Statin pleiotropic effects have been associated with both increased and decreased cancer risk. Despite this, several studies, summarized in Table 2, showed a fair antitumor effect of statins in both cellular and animal models of human cancer. Low levels of serum cholesterol may be associated with increased cancer risk and accelerated development of already initiated tumors (Kritchevsky and Kritchevsky, 1992). Indeed, statins, reducing cholesterol concentration, have been reported to stimulate TGF-β signaling and increase protumor factors (Chen et al., 2008). In various cell lines, lovastatin treatment, at concentrations higher than those used in humans, increased mitotic abnormalities interfering with development and function of centromeres, thus enhancing the risk of mutations and malignancies (Lamprecht et al., 1999).

Decreased cancer incidence may be attributed to statin-induced suppression of tumor growth, induction of apoptosis, and inhibition of angiogenesis. The intermediates of mevalonate pathway are essential for different cellular functions. Statins reduce not only cholesterol levels but also mevalonate synthesis and the production of dolichol, GPP, and FPP, as well as tumor cell growth in vitro and in vivo (Soma et al., 1992). Primary N-Ras-mutated acute myeloid leukemia (AML) cells were less sensitive to simvastatin than nonmutated AML cells, suggesting a Ras signaling-independent inhibition of cell proliferation (Clutterbuck et al., 1998). In primary cultured human glioblastoma cells, lovastatin inhibited Ras farnesylation and reduced proliferation and migration (Bouterfa et al., 2000). Moreover, lovastatin showed that inhibition of cyclin-dependent kinase 2 through a Ras-independent pathway accounted for growth inhibitory effects (DeClue et al., 1991). Inappropriate Ras signaling pathway activation has a critical function also in thyroid disorders. Indeed, it has been reported that geranylgeranylated Rho has important roles in cell proliferation and apoptosis beyond the control of cell migration. As statins inhibit both farnesylation and geranylgeranylation (and hence Ras and/or Rho activation), it seems plausible that they might potentially inhibit the malignant phenotype of tumor cells (Bifulco, 2008). Inhibition of Rho geranylgeranylation by lovastatin has been shown to exert growth-inhibitory and proapoptotic effects and to induce differentiation of human anaplastic thyroid carcinoma cells resistant to conventional therapies. Furthermore, inhibition of geranylgeranylation (but not farnesylation) has been suggested as the main mechanism regulating lovastatin-induced apoptosis (Wang et al., 2003; Zhong et al., 2005). By contrast, we found that the isoprenoid pathway was markedly altered in the FRTL-5 rat thyroid cell line upon transformation with K-*ras* (but not H-*ras*). This effect occurred via induction of farnesyltransferase activity, which resulted in the preferential farnesylation and functional activation of the oncogene product (Laezza et al., 1998). Treatment with lovastatin inhibited proliferation and induced apoptosis of K-*ras*-transformed thyroid cells through the modulation of the cellular redox state (Laezza et al., 2008). The preferential inhibition of a specific Ras isoform might therefore represent an alternative mechanism of lovastatin action and so provide a

TABLE 2
Targets of statins in cancer

Tumor (cell type)	Statin	Effect	Mechanism of action	Reference
Breast cancer (MCF-7, ZR75T, MDA-MB-157, Hs578T, T47D, MDA-MB-231)	Lovastatin	Inhibition of cell proliferation	Cell cycle arrest at G_1 phase; decrease of CDK2 activity through redistribution of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ from CDK4 to CDK2	Rao et al. (1998)
Breast cancer (MDA-MB-231)	Cerivastatin	Reduction of cell proliferation and invasiveness	Inhibition of RhoA-dependent cell signaling; down-regulation of cyclin D1, PCNA, c-myc, u-PA, MMP-9, u-PAR, PAI-1; up-regulation of $p21^{Waf1}$, $p19^{Ink4d}$, integrin $\beta 8$, Wnt-5a	Denoyelle et al. (2003)
Breast cancer (MCF-7, SKBr3, MDA-MB-231)	Fluvastatin, lovastatin, simvastatin	Inhibition of cell growth	Transient decrease in p-MEK1/2; increase of IκBα and p21; decrease of cyclin D1, Bcl-2, and Bcl-xL	Campbell et al. (2006)
Breast cancer (MCNeuA cell line injected in female neuTg mice)	Simvastatin, fluvastatin (orally administered)	Inhibition of tumor growth in vivo	Reduction of tumor volumes; induction of central necrosis; induction of caspase-3 cleavage	
Breast cancer (MCF-7, MDA-MB-231)	Simvastatin	Inhibition of cell proliferation	Induction of cell cycle arrest and apoptosis; activation of JNK and increased phosphorylation of c-Jun	Koyuturk et al. (2007)
Colon carcinoma (HCT116, SW480, LoVo, HT29)	Lovastatin	Inhibition of cell proliferation	Induction of apoptosis; decrease of bcl-2 and increase of bax protein expression	Agarwal et al. (1999)
Glioblastoma multiforme (primary cell cultures from biopsies)	Lovastatin	Inhibition of cell proliferation and migration	Reduction of MAPK activity; induction of apoptosis; disruption of actin cytoskeleton	Bouterfa et al. (2000)
Glioblastoma multiforme (U87, U251)		Inhibition of cell proliferation	Activation of ERK1/2, c-Jun, and p38; up-regulation of Bim and induction of apoptosis	Jiang et al. (2004)
Leukemia (Jurkat, CEM, IM9, U266)	Lovastatin, simvastatin, pravastatin, cerivastatin, atorvastatin	Inhibition of cell proliferation	Induction of apoptosis through cytosolic release Smac/DIABLO and activation of caspases 9, 3, and 8	Cafforio et al. (2005)
AML (OCI-AML-1, OCI-AML-2, OCI-AML-3, OCI-AML-4, and OCI-AML-5) and primary cell cultures from patients with AML	Lovastatin	Inhibition of cell proliferation	Induction of apoptosis	Dimitroulakos et al. (1999)
AML cell lines	Lovastatin (low doses)	Induction of differentiation	Down-regulation of bcl-2; increase of the leukocyte integrin CD11b and CD18 expression	Dimitroulakos et al. (2000)
Acute Promyelocytic Leukemia (NB4)	Atorvastatin, fluvastatin	Inhibition of cell proliferation	Induction of cell differentiation and apoptosis; activation of Rac1/Cdc42 and of JNK pathway	Sassano et al. (2007)
Leukemic progenitors (primary bone marrow–derived from patients with AML)	Fluvastatin		Inhibition of leukemic CFU-GM colony formation	
Promyelocytic leukemia (HL60 intravenously inoculated in SCID mice)	Simvastatin (subcutaneous continuous infusion)	Inhibition of cell proliferation in vivo	Reduction of the clonogenic cells in bone marrow and spleen of mice (Ras-independent mechanism).	Clutterbuck et al. (1998)
Promyelocytic leukemia (HL60)	Lovastatin	Inhibition of cell growth	Induction of apoptosis through release of mitochondrial cytochrome *c and caspase-3 activation.*	Wang et al. (2000)

TABLE 2—*Continued.*

Tumor (cell type)	Statin	Effect	Mechanism of action	Reference
Melanoma (A375M, SK-Mel 128, CHL, WM-166–4)	Atorvastatin	Alteration of melanoma cells morphology and inhibition of cell proliferation; inhibition of melanoma cell invasion	F-actin depolymerization and disassembly of stress fibers; up-regulation of RhoA and Rho exclusion from the membrane; increase of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$.	Collisson et al. (2003)
Melanoma (A375M injected in tail vein of SCID mice)	Atorvastatin (orally administered)	Inhibition of colonization and formation of metastatic lesions in the lung in vivo	Inhibition of the effects induced by RhoC overexpression.	
Myeloma (MCC-2)	Cerivastatin	Inhibition of cell proliferation	Induction of apoptosis through caspases activation; delocalization from membrane to cytosol of Rho and Ras	Cafforio et al. (2005)
Pancreatic cancer (PANC-1)	Fluvastatin, lovastatin	Inhibition of EGF-induced migration and invasiveness	Inhibition of RhoA translocation from cytosol to membrane; inhibition of actin stress fiber assembly	Kusama et al. (2001)
Prostate cancer (LNCaP)	Lovastatin	Inhibition of cell proliferation	Induction of apoptosis through proteolytic activation of caspase 7	Marcelli et al. (1998)
Anaplastic thyroid cancer (ARO)	Lovastatin	Inhibition of cell proliferation	Induction of apoptosis (higher doses) Induction of differentiation (lower doses)	Wang et al. (2003)
		Reduction of EGF-induced invasiveness	Reduction of phosphorylated p125 (FAK) and paxillin; inhibition of RhoA and Rac1 geranylgeranylation and membrane translocation	Zhong et al. (2005)
Thyroid cancer (KiMol)	Lovastatin	Inhibition of cell proliferation	Induction of apoptosis; modulation of the cellular redox state	Bifulco (2008); Laezza et al. (2008)
			Cytoskeletal disorganization	Bifulco (2005)

PCNA, proliferating cell nuclear antigen; u-PA, urokinase plasminogen activator; MMP-9, matrix metalloprotease-9; u-PAR, urokinase plasminogen activator receptor; PAI-1, plasminogen activator inhibitor-1; IκBα, inhibitor of nuclear factor-κBα; Smac/DIABLO, second mitochondria-derived activator of caspases/direct IAP binding protein with low PI; CFU-GM, colony-forming units-granulocyte macrophage; FAK, focal adhesion kinase; MCNeuA, mammary carcinoma from Neu transgenic mouse A.

useful selective chemotherapeutic tool for tumors harboring K-ras mutations (Bifulco, 2008). Furthermore, in Fisher rat thyroid cell line-5 cells, lovastatin induced cytoskeletal disorganization and disconnection of microtubules from the plasma membrane (Bifulco, 2005). Antiproliferative effects of statins involving G_1-S arrest are suggested to be attributable to the up-regulation of the cell-cycle inhibitors $p21^{WAF1/CIP1}$ and/or $p27^{KIP1}$ (DeClue et al., 1991; Hirai et al., 1997; Rao et al., 1998). In breast cancer cell lines, cerivastatin treatment modulated the expression of 13 genes that may contribute to the inhibition of both cell proliferation and invasion, either directly or indirectly, through the inhibition of RhoA-dependent cell signaling (Denoyelle et al., 2003). Statins also modify normal cell phenotype; however, these cells seem to be more resistant to statin antiproliferative effects than tumor cells (Hindler et al., 2006). Therefore, statins might inhibit the growth of a variety of tumor cell types, including gastric, pancreatic, and prostate carcinoma, as well as neuroblastoma, glioblastoma, adenocarcinoma, melanoma, mesothelioma, acute myeloid leukemia, and breast cancer. Statins exert proapoptotic effects in a wide range of tumor cell lines, but their sensitivity to statin-induced cell death significantly differs among different cell types. For instance, acute myeloid leukemia and neuroblastoma cells are very sensitive to statin-induced apoptosis (Dimitroulakos and Yeger, 1996; Dimitroulakos et al., 1999). These apoptotic mechanisms may involve inhibition of GPP, required for potential Rho-mediated cell proliferation. Lovastatin apoptotic effect was completely reverted by mevalonate and GGPP and only partially by FPP, whereas other products of the mevalonate pathway did not revert its effect in acute myeloid leukemia cells. In colon cancer cells, GGPP prevented lovastatin-induced apoptosis, whereas the cotreatment with FPP was ineffective. Moreover, lovastatin treatment up-regulated the proapoptotic proteins Bax and Bim (Agarwal et al., 1999a) and decreased the antiapoptotic Bcl2 protein (Dimitroulakos et al., 2000). These effects have been observed in both hematological and solid tumors. Lovastatin increased Bim protein levels and induced cell death through the phosphorylation of Erk1/2, c-Jun, and p38 in glioblastoma cells (Jiang et al., 2004). In addition, the antitumor effect of statins in breast cancer cells has been associated to the suppression of the MEK/ERK pathway with decreased NF-κB and adapter protein 1 DNA binding activities (Campbell et al., 2006). Moreover, simvastatin induced apoptosis in breast cancer cells via JNK pathway independently of their estrogen receptor or p53 expression status (Koyuturk et al., 2007). Thus, the antitumor effect of statins has been associated with the dual regulation of MAPK pathways involving both suppression of MEK/ERK activity and induction of JNK activity in breast cancer cells (Koyuturk et al., 2007) and in a similar way in leukemia cells (Sassano et al., 2007). Statins can also activate caspase proteases involved in programmed cell death. Lovastatin induced apoptosis in leukemia and prostatic cancer cells through activation of caspase-7 and caspase-3, respectively (Marcelli et al., 1998; Wang et al., 2000a) and cerivastatin caused cell death in human myeloma tumor cells by activating caspase-3, caspase-8, and caspase-9 (Cafforio et al., 2005).

Frick et al. (2003) reported multiple statin effects on blood vessel formation by inhibition of angiogenesis through down-regulation of proangiogenic factors, such as VEGF, inhibition of endothelial cell proliferation, and block of adhesion to extracellular matrix. Caveolin protein is essential to inhibit angiogenesis because it decreases eNOS, which is activated during angiogenesis; thus, endothelial cells with low caveolin concentrations may be more sensitive to the statin antiangiogenic effect (Brouet et al., 2001). High concentrations of statins can inhibit angiogenesis in a lipid-independent manner, and this effect can be reverted by mevalonate or GPP administration. (Weis et al., 2002). Cerivastatin inhibited endothelial proliferation at concentrations of 0.1 μM, whereas simvastatin induced the same effect at 2.5 μM and fluvastatin at 1 μM (Schaefer et al., 2004). On the other hand, statins can also stimulate angiogenesis through protein kinase B induction (Kureishi et al., 2000) and eNOS activation at low to mid-range concentrations (Brouet et al., 2001). In conclusion, as discussed above, according to the statin type and dose used, inhibition or stimulation of angiogenesis can occur. Along with the above-mentioned effects, statins may also impair tumor metastatic process by inhibiting cell migration, attachment to the extracellular matrix and invasion of the basal membrane. In this context, findings showed that statins are able to reduce endothelial leukocyte adhesion molecule, E-selectin (Nübel et al., 2004) and matrix metalloproteinase (MMP)-9 expression (Wang et al., 2000b), as well as the epithelial growth factor-induced tumor cell invasion (Kusama et al., 2001). In human pancreatic cells, fluvastatin attenuated EGF-induced translocation of RhoA from the cytosol to the membrane and actin stress fiber assembly without inhibiting the phosphorylation of EGF receptor or c-erbB-2. Fluvastatin and lovastatin inhibited invasion in a dose-dependent manner in EGF-stimulated cancer cells, and this inhibition was reverted by the addition of all-*trans*-geranylgeraniol (Kusama et al., 2001). Likewise, the anti-invasive effect of cerivastatin on highly invasive breast cancer cell lines has been associated with RhoA delocalization from the cell membrane, with a consequent disorganization of actin fibers and disappearance of focal adhesion sites. Moreover, cerivastatin was also shown to induce inactivation of NF-κB in a RhoA inhibition-dependent manner, resulting in decreased urokinase and matrix metalloproteinase-9 expression (Denoyelle et al., 2001). Atorvastatin inhibited in vitro the invasiveness of melanoma cells, through

negative modulation of geranylgeranylation, also reducing metastases formation in vivo (Collisson et al., 2003).

B. Statins and Cancer Risk Prevention

The worldwide use of statins as lipid-lowering drugs exponentially increased, in a short time, the number of statin consumers and consequently triggered growing concern about potential adverse effects in long-term users. Human data regarding the cancer risk associated with statin administration have highlighted conflicting results, and a large number of studies have analyzed the relationship between statin therapeutic regimen and cancer incidence. In this field, numerous potential misjudgments should be taken into account.

First, the onset of malignancies was mainly reported as a secondary endpoint in studies performed to evaluate lipid concentration and cardiovascular outcome. Data extrapolated from such trials lack sufficient hard information concerning clinical outcome, medical histories, presence of familial predisposition to cancer, and observational analysis in long-term use. Second, to ascertain their lipid-lowering efficacy, several statins, both hydrophobic and partially hydrophobic, have been tested, and the heterogeneous cancer types occurring frequently make it difficult to identify a real association between statin use and cancer risk rather than the lack of enough cases to detect significant differences or associations among users and nonusers.

The former studies suggested a potential carcinogenicity of statins, administered at doses higher than those usually used to treat hypercholesterolemia in human both in vitro and in vivo, in cancer cells, and in animal models. High doses of lovastatin (500 mg/kg day), but not doses lower than 180 mg/kg day, induced an increased incidence of hepatocellular and pulmonary cancer in animal models (MacDonald et al., 1988); in rodents, fluvastatin was found to be associated with thyroid cancer and forestomach papillomas (Robison et al., 1994). On the other hand, in mice and rats, the chemically induced colon carcinogenicity was reduced by both simvastatin and pravastatin (Narisawa et al., 1994; Narisawa et al., 1996), and pravastatin also decreased the number and volume of *N*-nitrosomorpholine-induced hepatic neoplastic nodules (Tatsuta et al., 1998).

Human clinical trials evaluating the cancer risk/prevention in statin users produced mixed (heterogeneous) and frequently conflicting results (Tables 3 and 4). The Atorvastatin versus Revascularization Treatment (AVERT) trial reported seven cases of cancer, three in the atorvastatin (80 mg) group and four in the angioplasty group (Pitt et al., 1999). No significant differences in cancer frequency were found comparing the simvastatin (28.5 mg)-treated patients with the placebo group in the Simvastatin/Enalapril Coronary Atherosclerosis Trial (SCAT) over a period of 4 years (Teo et al., 2000). Neither the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack (ALLHAT-LLT) trial (ALLHAT Officers, 2002) for the primary prevention of cardiovascular events nor the Long-term Intervention with Pravastatin in Ischemic Disease (LIPID) trial (LIPID Study Group, 1998) found significant differences in cancer risk between the pravastatin and the usual care for hypertension or pravastatin- and placebo-treated groups. In the Treating to New Targets (TNT) trial, LaRosa et al. (2005) analyzed the efficacy and safety of atorvastatin in 10,001 patients with stable coronary heart disease. Patients, randomly assigned to double-blind therapy, received either 10 or 80 mg of atorvastatin per day and were followed for 4.9 years. In this study, cancer (mainly lung and gastrointestinal) accounted for more than half the deaths from noncardiovascular causes in both groups, showing that cancer occurrence may not be associated with atorvastatin dose. Moreover, similar follow-up (4.8 years) of the patients included in the Incremental Decrease in End Points Through Aggressive Lipid Lowering (IDEAL) trial demonstrated no significant differences in the percentage of cancer cases occurring in patients treated with simvastatin or atorvastatin (20 and 80 mg/day, respectively) (Pedersen et al., 2005). Finally, treatment with 80 mg of atorvastatin compared with placebo was unable to increase the cancer incidence also in the Stroke Prevention by Aggressive Reduction in Cholesterol Levels (SPARCL) trial (Amarenco et al., 2007). In the randomized placebo-controlled Heart Protection Study (HPS) trial, no significant increase of cancer risk was found in simvastatin (40 mg/day)-treated patients compared with those in the placebo-treated group (Heart Protection Study Collaborative Group, 2002). Similar results were obtained after a 10-year follow-up period in the Scandinavian Simvastatin Survival Study (4S) for two simvastatin-treated groups (20 and 40 mg/day) compared with placebo (Strandberg et al., 2004). Despite this evidence that seems to suggest a neutral effect of statins in the cumulative incidence of cancer, several studies demonstrated an increase, or sometimes a decrease, in the occurrence of selective cancer types in statin users. In the Air Force Coronary Atherosclerosis Prevention Study (AFCAPS), the patients treated with lovastatin showed a significantly lower incidence of melanoma (approximately 50%) compared with the group treated with placebo (Downs et al., 1998). On the other hand, the Cholesterol and Recurrent Events (CARE) trial showed a significant increase (higher than 5%) in breast cancer that occurred in postmenopausal women treated with 40 mgof pravastatin compared with placebo (Sacks et al., 1996). In the same study, the statin-treated group showed a consistent but not significant reduction of colon cancer incidence, without affecting that of the new diagnosed melanoma (Sacks et al., 1996). Pravastatin 40 mg induced an increased cancer incidence in patients included in the Prospective Study of Pravastatin in the Elderly at Risk (PROSPER) study, and, in the same trial, breast cancer occurred preferentially in the pravastatin-treated group (Shepherd et al., 2002); however, more recently a meta-analysis of pravastatin and all statin trials performed in younger statin users have been unable to

TABLE 3
Randomized clinical trials reporting the effect of statin use on cancer risk

Study	Reference	Population	Duration	Statin		Results
				Name	Dose	
			months		*mg/day*	
AVERT	Pitt et al. (1999)	341	18	Atorvastatin	80	Seven cases of cancer (three in atorvastatin-treated and four angioplasty-treated patients)
SCAT	Teo et al. (2000)	460	48	Simvastatin/enalapril	28.5	No significant differences in simvastatin/enalapril-treated-group and placebo group
ALLHAT-LLT	ALLHAT Officers (2002)	10,355	97	Pravastatin	40	No significant differences in pravastatin-treated and usual care for hypertension
TNT	LaRosa et al. (2005)	10,001	57	Atorvastatin	10 or 80	Cancer occurrence may not be associated with atorvastatin dose
IDEAL	Pedersen et al. (2005)	8888	56	Simvastatin Atorvastatin	20 80	No significant differences in simvastatin- and atorvastatin-treated groups
SPARCL	Amarenco et al. (2007)	4731	57	Atorvastatin	80	No significant differences in atorvastatin-treated and placebo group
LIPID	LIPID Study Group (1998)	9014	73	Pravastatin	40	No significant differences in pravastatin-treated and placebo group
AFCAPS	Downs et al. (1998)	6605	62	Lovastatin	20–40	No significant differences in treated and placebo groups
HPS	Heart Protection Study Collaborative Group (2002)	20,536	60	Simvastatin	40	No significant differences in simvastatin-treated and placebo group
4S	Strandberg et al. (2004)	4444	120	Simvastatin	20 or 40	No significant differences in simvastatin-treated and placebo group
PROSPER	Shepherd et al. (2002)	5804	38	Pravastatin	40	Significant increase of cancer incidence in pravastatin-treated and placebo group especially breast cancer
CARE	Sacks et al. (1996)	4159	60	Pravastatin	40	Significant increase in breast cancer in postmenopausal women treated with pravastatin compared with placebo. No significant reduction of colon cancer incidence and no decrease in the new diagnosed melanoma
WOSCOPS	Shepherd et al. (1995)	6595	57	Pravastatin	40	Significant increase of overall cancers in treated and placebo group
WOSCOPS	Ford et al. (2007)	Survivors of previous trials [Shepherd et al. (1995)]	120	Pravastatin	40	No significant increased risk in pravastatin treated and placebo group

confirm these results. Finally, a symptomatic example of the need of cautious evaluation of these studies has been evidenced by the West of Scotland Coronary Prevention Study (WOSCOPS). In the first evaluation, the authors demonstrated that in men with hypercholesterolemia, 40 mg/day pravastatin increased the incidence of overall cancers (Shepherd et al., 1995); however, the prolonged 10-year follow-up period showed no increased risk in pravastatin consumers (Ford et al., 2007).

Observational studies taking as primary endpoint the diagnosis of malignancy and as second endpoint the evaluation of specific cancer types also investigated the potential correlation between the use of statins and cancer risk. A hospital-based case-control surveillance study was conducted in 1132 women with breast cancer, 1009 men with prostate cancer, and 2718 subjects admitted for condition unrelated to statin use. In this analysis, 1.5-and 1.2-fold increased risks for breast and prostate cancer, respectively, have been found (Coogan et al., 2002). Friis et al. (2005) performed a population-based case-control study using data from the Prescription Database of North Jutland County and the Danish Cancer Registry. In a population of 334,754 subjects, they compared overall and site-specific cancers occurring in 12,251 statin users with cancer occurring in nonuser subjects and in 1257 patients using other lipid-lowering drugs, during a total follow-up period of 3.3 years. Results showed that cancer incidence in statin-user group was lower than that observed in both the control group and in other lipid-lowering drug users. Moreover, no preferential site-specific cancers occurred in the examined groups. Similar results were obtained in a case-control study performed using the Quebec Administrtive Health Database (Blais et al., 2000). In a median follow-up period of 2.7 years, by comparing users of HMG-CoA reductase inhibitors with users of bile acid-binding resins to treat hypercholesterolemia, authors found a significant decrease (approximately 28%) of the new diagnosed cancers in statin-users. No increase of specific cancer was found preferentially associated with statin or resins consumer groups.

The PHARMO database, containing drug-dispensing records from community pharmacies and linked hospital discharge records for residents of eight Dutch cities, was used to evaluate the incidence of overall cancer by comparing subjects (3129) treated with statins (mainly simvastatin in this population) with people (16,976 control subjects) treated with other cardiovascular medications. Statin use has been associated with a 20% reduction in overall cancer risk, with a significant decrease for specific cancer subtype exclusive of renal carcinoma (Graaf et al., 2004).

More recently, the association between statin use and the occurrence of the ten most common types of neoplasia has been analyzed in a hospital-based case-control surveillance study (Coogan et al., 2007a). Hospitalized cancer patients (4913) have been compared with patients admitted for diagnosis other than cancer (3900). For all cancer types considered (breast, prostate, colorectal, lung, bladder, leukemia, pancreas, kidney, endometrial, and non-Hodgkin lymphoma), no significant differences were found among regular statin users compared with never-users. Moreover, duration of statin use, and a more selective analysis separately considering hydrophobic statin users and hydrophilic statin users, did not affect obtained results (Coogan et al., 2007a; Duncan et al., 2007).

The association between statin use and prostate cancer risk was studied in patients from the Veterans Affairs Medical Center in Portland, OR. Results demonstrated that statin use significantly reduced prostate cancer occurrence. Moreover, analyzing the correlation between statin assumption and the histological grade of the neoplasia, statin users showed a decreased risk of aggressive prostate cancer with Gleason score ≥ 7 (Shannon et al., 2005). It is noteworthy that data concerning 361,859 patients from the Kaiser Permanente Medical Care Program in Northern California showed an increased rate of overall cancers in statin users and a decreased, but not significant, rate for colon cancer in men and for liver and intrahepatic bile duct cancer in women. Moreover, in this population, statin users experienced an increased risk in stage 1 prostate cancer but not in more advanced stages of prostatic neoplasia (Friedman et al., 2008). More recently, a population-based case-control study in patients from the Taiwan National Health Insurance Research Database evaluated 388 prostate cancer cases and 1552 control subjects. Multiple logistic regression analyses demonstrated that the use of statins was associated with a significant increase in prostate cancer risk, and that increasing cumulative doses of statins were correlated with increasing prostate cancer risk (Chang et al., 2011).

The protective effect of statins toward colon cancer was evaluated by Poynter et al. (2005) using data from the Molecular Epidemiology of Colorectal Cancer (MECC) study, a population-based case-control study of patients who received a diagnosis of colorectal cancer in northern Israel. In particular, in 1953 patients with colorectal cancer and 2015 control subjects, the use of statins for at least 5 years (versus no use of statins) was associated with a significant reduction (47%) of the relative risk of colorectal cancer, persistent after adjustment for other risk factors (e.g., use of NSAID, presence or absence of family history of colorectal cancer, ethnicity, hypercholesterolemia). Moreover, the observed protective effect was specific for statins, because patients taking fibric-acid derivatives as cholesterol-lowering drugs showed a colorectal cancer risk similar to that observed in the control group. On the other hand, Coogan et al. (2007b) fail to find similar association in a case-control analysis of the Massachusetts Cancer Registry. In brief, among 1809 patients and 1809 matched control subjects, the use of statins for at least 3 months did not reduce the risk of colorectal cancer; nevertheless, the

TABLE 4
Observational studies of statins and the risk of developing cancer

All studies are case-control studies unless otherwise noted.

References	Population	Duration of Statin Use	Statins	Primary Endpoint	Results
		months			
Blais et al. (2000)	6721 Patients aged ≥65 years with prescription for lipid-lowering agents in the Regie de l'Assurance-Maladie du Quebec database	32	Lovastatin, pravastatin sodium, simvastatin	Diagnosis of any cancer	Decrease of overall cancer: RR, 0.72; 95%CI, 0.57–0.92
Graaf et al. (2004)	20,105 Patients with ≥1 prescription for cardiovascular drugs	48 (mean)	Simvastatin	Diagnosis of any cancer	Decrease of overall cancers, mainly renal cancer. Statin group vs. control group: OR, 0.8; 95%CI, 0.66–0.96 (adjusted); statins vs. lipid-lowering drugs: OR, 0.89; 95%CI, 0.56–1.41; renal cancer: OR, 0.27; 95%CI, 0.08–0.95
Friis et al. (2005)	334,754 Patients aged 30–80 years in the Prescription Database of North Jutland County and Danish Cancer Registry	39 (mean)	All statins	Primary diagnosis of cancer (overall and cancer specific)	Decrease of overall cancers in statin users vs. nonusers: RR, 0.86; 95%CI, 0.78–0.95
Coogan et al. (2002)	4859 Patients, of which 1132 women with breast cancer, 1009 men with prostate cancer, and 1331 women and 1387 men as controls from hospital-based Case-Control Surveillance Study of Drugs and Serious Illnesses				Increased risk of breast and prostate cancer. For breast cancer: OR, 1.5; 95%CI, 1.0–2.3; for prostate cancer: OR, 1.2; 95%CI, 0.8–1.7.
Coogan et al. (2007b)	3618 Patients with adenocarcinoma of the colon or rectum, and healthy control subjects from hospitals in Massachusetts and the Massachusetts Cancer Registry	1–120	All statins	Occurrence of colorectal cancer	Not reduced risk of colorectal cancer in statin users vs. nonusers: OR, 0.92; 95%CI, 0.78–1.09
Coogan et al. (2007a)	8813 Patients aged 40–79 years admitted to hospitals in New York, Philadelphia, and Baltimore	1–60	All statins	Occurrence of any of 10 cancers	No significant differences for 10 cancer types in statin use vs. nonuse: Breast cancer: OR, 1.2; 95%CI, 0.8–1.8. Prostate cancer: OR, 1.2; 95%CI, 0.9–1.7. Colorectal cancer: OR, 0.8; 95%CI, 0.5–1.2. Lung cancer: OR, 0.7; 95%CI, 0.4–1.1. Bladder cancer: OR, 1.3; 95%CI, 0.8–2.3. Leukemia: OR, 1.1; 95%CI, 0.6–2.0. Pancreatic cancer: OR, 0.7; 95%CI, 0.3–1.4. Kidney cancer: OR, 1.1; 95%CI, 0.6–1.9. Endometrial cancer: OR, 1.3; 95%CI, 0.7–2.4. Non-Hodgkin's lymphoma: OR, 1.2; 95%CI, 0.6–2.4.

TABLE 4—*Continued.*

References	Population	Duration of Statin Use	Statins	Primary Endpoint	Results
Poynter et al. (2005)	3968 Patients with colorectal cancer and healthy control subjects from northern Israel	>60	All statins	Occurrence of colorectal cancer	Significant reduction of risk of colorectal cancer in statin uses vs. nonusers: OR, 0.53; 95%CI, 0.38–0.74
Cauley et al. (2003)	7528 Women with mean age 77 years		All statins	Occurrence of breast cancer	Significant reduction of risk of breast cancer in combined statins group (RR, 0.28; 95%CI, 0.09–0.86) and among women who used other lipid-lowering drugs (RR, 0.37; 95%CI, 0.14–0.99) in comparison to nonusers.
Cauley et al. (2006)	156,351 Postmenopausal women aged 50–79 years	80	All statins	Occurrence of breast cancer	In breast cancer, no significant differences in statin use vs. nonuse: HR, 0.91; 95%CI, 0.80–1.05. Hydrophobic statin use was associated with a lower breast cancer incidence: HR, 0.82; 95%CI, 0.70–0.97.
Kumar et al. (2008)[a]	2141 Female patients listed in 2003 as incident cases of breast malignancy in the Kaiser Permanente Northern California Cancer Registry		Lovastatin, Simvastatin and Atorvastatin	Occurrence of hormone receptor phenotype of breast cancers	Fewer ER-PR-negative breast tumors of lower grade and stage in hydrophobic statin users. Moreover, statin use may influence the phenotype of tumors. OR,, adjusted for age, of developing an ER/PR-negative tumor was 0.63; 95%CI, 0.43–0.92 for statin use ≥1 year before breast cancer diagnosis compared with statin use <1 year (including nonuse). Breast cancers in patients with ≥1 year of statin use were more likely to be low grade (OR, 1.44) and less invasive stage (OR, 1.42).
Shannon et al. (2005)	302 Patients in the Portland Veterans Affairs Medical Center	2–35	All statins	Occurrence of prostate cancer	Significant reduction of risk of prostate cancer in statin use vs. nonuse: OR, 0.35; 95%CI, 0.20–0.64.
Friedman et al. (2008)	361,859 Patients enrolled for >20 years in the KPMCP and in the KPMCP Cancer Registry	59 (median)	Lovastatin, simvastatin or both	Occurrence of any cancer	No strong evidence of either causation or prevention of cancer by statins. After adjustment for smoking habit, data were consistent with a slight protective effect of statins for lung cancer
Chang et al. (2011)	483,733 Patients from the Taiwan National health Insurance Research Database aged ≥50 years and with a first-time diagnosis of prostate cancer		All statins	Occurrence of prostate cancer	Ever-use of any statin was associated with a significant increase in prostate cancer risk (OR, 1.55; 95%CI, 1.09–2.19
Khurana et al. (2007)	483,722 Patients in the VISN-16 database from south-central United States	>6	All statins	Occurrence of lung cancer	Reduced risk of lung cancer in statin users vs. nonusers: OR, 0.55; 95%CI, 0.52–0.59
Farwell et al. (2008)[a]	62,842 Patients aged ≥65 years in the Veterans Affairs New England health care system who were taking antihypertensive drugs	60 (median)	All statins	Occurrence of cancer excluding nonmelanoma skin cancer	Reduced risk developing cancers in statin users vs. nonusers. 95%CI 0.033–0.043 ($P < 0.001$)

OR, odds ratio; HR, hazard ratio.
[a] Retrospective.

occurrence of high-grade colorectal cancer was significantly lower among statin users than nonusers.

A multicenter prospective cohort study conducted at four community-based clinical centers in the United States evaluated the breast cancer incidence in a total of 7528 women (mean age, 77 years), divided into statin users, users of lipid-lowering agents other than statin, and nonusers. In this population, lipid-lowering drug users and statin users showed a reduction in the risk of breast cancer reaching 68 and 72%, respectively, compared with nonusers (Cauley et al., 2003). The same authors (Cauley et al., 2006) investigated associations among potency, duration of use, and type of statin used and risk of invasive breast cancer in a larger population of 156,351 postmenopausal women (50–79 years old) enrolled in the Women's Health Initiative. The average follow-up covered 6.7 years; unsurprisingly, no significant differences were found in breast cancer occurrence between user and nonuser patients. Nevertheless, the use of hydrophobic statins (i.e., simvastatin, lovastatin, and fluvastatin), but not of pravastatin and atorvastatin, was significantly associated with 18% reduction of breast cancer risk (Cauley et al., 2006).

Finally, a retrospective cohort analysis via the electronic pharmacy records from the Kaiser Permanente Northern California Cancer Registry explored the hormone receptor (both estrogen and progesterone receptors) phenotype in 2141 breast cancers. Among all patients, 387 used hydrophobic statins (mainly lovastatin) and showed proportionately fewer estrogen/progesterone receptor-negative tumors compared with nonusers (Kumar et al., 2008).

In patients enrolled in the Veterans Integrated Service Networks (VISN) 16 VA database, Khurana et al. (2007) studied the potential correlation between use of statins and lung cancer incidence, analyzing 483,733 patients. Among these patients, 163,662 were receiving statins and 7280 had a primary diagnosis of lung cancer. Results showed that statin use for at least 6 months, but not for shorter durations, was associated with a reduced risk of lung cancer. Moreover, in a retrospective cohort study of veterans performed by Farwell et al. (2008), the rate of lung, colon, and prostate cancer was found to be decreased in statin users. It is noteworthy that in these patients, simvastatin doses (10–40 mg) and risk of cancer occurrence showed a close dose-response relationship, because higher statin regimens correlated with the lowest occurrence of both lung and colorectal cancers (Farwell et al., 2008).

C. Statins in Cancer Treatment

To investigate the potential efficacy of statins in chemotherapy protocols, several studies evaluated, in vitro and in vivo, the combined effects of statins with drugs commonly used in cancer treatment. Statins were able to potentiate the antitumor effects of anthracyclines in both cellular and animal models. In mice, lovastatin synergistically potentiated doxorubicin-induced cytotoxicity in colon and breast carcinoma (Feleszko et al., 2000; Rozados et al., 2008) and showed an additive effect in lung cancer cell lines (Feleszko et al., 2000). Similar synergistic effects with different anthracyclines were also found for simvastatin and fluvastatin in human rhabdomyosarcoma cells (Werner et al., 2004) and breast cancer cell lines (Budman et al., 2007), respectively. Moreover, atorvastatin and mevastatin increased the sensitivity to anthracyclines of both lung cancer (Roudier et al., 2006) and human primary acute myeloid leukemia cell lines (Stirewalt et al., 2003), respectively. Several mechanisms have been proposed to explain the observed combinatorial effects (Fig. 2). First, statins suppress insulin-like growth factor 1 receptor glycosylation and its correct localization into the cell membrane (Girnita et al., 2000; Siddals et al., 2004) and inhibit NF-κB activation (Inoue et al., 2002; Wang et al., 2005). Both the reduced expression of insulin-like growth factor 1 receptor (Benini et al., 2001) and the inhibition of NF-κB (Arlt et al., 2001) sensitize tumor cells to doxorubicin. Moreover, statins, inhibiting the prenylation of RAS protein (Khosravi-Far et al., 1992), interfere with RAS-mediated pathways responsible for the resistance to doxorubicin and to several chemotherapeutic compounds (Jin et al., 2003). Because doxorubicin and statins induced an arrest of the cell cycle in the G_2 and G_1 phases, respectively (Sivaprasad et al., 2006; Javanmoghadam-Kamrani and Keyomarsi, 2008), the combined use of these drugs could exert a cumulative inhibitory effect in the cell cycle progression.

The potential interactions of statins with platinum compounds have been also tested. Lovastatin potentiated the antitumor effects of cisplatin in cellular and murine models of melanoma (Feleszko et al., 1998), increasing, at least partially, the apoptotic effect of oxaliplatin in human head and neck squamous cell carcinoma (SCC) (Mantha et al., 2003). In human colon cancer cell lines, the pretreatment with lovastatin increased the apoptotic death induced by cisplatin (Agarwal et al., 1999a). In combined use, simvastatin has been found able to reduce the liver toxicity induced by cisplatin treatment (Işeri et al., 2007). The main mechanism able to explain these interactions is the statin-induced inhibition of the MAPK/ERK kinase pathways (Fig. 2) (Nishida et al., 2005; Cerezo-Guisado et al., 2007). Finally, increasing evidence suggests that cisplatin-induced toxicity, mediated by cell-cycle arrest in G_1 phase (Donaldson et al., 1994), was highest in cells previously treated with compounds such as statins that were able to potentiate the block in G_1 phase (Sivaprasad et al., 2006; Javanmoghadam-Kamrani and Keyomarsi, 2008).

In human colon cancer cell lines (Agarwal et al., 1999a), but not in breast cancer cell lines (Mantha et al., 2003), the cytotoxic effects of 5-fluorouracil (5-FU) were potentiated by the combined use of lovastatin. Similar increases in 5-FU antiproliferative effects were also ob-

tained with atorvastatin or simvastatin combined with 5-FU in human non–small-cell lung cancer cell lines (Roudier et al., 2006) or in human myeloid leukemia cell lines (Ahn et al., 2008b), respectively. The statin-induced inhibition of NF-κB activation seems to be responsible for the increased sensitivity to 5-FU (Ahn et al., 2008b).

NSAIDs are associated with reduced colon cancer incidence, and in human colon cancer cell lines, celecoxib combined with lovastatin or atorvastatin induced a persistent cell cycle arrest in G_0/G_1 phase followed by an activation of the apoptotic process greater than that obtained with celecoxib alone (Feleszko et al., 2002; Swamy et al., 2002; Xiao et al., 2008). Atorvastatin-mediated inhibition of colon cancer cell growth also involved a decrease of the membrane-bound Rho-A in HT29 and HCT116 colon cancer cell lines (Yang et al., 2010).

It is noteworthy that the combinations of both sulindac with lovastatin and celecoxib with atorvastatin showed a significant inhibition of the cancer incidence and progression in experimental models of chemically induced colorectal carcinogenesis (Agarwal et al., 1999b; Reddy et al., 2006). The high extent of COX inhibitors antiproliferative effects induced by statins, involved several mechanisms, such as inhibition of kinase pathways, modulation of cyclin-dependent kinase activities, and arrest of cell cycle progression (Agarwal et al., 1999b; Zheng et al., 2007; Xiao et al., 2008; Guruswamy and Rao, 2009). Moreover, in the human HCT-116 colon cancer cell line, lovastatin and celecoxib suppressed caveolin-1 (Cav1) expression, impaired its membrane localization and inhibited Cav1-dependent cell survival pathways (Guruswamy et al., 2009). The inhibition of NF-κB and the synchronized arrest in different phases of the cell cycle have been also suggested as the main cellular mechanisms through which synergistic effects of statins (mainly lovastatin and simvastatin) and paclitaxel occurred (Holstein and Hohl, 2001a; Ahn et al., 2008b). These combinations efficaciously potentiated the paclitaxel-induced cytotoxic effects in human leukemic cells (Holstein and Hohl, 2001b; Ahn et al., 2008b) but not in human breast cancer cell lines and in head and neck SCC cell lines (Mantha et al., 2003).

The etoposide antiproliferative effects were increased by atorvastatin in both hepatoma and non–small-cell lung cancer cells (Roudier et al., 2006), potentially through the inhibition of PI3K/Akt pathways by mammalian target of rapamycin-mediated mechanisms triggered by statins (Krystal et al., 2002). Moreover, in leukemia cell lines, fluvastatin enhanced the apoptotic effects of both rapamycin and its analog RAD-001 (everolimus), two inhibitors of mammalian target of rapamycin (Calabro et al., 2008). In acute promyelocytic leukemia cell lines, the combination of low concentrations of ATRA with atorvastatin or fluvastatin resulted in a strong cell differentiation, and in retinoid-resistant cell lines, statins reverted the resistance to ATRA-induced differentiation (Sassano et al., 2007). It is noteworthy that, in NB4 human acute promyelocytic leukemia cells, several genes associated with differentiation and apoptosis were selectively induced by treatment with the atorvastatin and ATRA combination through direct or indirect activation of the JNK-mediated pathways (Sassano et al., 2009). Evidence demonstrated an increased efficacy of cytosine arabinoside used in combination with fluvastatin or mevastatin in both leukemic cell lines and in primary culture of cells obtained from patients with AML (Holstein and Hohl, 2001a,b; Lishner et al., 2001; Stirewalt et al., 2003; Roudier et al., 2006). In particular, an additive effect was demonstrated for mevastatin combined with cytosine arabinoside, whereas fluvastatin associated with cytosine arabinoside seemed to possess synergistic activity. In pancreatic cancer cell lines treated with gemcitabine and fluvastatin, similar results were obtained (Bocci et al., 2005). The antileukemic additive effects of statins seem ascribable to the inhibition of ERK1/2 kinases (Holstein and Hohl, 2001a,b).

The combined effect of statins and multikinase inhibitors has been also tested in several different tumor cell lines. In particular, lovastatin and sorafenib produced a synergistic cytostatic effect through induction of cell cycle arrest in G_1 phase (Bil et al., 2010). On the other hand, this combination showed a strong synergistic cardiotoxic effects in rat H9c2 cardiomyoblast cell line (Bil et al., 2010).

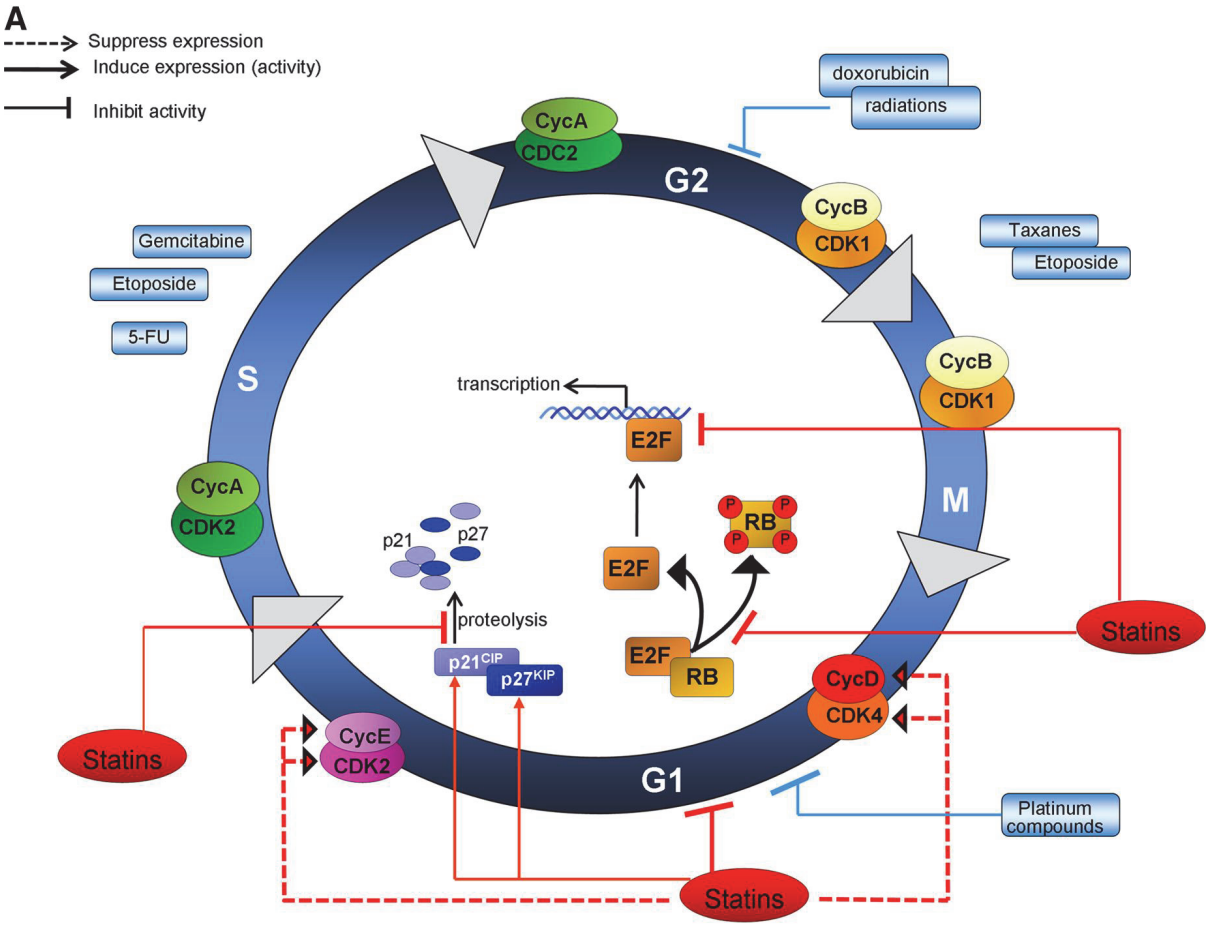
Finally, increasing evidence showed that statins enhanced the sensitivity of tumor cells to radiotherapy (Fritz et al., 2003). This effect was mediated by direct interference with RAS functions (Grana et al., 2002) and G_1 arrest of the cell cycle (Sivaprasad et al., 2006; Saito et al., 2008; Javanmoghadam-Kamrani and Keyomarsi., 2008), in addition to the arrest in G_2 phase induced by radiation.

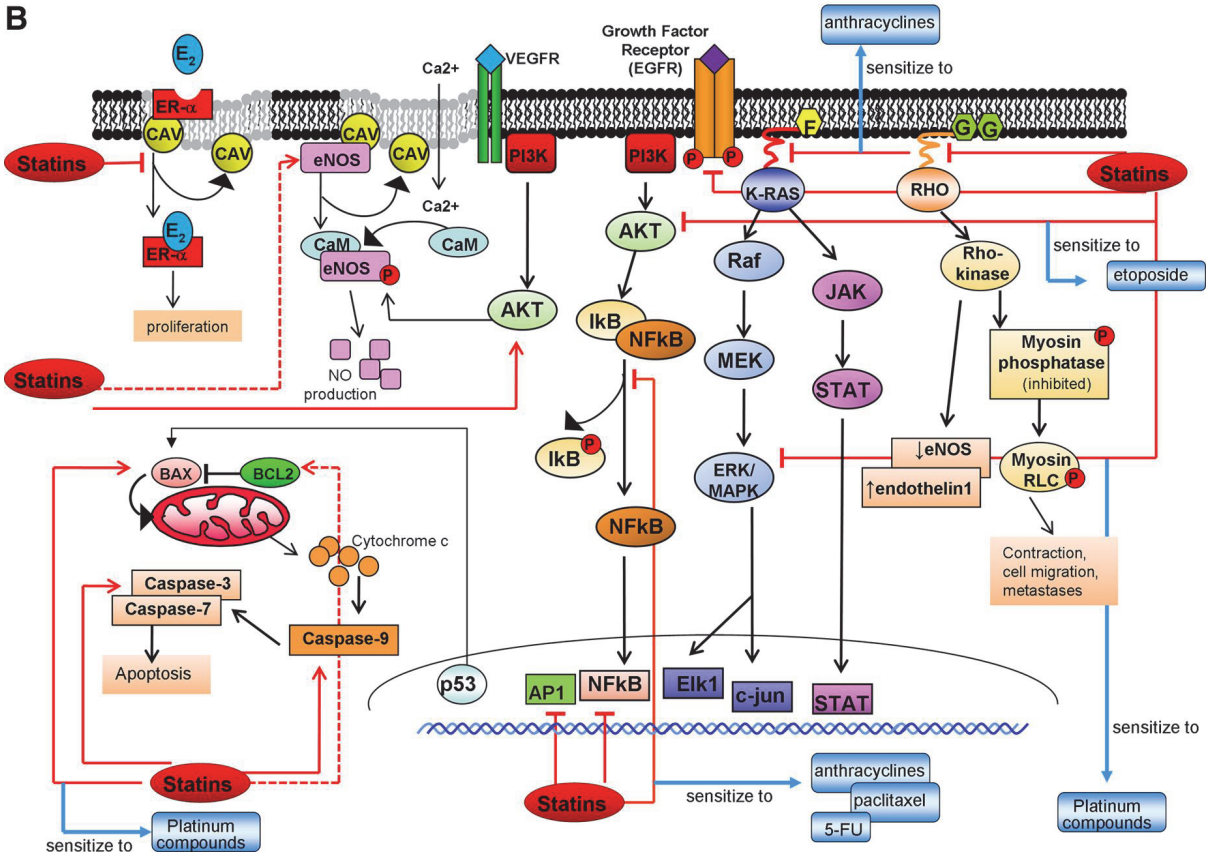
D. Clinical Trials: Monotherapy and Combined Therapy Using Statins in Human Cancer

In patients with cancer, the efficacy of the statins as chemotherapeutic drugs has been evaluated both in monotherapy and in combined therapy with currently used chemotherapeutic drugs (Table 5).

Lovastatin administered by mouth (2–45 mg/kg per day) for 7 days at monthly intervals, has been tested in patients with cancer for whom standard therapy failed or who harbored a disease for which no therapy was helpful (Thibault et al., 1996). Results showed that one patient (among 88 treated) affected by recurrent high-grade glioma, achieved a minor response. Moreover, patients treated with doses higher than 25 mg/kg per day experienced myopathy, which was counteracted by the coadministration of ubiquinone.

Because statins have been showed to increase the radiosensitivity of tumor cells, a group of patients with relapse after radiotherapy was treated with 30 mg/kg lovastatin per day consecutively administered for 7 days





and than repeated after 4 weeks. In the same study, patients first receiving the diagnosis of glioma were treated with radiotherapy combined with various doses of lovastatin. One patient was stable for more than 402 days; a minor response and a partial response were observed in two different patients (Larner et al., 1998). A similar therapeutic protocol (one daily administration of lovastatin 30 mg/kg, combined with ubiquinone, for 7 days, repeated at 4-week intervals) was used in patients with advanced unresectable gastric adenocarcinoma (Kim et al., 2001). Results showed that no patients achieved a response or a persistently stable disease.

Increasing doses of lovastatin (starting at 5 mg/kg per day for 2 weeks, every 21 days) were tested in advanced cancer of the head and neck (SCC) and of the cervix in a phase I-II study (Knox et al., 2005). The aim of the phase I study has been to identify safety, maximum tolerated dose, and recommended phase II dose of lovastatin. The scheduled treatment with 7.5 mg/kg lovastatin per day administered for 21 days, every 28 days, did not find an objective response but induced stable disease for more than 3 months in 23% of patients.

The efficacy of pravastatin in chemotherapy was tested in patients with unresectable hepatocellular carcinoma in a controlled randomized trial performed by Kawata et al. (2001). At diagnosis, patients underwent transcatheter arterial embolization and then were treated with oral 5-FU for 2 months. Among 91 patients initially enrolled in the study, 83 were then randomly assigned to control and pravastatin (20 mg daily for 2 weeks followed by 40 mg daily) groups. Both groups received concomitant 5-FU chemotherapy. Results showed that pravastatin slowly but significantly reduced the diameter of the main hepatic lesions 1 year after the start of the treatment. Moreover, the median survival was 18 months in the pravastatin group versus 9 months in the control group.

A significant prolonged survival has been also reported in a recent cohort study analyzing 183 patients with hepatocellular carcinoma (HCC) (Graf et al., 2008). All patients received palliative treatment with transarterial chemoembolization (TACE) and then were assigned to a treatment group (oral pravastatin at 20–40 mg/day; $n = 52$) or to a control group (treated with TACE alone; $n = 131$). Results showed that during the ≤5-year observation period, median survival was significantly longer in patients with HCC treated by TACE and pravastatin (20.9 months; 95% CI, 15.5–26.3; $p = 0.003$) than in those treated by TACE alone (12.0 months; 95% CI, 10.3–13.7). On the other hand, pravastatin failed to improve the median survival of patients with HCC analyzed in the randomized controlled trial performed by Lersch et al. (2004).

In a retrospective multivariate analysis, statin use significantly improved the response to neoadjuvant chemoradiation in patients with resectable nonmetastatic rectal cancer (Katz et al., 2005). Simvastatin has

Fig. 2. Cellular and molecular sites of action of statins. Potential positive and negative effects of combined use of statins and chemotherapeutic drugs. The figure shows the mechanisms involved in statin antitumor effects. Red arrows indicate the effect of statins on cell cycle phases and on specific components of the intracellular pathways; blue arrows indicate the site of action of chemotherapeutic drugs (A) and statin-mediated effects able to sensitize cancer cells to specific chemotherapies (B). A, statins inhibit the proliferation of cancer cell lines by G_1-phase cell-cycle arrest through increased expression of the cell-cycle kinase inhibitors $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ and inhibition of their proteolysis. Another mechanism by which statins inhibit cancer cell growth involves the down-regulation of cell-cycle-promoting mediators cyclin D1 (CycD), cyclin E (CycE), and cyclin-dependent kinase (CDK) 4 expression as well as the reduction of both levels and activity of CDK2. Finally, statins inhibit retinoblastoma protein (Rb) phosphorylation and consequently stabilize the transcriptionally inactive complex E2F-Rb. A direct inhibition of the E2F transcription factor activity has been also reported. The proliferative response of cancer cells is blocked by platinum compounds or anthracyclins combined to radiotherapies by arrest of G_1- or G_2-phase of the cell cycle, respectively. Statins showed a synergistic or an additive inhibitory effect on human cancer cell proliferation, when used in combination with these chemotherapeutic drugs, acting by several mechanisms at different phases of the cell cycle. B, in human cancer cell lines, statins control cancer cell growth by interfering with several intracellular pathways that differ according to cancer histological type, dose, and statin type (see section V.C for details). At least four potential antitumor mechanisms have been described, some of which also seem to be responsible for statin-induced sensitization of cancer cell lines to the treatment with chemotherapeutic drugs: inhibition of the small G-protein activities, modulation of several transduction pathways, induction of apoptosis, and destabilization of lipid rafts and caveolae. Moreover, statins can modulate the angiogenesis and impair the metastatic potential of tumor cells. Statins reduce the amount of farnesylated (mainly K-Ras) and geranylgeranylated (Rho and Rac) proteins localized in the plasma membrane by inhibition of the HMG-CoA reductase activity. Delocalization of the small G-protein Rho into the cytoplasm impairs the activation of the Rho-kinase pathway, which induces contraction, cell migration, metastatic processes, and modulation of the endothelin1 and eNOS. The statin-induced inhibition of K-Ras farnesylation improves the sensitivity of tumor cells to the anthracyclines by impairing the activation of the serine-threonine kinase Raf-1 and of the downstream effectors of the MAPK pathways involved in cell cycle progression and proliferation. Moreover, statins directly inhibit the activation of both Akt and MAPK/ERK kinases, sensitizing several human cancer cell lines to the effects of etoposide and platinum-derived compounds, or to anthracyclines, taxanes, and 5-FU by stabilizing NF-κB inactive cytoplasmic form associated with inhibitor of NF-κB (IκB). Finally, statins block the DNA-binding activity of the transcription factors adapter protein 1 (AP1) and NF-κB. Specifically, lovastatin inhibits EGF-induced EGFR autophosphorylation, whereas combination of lovastatin and gefitinib (a reversible selective EGFR-tyrosine kinase inhibitor) or lovastatin and cetuximab (a monoclonal antibody targeting the EGFR) results in enhanced cytotoxicity. Statin treatment suppresses the expression of the antiapoptotic Bcl2 protein and up-regulates that of the proapoptotic Bax, responsible for cytochrome *c* release from the mitochondria, leading to the activation of procaspase 9. Statins can also directly induce caspase 3, 7, and 9 activity. Lipid rafts and Cav1, a membrane protein localized in the cholesterol-rich domain named caveolae, regulate several signal transduction proteins, including steroid (both androgen and estrogen) receptors and the inactive form of eNOS. Signal transduction pathways involving Cav1 can be impaired by drugs that disperse plasma membrane cholesterol or disaggregate the lipid rafts. The palmitoylated cytosolic estrogen receptor α (ER-α) localizes to the plasma membrane associated with Cav1. The estradiol-induced activation of ER-α dissociates the receptor from Cav1 and triggers proliferation of breast cancer cells. Statins, impairing the ER-α–Cav1 association, inhibit ER-α localization to the plasma membrane and reduce the proliferation triggered by the estrogen-mediated nongenomic pathway in breast cancer cell lines. A similar mechanism of action has also been demonstrated for the membrane-associated androgen receptor in human prostate cancer cell lines. In endothelial cells, the activation of VEGFR, or the increase of cytosolic calcium through the activation of calmodulin (CaM), induces the release of eNOS from Cav1. eNOS binds to calmodulin, becomes phosphorylated by VEGFR-activated Akt, and produces NO, which triggers endothelial cell proliferation, migration, and vascular permeability, all processes involved in angiogenesis. Low doses of statins dissociate eNOS from Cav1, induce Akt activation, and stimulate angiogenesis. On the other hand, high doses of statins seem to inhibit the proangiogenic pathway.

TABLE 5
Human clinical trials reporting statin use in cancer therapy

Study	Statin	*n*	Tumor Type	Therapeutic Protocol	Results	Side Effects
Thibault et al. (1996)	Phase I:	88	38 Prostate	p.o. for 7 consecutive days in monthly cycles, increasing doses in the next cycle when well tolerated in the first	Minor response (45% reduction in tumor size maintained for 8 months) in one anaplastic astrocytoma	Incidence and severity of toxicity (mainly myopathy, nausea, diarrhea, fatigue, abdominal pain) increasing for dose level higher than 25 mg/kg
	Lovastatin (2–45 mg/kg/day)		24 Primary central nervous system 7 Breast 4 Colorectal 4 Ovary 3 Sarcoma 2 Lung 6 Others			Myalgias partially controlled by ubiquinone administration
Vitols et al. (1997)	Open nonrandomized pilot study: simvastatin (40 mg)	10	B-cell CLL previously untreated	p.o. 40 mg daily by a single oral dose for 12 weeks	No significant change in the clinical disease status during treatment; 40% of patients developed progressive disease during the subsequent year and 60% within 2 years after stopping simvastatin	No adverse reactions experienced during the treatment
Larner et al. (1998)	Phase I: lovastatin	18	Anaplastic glioma or glioblastoma multiforme	Lovastatin 30 mg/kg/day for 7 days at 4-week interval; recurrent disease after radiotherapy (n = 9) received lovastatin alone; newly diagnosed patients (n = 9) received radiotherapy plus lovastatin	One partial response; one minor response; one stable disease for a period longer than 400 days	Mild toxicity; mild pain in 2 patients
Kawata et al. (2001)	Randomized controlled trial: pravastatin (40 mg)	83	HCC pretreated with TAE, infusion of 30 mg doxorubicin) followed by oral 5-FU (200 mg daily) for 2 months	Pravastatin group (n = 41): p.o 20 mg/day for 2 weeks, followed by 40 mg/day (for 16.5 ± 9.8 months); control group (n = 42), not treated with any anticancer drugs.	No significant differences in Karnofsky performance status between treated and control group; slight improvement or stable status in the liver functions in treated group compared with control; in pravastatin-treated group, median survival higher than in control group (18 vs. 9 months)	No adverse reactions experienced during the treatment
Kim et al. (2001)	Phase II: lovastatin (35 mg/kg/day)	16	Locally advanced and metastatic adenocarcinoma of the stomach, previously treated with systemic chemotherapy	p.o. 35 mg/kg/day in four divided doses for 7 consecutive days in monthly cycles (median, two cycles) ubiquinone (p.o. 240 mg daily) coadministered with lovastatin	No significant responses	Anorexia in the 64% of patients; myalgia in two patients (12.5%)
Minden et al. (2001)	Case report lovastatin	1	Relapsed AML	40 mg/day	Partial control of the leukemic blast cells	Not reported

TABLE 5—*Continued.*

Study	Statin	*n*	Tumor Type	Therapeutic Protocol	Results	Side Effects
Lersch et al. (2004)	Randomized controlled trial: pravastatin (40–80 mg)	58	HCC previously treated with 3 × 200 μg/day octreotide for 2 months	Octreotide group (n = 30): 20 mg octreotide LAR every 4 weeks; pravastatin group (n = 20), 40–80 mg pravastatin; gemcitabine group (n = 8), 80–90 mg/m^2 over 24 h weekly in cycles of 4 weeks	No significant differences in tumor responses; pravastatin failed to prolong median survival	Not reported
Katz et al. (2005)	Retrospective multivariate analysis: all statins	349	Clinically resectable nonmetastatic rectal cancers; among these 33 statin users	Presurgery neoadjuvant chemoradiation (median dose, 50.4 Gy) and concurrent chemotherapy with 5-FU	No differences in clinical stages at time of diagnosis; in statin users, improved pathologic complete response rate after neoadjuvant chemoradiation	
Knox et al. (2005)	Phase I: Lovastatin (5–10 mg/kg/day)	26	14 HNSCC and 16 CC, advanced or recurrent	Lovastatin (5–10 mg/kg/day) for 2 weeks every 21 days	No significant responses; slight effect in disease stabilization	Muscle toxicity at 10 mg/kg/day for 14 days
Graf et al. (2008)	Cohort study: pravastatin	183	HCC patients selected for palliative treatment by TACE: 52 received TACE combined with pravastatin; 131 received chemoembolization alone	Pravastatin (20–40 mg/day)	In HCC treated by TACE and pravastatin, median survival was significantly longer than that in HCC treated by TACE alone (20.9 vs. 12.0 months)	Not reported
Kornblau et al. (2007)	Phase I: Pravastatin (40–1680 mg/day)	37	15 Newly diagnosed patients with AML; 22 salvage patients with AML	Pravastatin (40–1680 mg/day) administered p.o. once daily for 8 days; idarubicin (12 mg/m^2/day), intravenously, days 4–6; and cytarabine (1.5 g/m^2/day) by continuous infusion, days 4–7, coadministered with pravastatin	Among 15 newly diagnosed patients 11 experienced complete remission; in 9 of 22 salvage patients, a complete remission was obtained	No toxicity occurred at a frequency higher than that expected with the standard idarubicin-cytarabine protocols; no significant increase in the frequency and severity of toxicity associated with pravastatin dose escalation.
Lee et al. (2009)	Phase II: Simvastatin	49	Metastatic adenocarcinoma of the colon or rectum	Simvastatin (40 mg, p.o. once daily during the period of chemotherapy) coadministered with FOLFIRI (irinotecan 180 mg/m^2/90-min infusion; leucovorin 200 mg/m^2, 2-h infusion; 5-FU 400 mg/m^2 bolus injection followed by 2400 mg/m^2 as a 46-h continuous infusion), repeated every 2 weeks	Response rate (46,9%) and median survival time (21.9 months) similar to that obtained with FOLFIRI alone; modestly prolonged TTP (9,9 months) compared with FOLFIRI alone	No toxicity higher than that induced by FOLFIRI alone; no patients experienced myotoxicity or increase in serum creatine phosphokinase

CLL, chronic lymphocytic leukemia; TAE, transcatheter arterial embolization; CC, cervix carcinoma; HNSCC, head and neck SCC.

been tested in combination with folinic acid (leucovorin)/5-FU/irinotecan (FOLFIRI), a conventional second-line therapy used in colorectal cancer (Lee at al., 2009). Forty-nine patients affected by metastatic adenocarcinoma received 40 mg of simvastatin once daily by mouth during the period of FOLFIRI chemotherapy. In these patients, the overall responsive rate and the median survival were similar to that obtained with FOLFIRI alone. Moreover, the simvastatin-FOLFIRI combination treatment induced a slight increase in the time to progression (9.9 months; 95% CI, 6.4–13.3), and simvastatin did not increase the toxicity achievable with FOLFIRI alone. Finally, lovastatin and simvastatin were tested in patients affected by different histological types of leukemia. In 10 patients with chronic lymphocytic leukemia, oral simvastatin (40 mg daily for 12 weeks) induced no significant change in the clinical disease status, and 40% of the patients experienced a progression of the neoplasia during the subsequent year (Vitols et al., 1997). In a case report (Minden et al., 2001), lovastatin, at a dose double than that usually recommended for hypercholesterolemia, induced apparent control of the leukemic blast cells in a 72-year-old woman with relapsed AML. Because the AML blasts exposed to cytostatic agents increased their cellular cholesterol levels, which represents a mechanism able to induce chemoresistance, it is possible that statins, acting as HMG-CoA reductase inhibitors, improved the sensitivity to antitumor treatments. Encouraging results reported by a phase I study (Kornblau et al., 2007) seem to corroborate the effectiveness of statin use as adjuvant compounds in AML. Thirty-seven subjects (15 newly diagnosed and 22 salvage patients) received the Ida-HDAC regimen [idarubicin, 12 mg/m^2 per day, days 4–6, and high-dose cytarabine (HDAC), 1.5 g/M^2 per day, by continuous infusion, days 4–7], coadministered with pravastatin (40–1680 mg/day, by mouth, days 1–8). Complete remission was obtained in 73% (11/15) of new patients and in 41% (9/22) of salvage patients. Moreover, this scheduled treatment induced a toxicity similar to that expected with the standard Ida-HDAC protocols (Kornblau et al., 2007).

VI. Conclusions and Future Directions

Increasing evidence demonstrates the pleiotropic effects of statins, suggesting a potential use of these compounds beyond their lipid-lowering properties in several acute and chronic diseases. To date, in our opinion, the more promising applications of statins in human seem to be related to their antiinflammatory effects, mediated by both direct (via modulation of the immune-response) and indirect (via inhibition of platelet functions) mechanisms, and their ability to modulate bone metabolism.

A number of studies analyzed the cancer risk in statin users. The main difficulties in ascertaining the real role of statins in cancer occurrence are the lack of clinical and historical data for the examined patients; the presence of a consistent number of confounding variables, which produced conflicting results and accounted for unconvincing evidence; the moderate number of studies considering the cancer incidence as primary endpoint; and the heterogeneity in patient samples and in cancer types considered. Large, rigorous meta-analyses (Dale et al., 2006; Kuoppala et al., 2008) showed that statins have a neutral effect on cancer risk, and no type of cancer was affected by statin use. A meta-analysis evaluating the risk of colorectal cancer was performed by Bonovas et al. (2007). They found no evidence of association between statin use and risk of colorectal cancer either among randomized controlled trials (RR, 0.95; 95% CI, 0.80–1.13) or among cohort studies (RR, 0.96; 95% CI, 0.84–1.11), even if case-control studies suggested a slight reduction in the risk of colorectal cancer occurrence (RR, 0.91; 95% CI, 0.87–0.96). On the other hand, to date, no sufficient data are available to define the long-term effects of prolonged statin use up to 10 years and beyond.

Some authors have suggested a partial protective effect of statins on the occurrence of high-grade cancers, which account also for the favorable prognosis of the tumors and good response to therapies. In our opinion, this hypothesis suffers from a common confusing variable: patients taking statins are subjects that frequently undergo clinical and serological evaluations aimed to control therapy and chronic disease. The medical surveillance and the early evidence of the neoplasia is the real cause of the lack of high-grade tumors and, then, of the reduced number of relapse and of nonresponder status.

The potential efficacy of statins as therapeutic drugs has been also evaluated. At least two problems have to be considered. First, statins differ in their solubility and their hydrophobic/hydrophilic rate, which governs their biochemical function at extrahepatic sites (Duncan et al., 2005). In particular, hydrophilic pravastatin does not enter normal extrahepatic cells or malignant cells of extrahepatic origin, and this property could account for a reduced effect in cancer types other than HCC. Moreover, tumor tissues are frequently sites of edema, necrosis, vascular remodeling, all processes that could significantly modify the doses of statins able to penetrate tumor tissues. Second, statin doses able to induce both antiproliferative and antiangiogenic effects were higher than those used in lipid-lowering protocols. Several studies showed that lovastatin used at doses higher than 25 mg/kg per day (Thibault et al., 1996) or at 10 mg/kg per day for 14 days (Knox et al., 2005) induced severe muscle toxicity and frequently anorexia, nausea, diarrhea, fatigue, and abdominal pain, often only partially counteracted by a very modest anticancer effect.

An encouraging result has been reported by Kawata et al. (2001) in a randomized controlled trial performed in patients with HCC. The authors found that 40 mg/day pravastatin significantly increased the median survival (doubled in pravastatin-treated group compared with

untreated group). Similar results have been reported from the analysis of a cohort of 183 patients with advanced HCC treated with palliative TACE and pravastatin (Graf et al., 2008). On the other hand, Lersch et al. (2004) failed to replicate these results in HCC previously treated with octreotide for 2 months. Knox et al. (2005) found a disease stabilization of 23% in patients with cervical carcinoma or head and neck SCC treated with prolonged administration of lovastatin. The authors considered the obtained results encouraging, but stable disease (more than 2 years) was obtained in only one patient treated with EGFR inhibitor, despite progression of the disease, before taking lovastatin.

In metastatic colorectal cancers, 40 mg of simvastatin administered without resting during FOLFIRI chemotherapy (Lee et al., 2009) showed a weak cytostatic effect proved by a prolonged time to progression but did not improve the median survival. Fair results have been obtained in a phase I study performed in patients with AML treated with the combination Ida-HDAC and high doses of pravastatin (Kornblau et al., 2007). Among 37 patients enrolled in the study, 54% experienced a complete remission, and subjects receiving repeated cycles relapsed at a median period longer than 20 months.

In summary, clinical trials performed with statins used as anticancer treatment in human are largely heterogeneous and produced slight evidence of a real efficacy as adjuvant therapy. The statins possess mainly cytostatic but not cytotoxic effects on tumors, patients experienced relapse at the end of the treatment with high doses of statins, and the prolonged median survival seems not to be achievable in different types of cancer. However, it is relevant to consider that combining a specific statin with different chemotherapies or administering statins after several first-line treatments does not necessarily produce similar results. In our opinion, data obtained from trials in HCC, in AML, and partially in colon cancer deserve the planning of larger and homogeneous trials able to elucidate the tumor types, the therapeutic regimen, and the subgroup of patients that could really benefit from statins used as adjuvant drugs. Moreover, a large clinically and socially relevant goal will be to evaluate the role of statins in the possibility of overcoming resistance to biological therapies (e.g., cetuximab and bevacizumab) or at least to improve the responsiveness in tumors carrying Ras or ErbB2 activation.

Despite the partial or minor response obtained in clinical trials, in vitro evidence showed great anticancer potential for statins used in combination with chemotherapeutic compounds usually used in the clinical practice. In the last decade, a significant improvement in polychemotherapies has been obtained with the introduction of monoclonal antibodies, also known as biological agents. In human breast cancer cell lines, fluvastatin combined with trastuzumab (a monoclonal antibody against ErbB2) demonstrated a synergic cytotoxic effect; thus, their combination seems to represent a good chance to increase the incomplete efficacy achievable with trastuzumab alone in Her2-positive breast tumors (Budman et al., 2007). Moreover, daily oral intake of simvastatin or fluvastatin produced significant in vivo antitumor effects in the ErbB2-transformed Neu transgenic mouse A mammary cancer model through reduction of both proliferation and survival of the tumor cells (Campbell et al., 2006).

In human HCC cell lines, fluvastatin showed a synergistic antiproliferative effect with cetuximab, a monoclonal antibody targeting the EGFR (Huether et al., 2005). HCC cell lines carrying mutations of p53 are less sensitive to cetuximab treatment, but in these cellular models, the combined use of cetuximab and erlotinib or fluvastatin induced a significant reduction of the cell growth (Huether et al., 2005).

Finally, statins (mainly lovastatin) potentiated the antiproliferative effects of gefitinib, a potent tyrosine kinase inhibitor of EGFR, in SCC, non–small-cell lung cancer, colorectal cancer cell lines (Mantha et al., 2005), and glioblastoma-derived cell lines (Cemeus et al., 2008), probably through enhanced inhibition of the PI3K/Akt pathway.

The management of cancer patients frequently needs to accommodate, besides the tumor control and the choice of the therapeutic options, several concomitant diseases and complications triggered by the neoplasia, which represent the main cause of therapy disruption and the reduced quality of life. In this matter, statins could provide some benefit.

A new field of research is highlighting that statin use might confer protection against the risk of developing venous thromboembolism in patients with solid organ tumors, who are considered to be another high-risk population for the thrombotic events attributed to the hypercoagulable state caused by the disease and its treatments (Caine et al., 2002). In this context, a retrospective, case-control study reviewing 740 consecutive patients with a diagnosis of solid organ tumor suggested for the first time that in cancer patients, the use of statins decreased the odds ratio (0.33) of developing venous thromboembolism (95% CI, 0.19–0.57; $p \leq 0.05$) compared with nonstatin users (Khemasuwan et al., 2010). These preliminary data are encouraging but suffer from some limitations, so a prospective, randomized, placebo-controlled trial would provide further support and stronger evidence for this finding, making the statins a possible safe alternative anticoagulant medication to the commonly used warfarin for venous thromboembolism in cancer patients. In the same context, it has been proposed that statins, thanks to their potent antiplatelet and anti-inflammatory effects, together with the cytoreductive potential and restoration ability of endothelial dysfunction, may have potential clinical benefits in decreasing the thrombohemorrhagic complications in patients affected by classic Philadelphia chromosome-negative myeloproliferative disorders, polycythemia vera, essential thrombocythemia, and idiopathic myelofibro-

sis (Hasselbalch and Riley, 2006). Moreover, taking advantage of their antiplatelet functions, statins might also act as modulators of allograft outcome, potentially reducing the hypercoagulability seen in transplant recipients (Mehra et al., 2002).

Metastases to bone are a frequent progression of several tumors and pain associated with this localization of the neoplasia represent a heavy burden for patients. Bone can be affected by several neoplastic conditions, which can include both primary bone tumors and metastatic diseases. Bisphosphonates are a class of agents most frequently used to reduce these types of skeletal cancer-related events by inhibiting osteoclast activity. In the light of this evidence, statins, by inhibiting the same pathway, may be useful to decrease these skeletal cancer-related events. To date, statins have been demonstrated to exert antitumor effects on primary osteosarcoma cells, and very recently, *Cyr61* gene has been identified as a new target of this action (Fromigue et al., 2011). As proposed, simvastatin acts as an inhibitor of osteolysis, preventing skeletal metastasis in a mouse model of breast cancer skeletal metastasis of human mammary cancer cell MDA-MB-231, which expresses the mutant p53R280K. This effect has been associated with the decreased expression of CD44, which highly correlates with the level of oncogenic p53 (Mandal et al., 2011) and the invasive potential of the tumor.

In conclusion, despite the inconclusive results obtained in human by the little phase I-II studies performed to date, the statins could represent a fair possibility to improve adjuvant therapies at least in some cancer types, such as HCC, colorectal cancer, and AML, but this hypothesis needs to be corroborated by large and well planned clinical trials.

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Authorship Contributions

Participated in research design: Gazzerro and Bifulco.

Performed data analysis: Gazzerro, Proto, Gangemi, Malfitano, Ciaglia, Pisanti, Santoro, and Laezza.

Wrote or contributed to the writing of the manuscript: Gazzerro, Proto, Gangemi, Malfitano, Ciaglia.

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