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PHARMACOLOGICAL CHARACTERIZATION OF THE SIGNAL TRANSDUCTION PATHWAYS MODULATED BY A_3 RECEPTORS IN CANCER CELLS: POSSIBLE TARGETS FOR THERAPEUTIC INTERVENTION

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CHAPTER 1

ADENOSINE AND CANCER

Adenosine is a nucleoside composed of a molecule of adenine attached to a ribose sugar molecule (ribofuranose) via a β-N9-glycosidic bond (Figure 1).

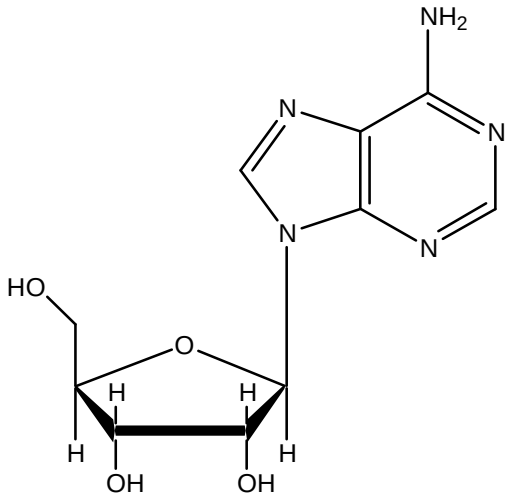


Figure 1 – Chemical structure of Adenosine

Adenosine is a primordial signaling molecule present in every cell of the human body that mediates its physiological functions by interacting with 4 subtypes of G-protein-coupled receptors, termed A_1, A_{2A}, A_{2B}, and A_3. These receptors are widely distributed throughout the body. A_{2A} and A_{2B} receptors are coupled to adenylate cyclase activity, and their stimulation increases the intracellular cyclic adenosine monophosphate (cAMP) concentration while A_1 and A_3 receptor stimulation decreases cAMP concentration and raises intracellular Ca^{2+} levels by a pathway involving phospholipase C (PLC) activation (Abbracchio et al., 1995; Fredholm et al., 2001a). Adenosine effects are widespread and pleiotropic. The cellular response to this autacoid strictly depends on the expression of the different adenosine receptor subtypes, which can be coexpressed by the same cell and serve as active modulators in signal transduction. Understanding the interactions between them could provide critical information for determining the mechanism involved in cellular survival. Adenosine receptors have

been actively studied as potential therapeutic targets in several disorders such as Parkinson's disease, schizophrenia, analgesia, and ischemia. Adenosine has been shown to be a crucial factor in determining the cell progression pathway, either in the apoptotic or in the cytostatic state (Spychala, 2000). At present, however, few therapeutic candidates in the fight against cancer are available from the ''adenosinergic system.''

1.1 Introduction

One of the difficulties in treating most of the common cancers (colon, lung, breast, prostate, etc.) is that they form solid tumors. The individual cancer cells, being different from normal cells, form a tissue mass that behaves in a radically different way from normal tissues in the body. This is because the major cell population (the cancer cells) has grown in a way that is out of step with all of the other cells that would normally form a supportive network. In particular, the growth of the cancer is not coordinated with the development of a proper blood supply. The vascular net, work of a tumor, is usually inadequate, the blood vessels are often too few in number, the network is improperly branched, and their calibre is not well controlled. This means that the blood supply is inadequate. Consequently, most solid tumors do not receive sufficient oxygen and the cells are hypoxic. Specifically, hypoxia is conducive to adenine nucleotide breakdown, which is responsible for the adenosine release (Vaupel et al., 1989, 2001). As a consequence, adenosine accumulates to high levels in hypoxic tissues. In particular, it is recognized that significant levels of adenosine are found in the extracellular fluid of solid tumors (Blay et al., 1997), suggesting a role of adenosine in tumor growth. Adenosine, released from hypoxic tissue, is thought to be an angiogenic factor that links altered cellular metabolism, caused by oxygen deprivation, to compensatory angiogenesis. Angiogenesis (or neovascularization) begins with the migration of endothelial cells, originating from capillaries, into the tissue being vascularized. Adenosine has been reported to stimulate or inhibit the release of angiogenic factors depending on the cell type examined (Burnstock, 2002; Feoktistov et al., 2002). On one hand, adenosine is known to cause the synthesis of vascular endothelial growth factor (VEGF) (Grant et al., 1999) and increases the proliferation of endothelial cells obtained from the aorta (Van Daele et al., 1992), coronary vessels, and retina (human retinal endothelial cells, HREC) (Burnstock, 2002). In particular,

adenosine has been shown to induce the DNA synthesis in cultures of human umbilical vein endothelial cells (HUVEC) (Ethier et al., 1993; Rathbone et al., 1992a,b; Sexl et al., 1995, 1997; Ethier & Dobson, 1997). Furthermore, adenosine has been shown to stimulate canine retinal microvascular endothelial cell migration and tube formation (Lutty et al., 1998; Lutty & McLeod, 2003). On the contrary, adenosine has been reported to inhibit growth of human aortic smooth muscle cells (Dubey et al., 1998a, 1998b), cardiac fibroblasts (Dubey et al., 2001), and vascular smooth muscle cells (Dubey et al., 2000).

An increase in proliferation, unless repaired or balanced by an increase in apoptosis, results in hyperproliferative disease and cancer. Apoptosis, also called programmed cell death, progresses through a series of well-regulated morphological and biochemical phases, including chromatin condensation (Kerr et al., 1994) and caspase activation (Hale et al., 1996; Leist & Jaattela, 2001). Failure to undergo apoptosis has been implicated in pathological situations, including tumor development (King & Cidlowski, 1995).

Adenosine promotes wound healing (keratinocytes proliferation) and mediates angiogenesis (endothelial cell proliferation) in response to tissue injury (Montesinos et al., 2002), but it has been implicated in the induction of apoptosis in several cell types such as rat brain astroglial cells (Ceruti et al., 1997), human thymocytes (Szondy, 1994), rat microglia cells (Schubert et al., 2000), arterial smooth muscle cells (Peyot et al., 2000), pulmonary artery endothelial cells (Dawicki et al., 1997), and sympathetic neurons (Wakade et al., 2001). Concerning the nervous system-derived cells, adenosine has been demonstrated to protect damaged neuronal cells against cell death (Ongini et al., 1997).

In the human leukemia HL60, human melanoma A375, and human astrocytoma cells, adenosine at millimolar concentrations caused apoptosis. It seems likely that apoptosis is mediated by the intracellular actions of adenosine rather than through surface receptors (Tanaka et al., 1994; Abbracchio et al., 2001; Merighi et al., 2002a). It has been argued that the effect of high adenosine concentration might be subsequent to uptake of adenosine by the cell and intracellular accumulation of AMP, leading to caspase activation (Schrier et al., 2001).

Other in vitro studies have shown that adenosine exerts inhibitory effects on tumor cell growth. In human epidermoid carcinoma A431 cells, adenosine evoked a biphasic response in which a concentration of 10 µM produced inhibition of colony formation;

however, at concentrations up to 100 µM, this inhibition was progressively reversed (Tey et al., 1992). Moreover, adenosine inhibited Nb2-11C lymphoma cell proliferation at concentrations of 5 – 25 µM and induced in a dose-dependent manner an arrest of the cells in the G_0/G_1 phase of the cell cycle and indeed a decrease in the telomeric signal, thus suggesting a cytostatic rather than an apoptotic effect (Fishman et al., 2000a, 2000b, 2001). Adenosine has been shown to interfere with the proliferation of many cell types: its introduction at micromolar concentrations to cultures of tumor proliferating cells, e.g., lymphoma, prostate carcinoma, or leukemia cells, markedly inhibited their proliferation whilst stimulated the proliferation of normal cells, such as bone marrow cells or fibroblasts (Fishman et al., 1998; Fishman et al., 2000a, 2000b). Exogenous and endogenous adenosine has been shown to inhibit both collagen production and cellular hypertrophy induced by fetal calf serum (Dubey et al., 1998a, 1998b). In many cases, tumor-induced immune suppression is mediated by soluble inhibition factors or cytokines elaborated by the tumor cells. Extracellular fluid of solid carcinomas contains immunosuppressive concentrations of adenosine, suggesting that this autacoid constitutes an important local immunosuppressant within the microenvironment of solid tumors.

Antigen-presenting cells such as dendritic cells and macrophages are specialized to activate naive T-lymphocytes and initiate primary immune responses. Adenosine inhibits interleukin- 12 (IL-12) and tumor necrosis factor-α (TNF-α) production in dendritic cells and in macrophages impairing T-cell priming (Hasko et al., 2000, 2002; Panther et al., 2001) and suppressing the anticancer immune response. Furthermore, adenosine impairs the induction and expansion of cytotoxic T-lymphocytes and the antitumor activity of natural killer cells (Williams et al., 1997a, 1997b; Hoskin et al., 2002).

In vivo studies have shown that adenosine exerts a profound inhibitory effect on the induction of mouse cytotoxic T-cells, without substantially affecting T-cell viability (Hoskin et al., 1994, 2002). In vitro studies confirmed that adenosine had profound effects on immune cells and has been implicated in the intrathymic apoptotic deletion of T-cells during development (Barbieri et al., 1998). Furthermore, the spontaneous proliferation of thymocytes after 20–25 hours of culture was significantly increased by the presence of adenosine deaminase (ADA), the enzyme that removes extracellular adenosine from the culture medium (Sandberg, 1983). In addition, in murine bone marrow-derived macrophages, adenosine inhibited macrophage colony stimulating

factor-dependent proliferation (Xaus et al., 1999).

Adenosine sustains a complex role in the immune system activity, because when given to mice pretreated with cyclophosphamide it demonstrated a myeloprotective effect by restoring the number of white blood cells and the percentage of neutrophils as compared with normal values. Furthermore, it has been demonstrated that the elevation of the extracellular adenosine concentrations induced a radioprotective effect in mice by the stimulation of hematopoiesis in the bone marrow and the spleen (Pospisil et al., 1995, 1998). In support of this myelostimulatory role, it has been demonstrated that adenosine enhances cycling of the hematopoietic progenitor cells (Pospisil et al., 2001).

In conclusion, adenosine can drive neovasculogenesis and immune system activity, thus affecting regional tumor control. Interactions between adenosine signaling for proliferation and cell death also occur (Jacobson et al., 1999). The ability of adenosine to specifically inhibit tumor cell growth in vitro and in vivo suggests that the activation and/ or blockade of the pathways downstream of adenosine receptors may contribute to tumor development. Furthermore, the extracellular adenosine concentration may be a crucial factor in determining the cell progression pathway, either in the apoptotic or in the cytostatic state.

Adenosine receptors and cancer

A_1 adenosine receptors

The role of A_1 adenosine receptors in tumor development is unknown and debatable. A_1 receptors have been associated to carcinogenesis by previous investigations where the expression of this receptor has been demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) in colorectal adenocarcinomas and peritoneal colon tissues (Khoo et al., 1996). A_1 receptors have been detected also in the human leukemia Jurkat and human melanoma A375 cell lines (Gessi et al., 2001; Merighi et al., 2001).

A_1 adenosine receptors can be coupled to different pertussis toxin-sensitive G proteins, which mediate inhibition of adenylate cyclase and regulate calcium and potassium channels, as well as inositol phosphate metabolism (Fredholm et al., 2001). A_1 and A_{2A} adenosine receptors can be found presynaptically and modulate neurotransmitter release. Presynaptic A_1 adenosine receptors are the prototype of GPCRs, the stimulation of which decreases the probability of neurotransmitter release. The main mechanism of A_1 receptor-mediated inhibition of exocytosis is a direct inhibitory effect on voltage-

dependent Ca^{2+} channels (Moore et al., 2003). Recent studies suggest an anti-inflammatory role for chronic A_1 receptor activation by high levels of adenosine in the lung, a surprising and important finding in light of the fact that A_1 receptor antagonists are being investigated as a potential treatment for asthma (Sun et al. 2005)

Recently, it was reported that A_1 receptors act as antiapoptotic and prosurvival mediators protecting human proximal tubular cells from the direct cytotoxic effect of H_2O_2 (Lee& Emala, 2002). After exposure to H_2O_2, intracellular ATP decreases significantly and the A_1 receptor activation may attenuate this component of cellular injury. Furthermore, A_1 receptors promote ethanol activation of PI3K/Akt pathway in cultured HUVEC, exerting an antiapoptotic activity (Liu et al., 2002). In contrast, adenosine-induced apoptosis in primary cultured rat astrocytes and in C6 glial cells was not sustained by A_1 receptors (Appel et al., 2001; Di Iorio et al., 2002). Similarly, it has been recently demonstrated that adenosine-induced alteration of cell proliferation and cell death was not mediated by A_1 receptors expressed in A375 human melanoma cells (Merighi et al., 2002a). Even considering that the negative results may be due to low A_1 receptor expression and/or uncoupling with Gi proteins, the absence of a proapoptotic or deleterious effect of A_1 stimulation does not exclude the idea that A_1 can play a role in carcinogenesis and tumor development. In particular, cisplatin, a widely used antineoplastic agent, up-regulates the A_1 receptor in the rat kidney (Bhat et al., 2002). However, antagonists of A_1 receptor exerted opposite effects reducing (Bhat et al., 2002) and exacerbating (Heidemann et al., 1989; Knight et al., 1991) cisplatin-induced nephrotoxicity. In the light of this, A_1 receptors could act by exerting an antiapoptotic and prosurvival activity on normal cell survival under critical environment. Moreover, in an experimental approach using an A_1 receptor deficient mouse as a tumor host, the importance of the microglial cells for mediating the A_1 receptor anticancer effect is highlighted (Synowitz et al. 2006). Metalloproteinase MMP-9 and MMP-12 are significantly elevated in A_1 adenosine receptor-deficient mice (Tsutsui et al. 2004). Indeed, MMPs play an important role in glioblastoma progression and, as was recently demonstrated, the expression of MMPs by microglia has an impact on tumor growth (Markovic et al. 2005). Matrix degradation by MMPs is an important prerequisite for glioblastoma invasion (Rao 2003). A_1 receptor activation on microglia/macrophages inhibits not only the production of cytokines like interleukin-1β but also matrix MMPs like MMP-12 (Tsutsui et al. 2004). Further studies are necessary to corroborate an active prosurvival function of A_1 receptors and their involvement with tumors.

A_{2A} adenosine receptors

A_{2A} adenosine receptors have been found on cell membranes of different human tumor cells: SH-SY5Y neuroblastoma, NG108-15 neuroblastoma × glioma hybrid, U937 monocytic lymphoma, Jurkat T-cell leukemia, A375 melanoma, and A431 epidermoid cells (Tey et al., 1992; Van der Ploeg et al., 1996; Mundell & Kelly, 1998; Gessi et al., 2001; Mayne et al., 2001; Merighi et al., 2001; Hillion et al., 2002). However, the possible physiological role ascribed to A_{2A} adenosine receptors in cytoprotection (i.e., protection against apoptosis and toxic insults) remains to be evaluated.

It has to be remarked that A_{2A} receptors contribute significantly to the antiischemic action of adenosine. The evidence for this comes from studies performed on the rat PC12 cell line used as a model for evaluating adenosine effect that has been attributed to the endogenous A_{2A} receptor.

Stimulation of the A_{2A} adenosine receptors counteracts the inhibition of neurite outgrowth due to MAPK blockade (Cheng et al., 2002). Stimulation of the A_{2A} adenosine receptor alone also activates the Ras/Raf-1/MEK/ERK signaling through PKA-dependent and PKA-independent pathways via Src- and Sos- mediated mechanisms, respectively (Schulte and Fredholm, 2003). Interestingly, phosphorylation/activation of CREB has been shown to compete with nuclear factor-κB (NFκB) p65 for an important co-factor, CBP. Phosphorylated CREB was therefore proposed to mediate the anti-inflammatory effect of the A_{2A} adenosine receptor and inhibition of NFκB by A_{2A} adenosine receptor activation during acute inflammation in vivo was demonstrated (Fredholm et al., 2007).

Treatment with an adenosine A_{2A} agonist results in a reduction in neuronal apoptosis and a decrease in spinal cord reperfusion inflammatory stress during rabbit spinal cord reperfusion (Cassada et al., 2001). It is possible to achieve infarct reduction with adenosine, inhibiting apoptosis. A recent study suggested that inhibition of apoptosis by adenosine at reperfusion involves the alterations in antiapoptotic Bcl-2 and proapoptotic Bax proteins and neutrophil accumulation, primarily mediated by an adenosine A_{2A} receptor (Zhao et al., 2001).

Despite this antiapoptotic role on normal tissue, A_{2A} receptors on T-cell surface may play immunosuppressive role in conditions leading to an increase of adenosine concentrations, as found in large solid tumors where hypoxic conditions are known to cause accumulation of extracellular adenosine, which in turn could inhibit incoming antitumor cytotoxic T-lymphocytes from destroying the tumor (Koshiba et al., 1997).

Adenosine derivatives may induce apoptosis of human peripheral blood mononuclear cells through an A_{2A}-like extracellular membrane receptor (Barbieri et al., 1998). At the same time, however, activation of adenosine A_{2A} receptor has been shown to delay apoptosis in human neutrophils (Walker et al., 1997) and promote cell death of human melanoma cells (Merighi et al., 2002a). In contrast, adenosine-induced apoptosis in primary cultures of rat astrocytes and in C6 glial cells is not sustained by A_{2A} receptors (Appel et al., 2001). Further evidences from in vivo studies suggest that blockade of A_{2A} provides neuroprotection and moreover supports the view that A_{2A} stimulation is detrimental in neurons and thymocytes (Ongini & Schubert, 1998; Apasov et al., 2000). Conversely, theophylline has an immunomodulatory action on neutrophil apoptosis via a mechanism involving A_{2A} antagonism that increases granulocyte apoptosis (Yasui et al., 2000). The seminal observations of Ohta and Sitkovsky (2001) clearly established a role for the A_{2A} adenosine receptors in protecting host tissue from destruction by overexuberant immune responses. Considering that the tumor microenvironment contains relatively high levels of extracellular adenosine, data is emerging to support the hypothesis that tumor-derived adenosine is one mechanism by which tumors evade immune destruction (Blay et al. 1997; Ohta et al. 2006).

The immunosuppressive role and the ability to protect against ischemia suggests that A_{2A} receptor activation improves hypoxic tumor cell survival and immunoescaping. Furthermore, adenosine promotes wound healing and mediates angiogenesis in response to tissue injury via occupancy of A_{2A} receptors (Montesinos et al., 1997, 2002). Recently, synergistic up-regulation of VEGF expression in murine macrophages by adenosine A_{2A} receptor agonists and endotoxin was reported (Leibovich et al., 2002). A_{2A} receptors stimulate endothelial and melanoma cell proliferation (Sexl et al., 1995, 1997; Merighi et al., 2002a). The results presented above provide further evidence for an active role of A_{2A} receptors in tumor growth.

A_{2A} receptors have been shown to increase erythropoietin production in hepatocellular carcinoma cells (Hep3B) in culture and in vivo in rats and in mice under normoxic and hypoxic conditions (Nagashima & Karasawa, 1996; Fisher & Brookins, 2001). The discrepancy of A_{2A} effects between different cellular systems is attractive. Most likely, A_{2A} effects are linked to cell-specific factors or to the different role exerted by a similar signaling pathway downstream A_{2A} receptor in different cell types.

Further studies are needed to verify if A_{2A} adenosine receptors are crucially involved in both positive and negative regulation of cell survival depending upon the cell type,

degree of receptor activation, and/or coupling to different transduction mechanisms.

A_{2B} adenosine receptors

The A_{2B} adenosine receptor properties are still to be fully clarified. The distribution of the gene transcript indicates that the A_{2B} receptor is expressed in many tissues. At present, it is clear that the receptor is activated only at exceptionally high concentrations of adenosine, i.e., under pathophysiological rather than physiological conditions (Fredholm et al., 2001b). Recent studies have demonstrated that extracellular adenosine induces apoptosis of human arterial smooth muscle cells via A_{2B} receptor, involving a cAMP-dependent pathway (Peyot et al., 2000). On the contrary, adenosine prevents the death of mesencephalic dopaminergic neurons by a mechanism that seems to involve A_{2B} receptor stimulation (Michel et al., 1999). However, in this study, it was not excluded that the activation of an intracellular signaling pathway occurs in target cells without receptor mediation. Functional A_{2B} adenosine receptors have been found in fibroblasts and various vascular beds, hematopoietic cells, mast cells, myocardial cells, intestinal epithelial and muscle cells, retinal pigment epithelium, endothelium, and neurosecretory (Gessi et al., 2005). Although activation of adenyl cyclase is arguably an important signaling mechanism for A_{2B} adenosine receptors, this is not necessarily the case for A_{2B} adenosine receptors, as other intracellular signaling pathways have been found to be functionally coupled to these receptors in addition to adenyl cyclase. In fact activation of A_{2B} adenosine receptors can increase phospholipase C in human mast cells and in mouse bone marrow-derived mast cells. A_{2B} adenosine receptor activation also elevates inositol triphosphate (IP3) levels, indicating this receptor can couple also to Gq-proteins. A_{2B} adenosine receptors have been implicated in the regulation of mast cells secretion and gene expression, intestinal function, neurosecretion, vascular tone and in particular asthma (Varani et al., 2005). Although the A_3 adenosine receptor subtype is involved in the release of angiogenic factors, in some cases the A_{2B} adenosine receptor also seems to be responsible for the release of a certain subset of cytokines (Feoktistov et al. 2003; Merighi et al. 2007). A_{2B} adenosine receptors are expressed in human microvascular endothelial cells, where they play a role in the regulation of the expression of angiogenic factors like vascular endothelial growth factor (VEGF), IL-8, and basic fibroblast growth factor (bFGF) (Feoktistov et al. 2002).Adenosine activates the A_{2B} adenosine receptor in HREC, which may lead to

neovascularization by a mechanism involving increased angiogenic growth factor expression (Grant et al., 1999). In regard to this, A_{2B} adenosine receptor inhibition may offer a way to inhibit retinal angiogenesis and provide a novel therapeutic approach to the treatment of diseases associated with aberrant neovascularization, such as diabetic and prematurity retinopathy (Grant et al., 2001).

Adenosine causes inhibition of cardiac fibroblasts growth and of aortic and vascular smooth muscle cells by the activation of A_{2B} adenosine receptors (Dubey et al., 1998a, 1998b, 1999, 2000, 2001) while increases the proliferation of rat arterial endothelial cells via A_{2B} receptors (Dubey et al., 2002). Furthermore, exogenous and endogenous adenosine inhibits both collagen production and cellular hypertrophy induced by fetal calf serum, and this is most likely via the activation of A_{2B} receptors (Dubey et al., 1999). Thus, considering these facts, A_{2B} adenosine receptors may play a critical role in regulating cardiac remodelling associated with cardiac fibroblast proliferation. Pharmacological or molecular biological activation of A_{2B} adenosine receptors may prevent cardiac remodelling associated with hypertension, myocardial infarction, and myocardial reperfusion injury after ischemia. It is interesting to note that in contrast to smooth muscle cells A_{2B} receptors induce growth of endothelial cells (Grant et al., 1999).

We excluded a role for A_{2B} in the adenosine-induced proliferation of human melanoma cells (Merighi et al., 2002a). However, further studies are needed to assess the effect of A_{2B} stimulation in cancer cell development, proliferation, and diffusion. Moreover, data demonstrating A_{2B} receptor-mediated modulation of neovascularization may have interesting implications in the identification of novel drugs that may be utilized to increase endogenous protection against tumors.

A_3 adenosine receptors

The A_3 adenosine receptor is the only adenosine subtype which was cloned before its pharmacological identification. It was originally isolated as an orphan receptor from rat testis, having 40% sequence homology with canine A_1 and A_{2A} subtypes (Meyerhof et al., 1991) and was identical with the A_3 adenosine receptor later cloned from rat striatum (Zhou et al., 1992). Homologs of the rat striatal A_3 adenosine receptor have been cloned from sheep and human, revealing large interspecies differences in A_3 adenosine receptor structure. Recently equine A_3 adenosine receptor has been cloned

and pharmacologically characterized. Sequencing of the cDNA indicated that it has a high degree of sequence similarity with that of other mammalian A_3 adenosine receptor transcripts, including human and sheep (Brandon et al., 2006). Adenosine A_3 receptors have been shown to couple to classical or G protein dependent second-messenger pathways through activation of both Gi family and Gq family G proteins (Palmer et al. 1995; Merighi et al. 2003; Haskó and Cronstein 2004). Therefore, A_3 receptor stimulation inhibits adenylyl cyclase, resulting in a reduction of intracellular cAMP levels (Zhou et al. 1992; Varani et al. 2000). Furthermore, the abilities of recombinant A_3 receptors in transfected CHO cells (hCHO-A_3) to inhibit cAMP accumulation and endogenous A_3 receptors in rat basophilic leukemia cells (RBL-2H3; a cultured mast cell line) to stimulate PLC are abolished by pretreatment with pertussis toxin (Zhou et al. 1992; Ali et al.1990). This is consistent with a functional coupling of this receptor to Gi family G proteins. Furthermore, adenosine A_3 receptor signaling can increase phosphatidylinositol-specific phospholipase C (PLC) activity (Abbracchio et al. 1995; Ali et al.1990; Ramkumar et al. 1993) and cause Ca^{2+} to be released from intracellular stores (Fossetta et al. 2003; Shneyvays et al. 2004, 2005; Englert et al. 2002; Gessi et al.2001, 2002; Merighi et al. 2001). Presumably, the pertussis toxin-sensitive A_3 receptor-stimulated increase in inositol 1,4,5-triphosphate production in RBL-2H3 cells is due to increased levels of dissociated Gi-derived bg-subunits activating phosphatidylinositol-specific phospholipase C-b isoforms, an interaction that has been demonstrated both in intact cells (Hawes et al. 1994) and with purified components (Hepler et al. 1993). However, in experiments using the rat A_3 receptor stably expressed in a CHO cell line a functional interaction with G-proteins belonging to the Gq/11 family was demonstrated. Although derived from experiments using a heterologous expression system, this result suggests that at least in some instances the A_3 receptor mediated activation of PLC has a pertussis toxin-insensitive element (Iredale and Hill 1993; Palmer et al. 1995). Perhaps consistent with this finding was the observation that inosine produced an increase in cytosolic calcium in hepatocytes. This effect could be blocked using an A_3 selective antagonist, but was independent of a decrease in cAMP levels (Guinzberg et al. 2006). Recently, it has been shown that the A_3 receptor signals via PLC-b2/b3 to achieve its protective effect on skeletal muscle (Zheng et al. 2007). Because of their selective tissue distribution and the development of specific A_3 adenosine receptor agonists and antagonists for them, A_3 adenosine receptors have recently attracted considerable interest as novel drug targets.

Recent data demonstrated that activation of A_3 adenosine receptor is crucial for cardioprotection during and following ischemia–reperfusion and it has been suggested that a consistent part of the cardioprotective effects exerted by adenosine, once largely attributed to the A_1 receptor, may now be in part ascribed to A_3 adenosine receptor activation (Headrick & Peart, 2005). Even though there is a low expression of A_3 adenosine receptor in myocardial tissue, a number of studies have demonstrated that acute treatment with agonists induced protective "anti-ischemic" effects (Auchampach et al., 1997a; Tracey et al., 1997; Thourani et al., 1999a; Ge et al., 2006; Xu et al., 2006). The molecular mechanism of A_3 adenosine receptor cardioprotection has been attributed to regulation of mitochondrial (mito) KATP channels (Thourani et al., 1999b; Shneyvays et al., 2004; Peart & Headrick, 2007). In addition Shneyvays et al. (2005) demonstrated that in cultured rat myocytes Cl-IB-MECA delayed the dissipation of the mitochondrial membrane potential ($\Delta\psi$) and decreased the elevated intracellular calcium concentrations induced by hypoxia. These effects prevented irreversible cardiomyocyte damage and confirmed previous results showing that A_3 adenosine receptor activation protected cardiomyocytes treated with doxorubicin via inhibition of calcium overload and prevented cardiomyocyte death during incubation in high extracellular calcium concentrations (Shneyvays et al., 2001, 2004). As for the timing of cardioprotection, some studies have indicated that protection occurred post-ischemia, through inhibition of neutrophil-induced reperfusion injury or inhibition of myocyte apoptotic cell death (Jordan et al., 1999; Maddock et al., 2002), while others found that preischemic A_3 activation was effective and necessary for cardioprotection (Thourani et al., 1999a). Auchampach et al. demonstrated that A_3 agonism was able to trigger an anti-infarct response with either pre- or postischemic treatment (Auchampach et al., 2003). Moreover, it has been reported that A_3 adenosine receptor activation is able to mimic or induce myocardial preconditioning, meaning that transient stimulation of the A_3 before induction of ischemia leads to both an early and a delayed protection (Peart & Headrick, 2007).

It has been demonstrated that A_3 adenosine receptor triggers a prosurvival signal on human melanoma cells and that blockade of A_3 receptors by selective antagonists induces deleterious consequences (Merighi et al., 2002a). Despite the prosurvival and antiapoptotic role of A_3 adenosine receptor, different authors have shown that at the same time low concentrations of the A_3 receptor agonists have protective effects while, in contrast, high concentrations of the agonists for A_3 receptor can induce apoptosis.

Exposure of various cell types to different A_3 receptor agonists showed inhibition of cell proliferation in a dose-dependent manner, thus suggesting a cytostatic rather than apoptotic effect, mediated through A_3 adenosine receptors (Fishman et al., 1998, 2000b; Brambilla et al., 2000; Merighi et al., 2002a). In three different experimental tumor models in mice, including syngeneic (B16-F10 melanoma in C57Bl/6J mice) and xenograft models (HCT-116 human colon carcinoma and PC3 human prostate carcinoma in nude mice), A_3 receptor agonists, IB-MECA and Cl-IBMECA, inhibited tumor growth when administered orally (Fishman et al., 2002a). IB–MECA enhanced the cytotoxic effect of chemotherapy when tested in 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan thiazolyl (MTT) and colony formation assays. A combined treatment of 5-flurouracil plus IB–MECA yielded higher growth inhibition of HCT-116 human colon carcinoma cells in comparison to the chemotherapy alone (Bar-Yehuda et al. 2005). In particular, A_3 receptor agonists exhibited a myelostimulatory effect both in vitro and in vivo, by inducing G-CSF production, which led to a stimulatory effect on bone marrow cells (Fishman et al., 2000a).

The interest in the elucidation of A_3 receptor involvement in inflammation is attested by the large amount of experimental work carried out in cells of the immune system and in a variety of inflammatory conditions. However, as in the SNC or in the cardiovascular system the A_3 receptor subtype appears to have a complex or “enigmatic” role, as both proinflammatory and antiinflammatory effects have been demonstrated. One of the first evidence for a role of A_3 receptor in increasing inflammation derived by studies in mast cells where it was found that its activation was responsible for release of allergic mediators (Ramkumar et al., 1993; Fozard et al., 1996). In addition, it has been reported that A_3 receptor mRNA was higher in lung tissue of patients with airway inflammation and that A_3 receptor activation mediates rapid inflammatory cell influx into the lungs of sensitized guinea pigs (Walker et al., 1997; Spruntulis & Broadley, 2001). It has been reported that A_3 receptor activation in RBL-2H3 mast cells inhibits apoptosis and may have a profound effect on survival of inflammatory cells expressing A_3 receptors in inflamed tissues, thus contributing to inflammatory cell expansion (Gao et al., 2001). Moreover, antigen-dependent degranulation of bone marrow-derived mast cells was found to be mediated by A_3 receptor (Reeves et al., 1997), and the ability of Cl-IB-MECA to potentiate antigen-dependent mast cells degranulation was lost by using mice lacking A_3 receptor, suggesting a role for antagonists as antiasthmatic agents (Salvatore et al., 2000).

The double role of A_3 agonists on tumor and normal cells is very interesting. These receptors inhibit the growth of various tumor cells while promote the proliferation of bone marrow cells (Ohana et al., 2001; Merighi et al., 2002a).

In a set of experiments conducted by Gessi et al., low-concentration (100 nM) of Cl–IB–MECA stimulated the proliferation of some cancer cell lines such as Caco-2, DLD1, and HT29 human colon carcinoma cell line (Gessi et al. 2007).

A_3 adenosine receptor stimulation is able to impair cancer cell proliferation and shows an intriguingly myeloprotective effect, increasing bone marrow proliferation. Even if the stimulation induces antiapoptotic signals in cancer and normal cells, A_3 adenosine receptor agonists represent an attractive opportunity to develop new combined anticancer therapy with conventional chemotherapeutic drugs.

Table 1. Effects mediated by A_1 adenosine receptors

Cell type/tissue	Animal species	Effect	References
Cardiomyocytes	Rat	Inhibition of cell death	Safran et al., 2001
Cervical lymphocytes	Rat	Inhibition of proliferation	Colquhoun & Newsholme, 1997
MOLT 4 leukaemia cell line	Human	Inhibition of proliferation	Colquhoun & Newsholme, 1997
T47D breast tumor cell line	Human	Inhibition of proliferation	Colquhoun & Newsholme, 1997
Hs578T breast tumor cell line	Human	Inhibition of proliferation	Colquhoun & Newsholme, 1997
MCF-7 breast tumor cell line	Human	Inhibition of proliferation	Colquhoun & Newsholme, 1997
HUVEC, umbilical vein endothelial cells	Human	Antiapoptotic	Liu et al., 2002
Kidney	Rat	Increase of cisplatin-induced nephrotoxicity	Heidemann et al., 1989; Knight et al., 1991
Kidney	Rat	Reduction of cisplatin-induced nephrotoxicity	Bhat et al., 2002
Microglial cells	Rat	Anti-cancer effect	Synowitz et al., 2006
Microglia/macrophages	Mouse	Inhibition of cytokines and MMP-12	Tsutsui et al., 2004
HK-2 proximal tubular cell line (kidney)	Human	Antiapoptotic, prosurvival	Lee & Emala, 2002
A2058 melanoma cell line	Human	Increase of chemotaxis	Woodhouse et al., 1998
TM4 Sertoli-like cell line	Mouse	Inhibition of proliferation	Shaban et al., 1995

Table 2. Effects mediated by A_{2A} adenosine receptors

Cell type/tissue	Animal species	Effect	References
Spinal cord	Rabbit	Reduction of apoptosis	Cassada et al., 2001
Neurons	Gerbil, rat	Increase of ischemic injury	Ongini & Schubert, 1998
Granulocyte	Human	Reduction of apoptosis	Yasui et al., 2000
Macrophages	Mouse	Up-regulation of VEGF expression	Leibovich et al., 2002
Macrophages, T cells and dendritic cells	Human	Limitation to the effector cell function	Naganuma et al., 2006
A375 melanoma cell line	Human	Increase of cell death, stimulation of cell proliferation	Merighi et al., 2002a, 2002b
Skin	Mouse	Increase of wound healing and angiogenesis	Montesinos et al., 1997, 2002
HUVEC, umbilical vein endothelial cells	Human	Stimulation of cell proliferation	Sexl et al., 1995, 1997
Thymocytes	Mouse	Induction of cell death	Apasov et al., 2000
Liver	Rat	Reduction of ischemia-reperfusion injury	Harada et al., 2000
Blood	Rat	Increase of erythropoietin production	Nagashima & Karasawa, 1996
Blood	Mouse	Increase of erythropoietin production	Fisher & Brookins, 2001
Hep3B cell line	Human	Increase of erythropoietin production	Fisher & Brookins, 2001

Table 3. Effects mediated by A_{2B} adenosine receptors

Cell type/tissue	Animal species	Effect	References
Bone marrow-derived macrophages	Mouse	Inhibition of proliferation	Xaus et al., 1999
HMEC-1, microvascular endothelial cells	Human	Modulation of expression of angiogenic factor	Feoktistov et al., 2002
HUVEC, umbilical vein endothelial cells	Human	Modulation of expression of angiogenic factor	Feoktistov et al., 2002
HREC, retinal endothelial cells	Human	Neovascularization, increase of proliferation, increase of angiogenic growth factor expression	Grant et al., 1999, 2001
Cardiac fibroblasts	Rat	Inhibition of growth	Dubey et al., 2001
Aortic smooth muscle cells	Human	Inhibition of growth	Dubey et al., 1998a, 1998b
Vascular smooth muscle cells	Rat	Inhibition of growth	Dubey et al., 1999, 2000
Arterial smooth muscle cells	Human	Induction of apoptosis	Peyot et al., 2000
Arterial endothelial cells	Rat, porcine	Stimulation of cell proliferation	Dubey et al., 2002
Mesencephalic dopaminergic neurons	Rat	Reduction of cell death	Michel et al., 1999
U373 astrocytoma cell line	Human	Increase of interleukin-6 mRNA and protein synthesis	Fiebich et al., 1996

Table 4. Effects mediated by A_3 adenosine receptors

Cell type/tissue	Animal species	Effect	References
Astrocytes	Rat	Induction of cell death	Abbracchio et al., 1998
Astrocytoma	Human	Changes in cytoskeleton	Abbracchio et al., 1997, 2001
Forebrain	Gerbil	Neuroprotective action	Von Lubitz et al., 2001
Cardiac myocytes	Rat	Induction of apoptosis	Shneyvays et al., 1998, 2000
Cardiac myocytes	Rabbit	Cardioprotection	Liu et al., 1994
Cardiac myocytes	Rat	Reduction of doxorubicin-induced cardiotoxicity	Shneyvays et al., 2001, 2002
Cardiac myocytes	Rat	Cardioprotection	Safran et al., 2001
Cardiac myocytes	Rabbit	Cardioprotection	Tracey et al., 1998
Heart	Mouse	Cardioprotection	Cross et al., 2002
2H3 basophilic leukemia mast cells	Rat	Reduction of apoptosis	Gao et al., 2001
PBMC	Human	Induction of cell death	Barbieri et al., 1998
U937 and HL60 myeloid and lymphoid cell lines	Human	Induction of cell death	Kohno et al., 1996;
K562 leukemia cell line	Human	Inhibition of proliferation	Fishman et al., 2002a
Nb2-11C lymphoma cell line	Rat	Inhibition of proliferation	Fishman et al., 2000b
Yac-1 lymphoma cell line	Mouse	Inhibition of proliferation	Fishman et al., 2002a
MCF-7, MDA-MB468 breast	Human	Inhibition of tumor cell growth	Panjehpour & Karami-Tehrani, 2004
U87MG, A172 glioblastoma	Human	Expression of HIF-1α and hypoxic cell survival	Merighi et al., 2006, 2007a,b
HT29, Caco2, DLD1 colon	Human	Inhibition of cAMP, stimulation of cell proliferation, VEGF and HIF-1α	Gessi et al., 2007; Merighi et al., 2007b

Adenosine-sustained ways that could prime tumor development

A schematic diagram illustrating possible signaling pathways via A_1, A_{2A}, A_{2B}, and A_3 adenosine receptor stimulation is shown in Figures 2 and 3.

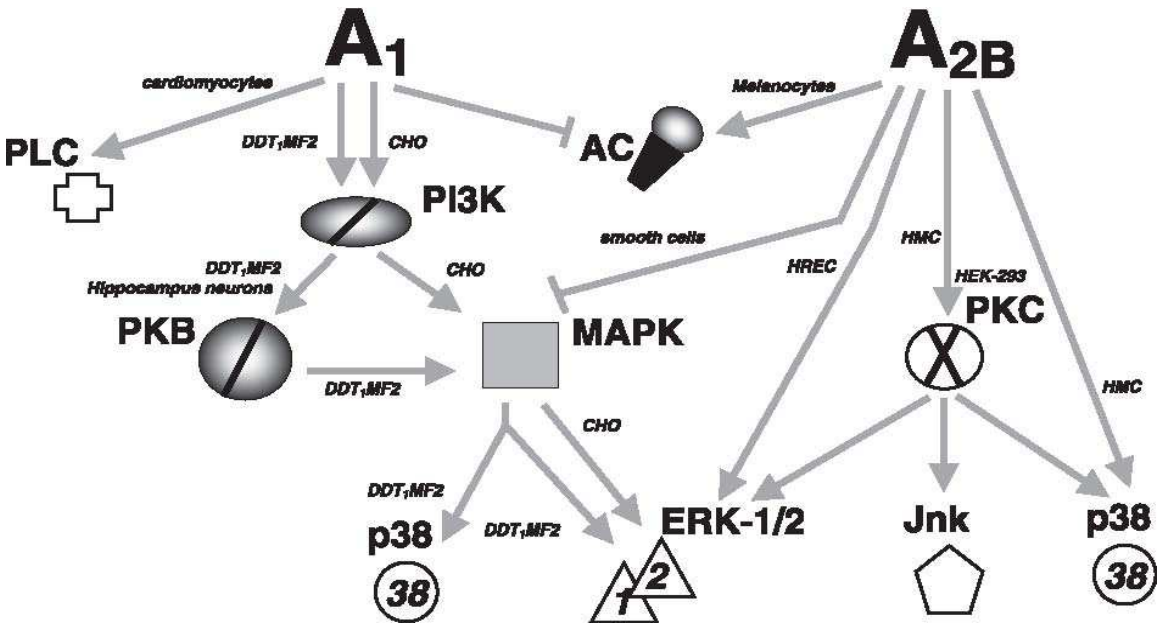


Figure 2. Schematic diagram illustrating possible signaling pathways via A_1 and A_{2B} adenosine receptor stimulation. AC: adenylate cyclase; CHO: Chinese hamster ovary cells; DDT1MF-2: hamster vas deferens smooth muscle cells; ERK1/2: extracellular signal-regulated kinases 1 and 2; HEK-293: human embryonic kidney cells; HMC: human mast cells; HREC: human retinal endothelial cells; JNK: c-Jun N-terminal kinase; MAPK: mitogen-activated protein kinases; PI3K: phosphoinositide 3-kinase; PKB: protein kinase B; PKC: protein kinase C; PLC: phospholipase C; p38: p38 MAP kinase.

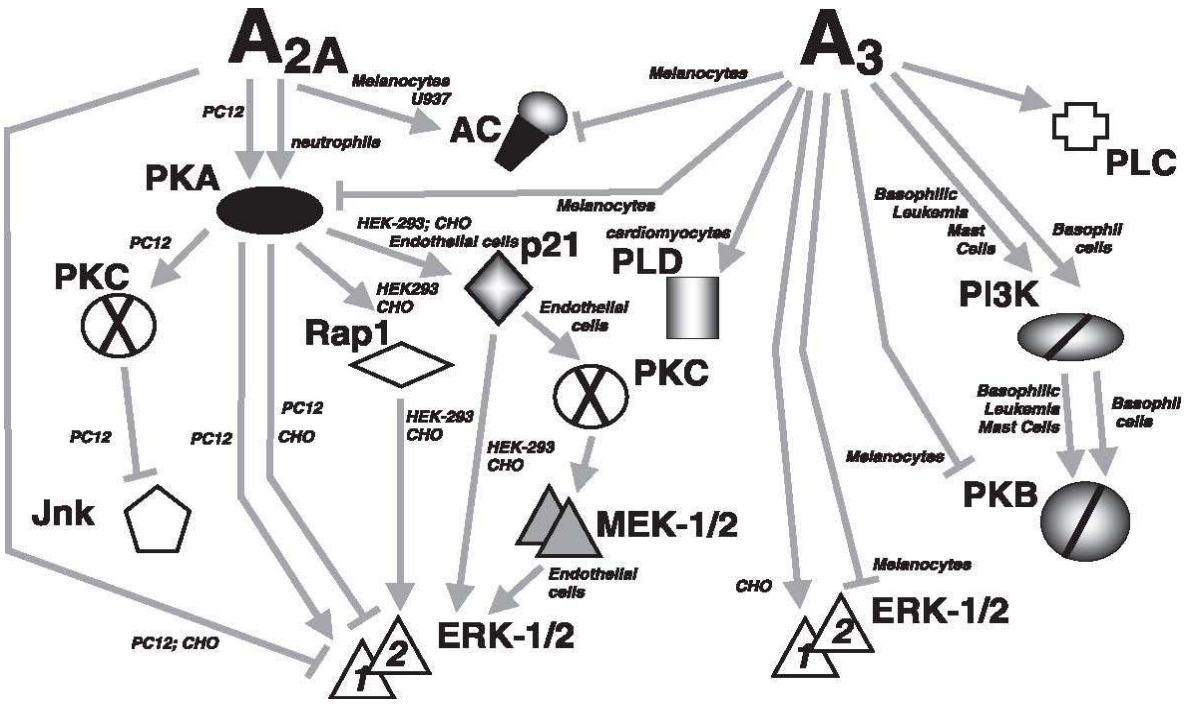


Figure 3. Schematic diagram illustrating possible signaling pathways viaA_{2A} and A_3 adenosine receptor stimulation. AC: adenylate cyclase; CHO: Chinese hamster ovary cells; ERK1/2: extracellular signal-regulated kinases1 and2; HEK-293: human embryonic kidney cells; JNK: c-Jun N- terminal kinase; MEK-1/2: MAP kinase kinases 1 and 2; PC12: pheochromocytoma cell line; PI3K: phosphoinositide 3-kinase; PKA: protein kinase A; PKB: protein kinase B; PKC: protein kinase C; PLC: phospholipase C; PLD: phospholipase D; p21: smallG protein, p21(ras); rap 1: small G protein rap1; U937: monocytic lymphoma cell line.

Two potential interesting mechanisms of signal transduction by the adenosine A_1 receptor have been investigated: the mitogen-activated protein kinase (MAPK) family and the protein kinase B, also known as Akt/PKB. The MAPK family consists of the p42/p44 MAPK and the stress-activated protein kinases, c-Jun N-terminal kinase (JNK), and p38 MAPK. Adenosine A_1 receptors expressed in Chinese hamster ovary (CHO) cells can activate extracellular signal-regulated kinase (ERK) 1/2 at physiologically relevant concentrations of the endogenous agonist (Schulte & Fredholm, 2000). This activation is sensitive to the phosphoinositol-3-kinase (PI3K) inhibitors wortmannin and LY294002 (Dickenson et al., 1998). In particular, the adenosine A_1 receptor agonist N^6-cyclopentyladenosine stimulated p42/p44 MAPK and p38 MAPK phosphorylation in the hamster vas deferens smooth muscle (DDT1MF-2) cells in a time-and concentration-dependent manner. No increase in JNK phosphorylation was observed following adenosine A_1 receptor activation (Robinson & Dickenson, 2001).

Furthermore, A_1 adenosine receptor stimulation in the DDT1MF-2 cells increases protein kinaseB (PKB) phosphorylation througha PTX-and PI-3Ksensitive pathway (Germack & Dickenson, 2000). Recently, it has been suggested that the A_1 receptor-mediated signal transduction pathway results in the activation of PKB also in the rat hippocampus in vitro and in vivo (Gervitz et al., 2002). The ability of A_1 receptors to activate ERK and PKB downstream may explain the antiapoptotic and prosurvival activity of A_1 agonist.

A_{2A} receptor activation may not only stimulate but also inhibit ERK phosphorylation. Activation of guinea pig A_{2A} receptors expressed in CHO cells inhibited thrombin-induced ERK1/2 activation (Hirano et al., 1996). In PC12 cells, activation of endogenously expressed A_{2A} receptors inhibits nerve growth factor (NGF)-induced ERK1/2 phosphorylation (Arslan et al., 1997). On the other hand, the A_{2A} receptor can lead to ERK1/2 activation in the absence of NGF (Arslan & Fredholm, 2000).

In CHO and HEK293 cells heterologously transfected with the human A_{2A} receptor, the capacity to activate MAP kinase via at least two signaling pathways was shown to be dependent on two distinct small G proteins, namely rap1 and p21 (ras) (Seidel et al., 1999).

Importantly, it has been demonstrated that the stimulation of A_{2A} receptors has a protective mechanism, enhancing protein kinase A (PKA) activity and activating novel protein kinase C isozymes in PC12 cells and thus preventing apoptosis in serum-deprived cells (Huang et al., 2001). In particular, a serine-threonine phosphatase appears to act downstream of the PKA to facilitate survival of serum-starved cells upon A_{2A} receptor stimulation. In addition, the selective A_{2A} receptor agonist 2-[p-(2-carboxyethyl)-phenethyl-amino]-5'-N-ethyl-carboxamidoadenosine (CGS 21680) reversed the DNA fragmentation and cell death induced by serum deprivation and also significantly reduced phosphorylation of the stress-activated kinases JNK1 and JNK2, which are implicated in apoptosis (Huang et al., 2001). In this cell system, although MAPK was activated by stimulation of A_{2A} receptors, blocking the MAPK pathway did not alter A_{2A} receptor-mediated protection against apoptosis. In addition, in human neutrophils, A_{2A} receptor stimulation delayed apoptosis, presumably via a PKA-dependent mechanism (Walker et al., 1997). Furthermore, recently, it has been shown that adenosine by A_{2A} stimulation might protect cells against hypoxia via PKA-sustained signaling (Kobayashi et al., 1998; Kobayashi & Millhorn, 1999).

Activation of A_{2A} receptors in U937 cells inhibited TNF-α production (Mayne et al.,

2001). In particular, there is the possibility that adenosine may inhibit nuclear factor-kB (NF-kB)-mediated TNF-α gene transcription in monocytes by increasing cAMP levels via A_{2A} receptor stimulation (Harada et al., 2000), thus supporting the idea of a protective role for A_{2A} receptor, which is able to delete the negative effects of TNF-α in intracerebral hemorrhage. Characterizing these mechanisms could prove helpful in formulating realistic and effective strategies of therapeutic intervention.

Despite the prosurvival and antiapoptotic signaling pathways, A_{2A} receptor stimulation leads also to impaired vitality and induces cell death. Different molecular mediators are surely involved in different cell lines that may have promising effect on the modulation of tumor cell viability.

It seems possible that A_{2B} receptors may play a very important role in cell proliferation and/or differentiation. These effects are demonstrated in vascular smooth muscle cell growth, where adenosine is antiproliferative by activating A_{2B} receptors coupled to inhibition of MAP kinase activity (Dubey et al., 2000). On the contrary, the adenosine A_{2B} receptor is peculiar in that it can activate ERK1/2 (Gao et al., 1999), JNK, and p38 (Feoktistov et al., 1999) in other different cell systems. Activation of the ERK and p38 MAPK pathways is an essential step in adenosine A_{2B} receptor-dependent stimulation of interleukin-8 (IL-8) production in human mast cells (HMC-1). Adenosine also has a synergistic effect with VEGF on retinal endothelial cell migration and capillary morphogenesis in vitro (Lutty et al., 1998). What is interesting is that proliferation, migration, and ERK activation in HREC cells are mediated through A_{2B} adenosine receptor stimulation (Grant et al., 2001).

One of the different mechanisms through which A_3 adenosine receptors are able to inhibit cell proliferation was found to involve inhibition of telomerase activity and a cell cycle arrest in the G_0/G_1 phase, leading to a cytostatic effect (Fishman et al., 1998, 2000; Brambilla et al., 2000). Furthermore, it has been demonstrated that the antigrowth signal exerted by A_3 receptors blocks cells into G_1 late cell cycle phase (Merighi et al., 2002a). In addition, recent studies have indicated that the ability of the A_3 agonist, N^6-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (IBMECA), to decrease the levels of PKA, a downstream effector of cAMP, and PKB/Akt in melanoma cells results in the down-regulation of the Wnt signaling pathway. This pathway is generally active during embryogenesis and tumorigenesis to increase cell cycle progression and cell proliferation (Fishman et al., 2002b).

In contrast, there is evidence that A_3 adenosine receptor activation triggers

phosphorylation of PKB/Akt, protecting rat basophilic leukemia 2H3 mast cells from apoptosis by a pathway involving the βγ subunits of Gi and phosphoinositide 3-kinase (PI3K)-b. This process is blocked by pertussis toxin and wortmannin (Gao et al., 2001). It has been demonstrated that A_3 receptors are involved in the control of cytoskeletal rearrangement (Abbracchio et al., 2001) and in the intracellular distribution of the antiapoptotic protein Bcl-XL (Abbracchio et al., 1997), which are events that may be at the basis of cell survival modulation by this receptor. Concerning the cardioprotective effects, A_3 receptors appear to be coupled via Rho A in the activation of phospholipase D (Lee et al., 2001).

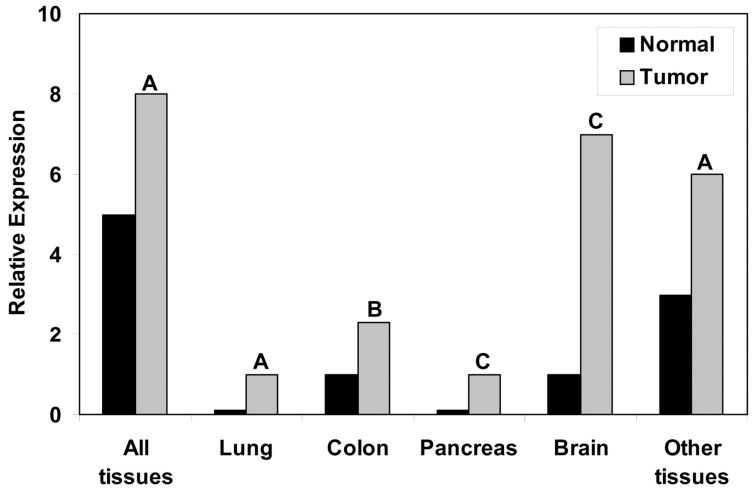
Physiological concentrations of adenosine have been demonstrated to cause an increase in phosphorylation of ERK1/2 after 5 min in CHO cells transfected with any one of the four adenosine receptors. Levels of adenosine reached during ischemia (3 μM) induce a more pronounced, but still transient, activation of ERK1/2. Thus, all the human adenosine receptors transfected into CHO cells are able to activate ERK1/2 at physiologically relevant concentrations of the endogenous agonist. In particular, it has been established that activation of human A_3 receptors expressed in CHO cells stimulates a rapid, transient increase in MAPK activity. Both 5'-N-ethyl-carboxamidoadenosine (NECA) and the endogenous agonist (adenosine) lead to a time- and dose-dependent increase in ERK1/2 phosphorylation, at concentrations as low as 10 –30 nM (Schulte&Fredholm, 2000). Furthermore, recently, it has been shown that MAPK activation is involved in A_3 receptor regulation, both contributing to direct phosphorylation and controlling G protein-coupled receptor (GPCR) kinase protein membrane translocation, which are involved in GPCR phosphorylation. Thus, an active MAPK pathway appears to be essential for A_3 receptor phosphorylation, desensitization, and internalization (Trincavelli et al., 2002). On the contrary, we have recently demonstrated that in the human melanoma A375 cell line A_3 antagonists are able to improve MEK activity: these results emphasize the role of A_3 as inhibitors of ERK activation (Merighi et al., 2002a). In agreement to what reported by Fishman et al. (2002b), in melanoma murine cells, we have found that N^6-(3-iodobenzyl)2-chloroadenosine-5'-*N*-methyluronamide (Cl-IB-MECA) was unable to activate ERK phosphorylation. Finally, A_3 adenosine receptor agonists, due to their high bioavailability, are good agents to combat cancer because they exert chemoprotective effects (cardioprotective and neuroprotective) on normal tissues. Further knowledge about protective mechanisms evoked by adenosine receptor stimulation and/or blockade

may help to facilitate the clinical application of adenosine receptor agonists and/or antagonists in the treatment of tumor disorders.

A_3 receptor as a tumor cell marker

The possibility that adenosine plays a role in the progression of cancer has aroused considerable interest for several years (Merighi et al., 2004; Fishman et al., 2002; Bar-Yehuda et al., 2001). Since the observation that adenosine could be detected in the interstitial fluid surrounding a carcinoma (Blay et al., 1997), numerous reports have shown the effects and the possible mechanism of action of this nucleoside on tumor cell growth (Ohana et al., 2002; Merighi et al., 2001; Barry et al., 2000). Several studies seem to indicate an emerging role for the A_3 receptor as a good candidate for the identification of tumor cells (Gessi et al., 2001a; Merighi et al., 2002; Suh et al., 2001; Gessi et al., 2001b). In more recent studies, a comparison between A_3 adenosine receptor expression in tumor vs. adjacent and relevant normal tissues supported the assumption that the receptor is upregulated in different types of malignancies. Recently, A_3 receptor in solid tumors was analyzed, leading to robust findings showing overexpression of the A_3 receptor in tumor tissues vs. low expression in the adjacent normal tissues. Furthermore, there is substantial evidence showing that A_3 adenosine receptor expression level is directly correlated to disease severity (Gessi et al. 2004; Madi et al. 2004). Low A_3 receptor mRNA expression level was reported as a general characteristic of various normal cell types (Auchampach et al., 1997), whereas in tumor cell lines such as melanoma, lymphoma, pineal gland, colon, and prostate carcinoma prominent receptor level was recorded (Ohana et al., 2003; Fishman et al., 2003; Madi et al., 2003; Gessi et al., 2001; Merighi et al., 2001; Suh et al. 2001, Trincavelli et al., 2002). In a recent study, Bar-Yehuda et al. showed that A_3 receptor mRNA expression is upregulated in HCC tissues in comparison to adjacent normal tissues (Bar-Yehuda et al., 2008). Remarkably, upregulation of A_3 adenosine receptor was also noted in peripheral blood mononuclear cells (PBMCs) derived from the HCC patients compared to healthy subjects. These results further show that A_3 receptor in PBMCs reflect receptor status in the remote tumor tissue (Bar-Yehuda et al., 2008). Moreover, the high expression level of the A_3 receptor was directly correlated to overexpression of NF-κB, a transcription factor for the A_3 receptor.

Dixon *et al.* detected A_3 receptor message only in the testis tissue using *in situ* hybridization, but found widespread distribution after amplification of the message using PCR. Carre *et al.* examined A_3 receptor expression in nonpigmented ciliary epithelial cells and found that to establish identity of the A_3 receptor message, two rounds of PCR amplifications were needed, suggesting that the message is present in low copy number. Atkinson *et al.* studied expression of A_3 receptor by Northern blot analysis in 35 different human normal tissues. This study revealed that hA_3 receptor is widely expressed at low to moderate levels. Most abundant levels were found in a number of discrete loci in the central nervous system with low expression in spleen and small intestine. A recent study (Madi et al., 2004) shows that a high A_3 receptor mRNA expression level is found in colon and breast tumor tissues in comparison with the normal adjacent and normal relevant tissue derived from healthy subjects. Remarkably, a higher mRNA expression level was detected in the regional lymph node metastases in comparison with the primary tumor tissue. In addition, a high A_3 mRNA receptor level was also detected in other solid tumors including melanoma, colon, breast, renal, ovarian, small cell lung, and prostate carcinoma. This is the first study in which it was compared side by side A_3 receptor expression level in tumor *versus* normal tissue, demonstrating that the message is higher in the malignant tissue. A support for this finding came from a search conducted in different sources of database, showing a 2.3-fold increase in the expression of A_3 adenosine receptor in human colon adenoma *versus* normal colon tissue using microarray analysis (Princeton University database). A search in the CGAP (The Cancer Genome Anatomy project; SAGE Genie; Virtual Northern Legend) based on serial analysis of gene expression revealed that A_3 receptor was abundant in brain, kidney, lung, germ cells, placenta, and retina but brain, lung, and pancreatic tumors expressed more A_3 receptor in the malignant than the normal relevant tissues. A search in Expression Viewer (HUGO-Gene Nomenclature Committee/CleanEX) based on expressed sequence tags revealed that the relative expression for A_3 receptor was 1.6-fold higher in all of the cancer tissues compared with normal tissues. A summary of A_3 receptor expression from the various database searches is presented in Fig. 4.



Madi et al., 2004

Figure 4. A_3 adenosine receptor expression in tumor *versus* normal relevant tissue based on (*A*) expressed sequence tags (expression viewer, HUGO-Gene Nomenclature Committee/CleanEx); (*B*) microarray analysis, Princeton University database; (*C*) serial analysis of gene expression, the Cancer Genome Anatomy Project.

Interestingly, tumor and normal cells respond differentially to activation of A_3 receptor by a synthetic agonist. Inhibition of tumor cell growth both *in vitro* and *in vivo* was observed in melanoma, colon, and prostate carcinoma (Fishman et al., 2002; Ohana et al., 2003; Fishman et al., 2003; Madi et al., 2003; Fishman et al., 2001). On the other hand, the proliferation of normal cells such as murine or human bone marrow was stimulated on cell activation with an A_3 receptor agonist (Ohana et al., 2001; Bar-Yehuda et al., 2002). This differential effect may be explained by the high *versus* low A_3 receptor expression level in tumor and normal cells, respectively. The association between A_3 receptor expression level and functionality was discussed earlier. Black *et al.* transfected the A_3 receptor gene in cardiomyocytes and tested the effect of gene dosage on protection against ischemia. Interestingly, gene overexpression reversed the protective effect demonstrating that the level of receptor expression plays a role in determining cell response to receptor activation. Dougherty *et al.* found that increased expression of the A_3 receptor adenosine receptor in cardiac myocytes caused an enhanced cardioprotective effect by improving the myocyte sensitivity to the endogenous adenosine, which, in turn, induces the protective effect. Dhalla *et al.*

suggested that an agonist is more efficacious or potent where the receptor number is high. Thus, receptor expression is cell type specific and reflects the response to a given agonist.Several studies have compared receptor expression profiles in tissues and peripheral blood cells from normal and pathological conditions and found a positive association or trend (Brodde et al., 1987; Varani et al., 1999; Varani et al., 2002). In light of these results, we therefore propose that A_3 protein could be required during all stages of cancer development, with a major role in cancer aggressiveness and it may raises the possibility of identifying a diagnostic and prognostic cancer index since the observations performed in peripheral circulating blood cells. Interestingly A_3 receptor expression of circulating blood cells normalizes after surgical treatment. The high A_3 receptor expression in neoplastic cells may be attributed to high adenosine level in the microenvironment of the tumor, released by necrotic or hypoxic cells. During homeostasis, the physiological levels of adenosine do not reach the concentrations needed to activate A_3 receptor. A_3 receptor has the lowest affinity to the natural ligand adenosine, which is ~1 μM (Schulte and Fredholm, 2002). Therefore, it may be suggested that the elevation in the extracellular adenosine concentration may trigger more receptor expression by the tumor cells. In addition, it may happen that in tumor cells, overexpression of transcription factors, responsible for A_3 receptor expression, takes place, resulting in up-regulation of receptor mRNA and protein levels. To conclude, high mRNA and protein A_3 receptor expression level was detected in various tumor cell types, classifying this Gi protein receptor to the family of other receptors, such as the epidermal growth factor, and suggesting it as both diagnostic and therapeutic target.

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AIM OF THE THESIS

The possibility that adenosine plays a role in the progression of cancer has aroused considerable interest for several years. Since the observation that adenosine could be detected in the interstitial fluid surrounding a carcinoma numerous reports have shown the effects and the possible mechanism of action of this nucleoside on tumor cell growth. Adenosine modulates a variety of cellular functions via occupancy of four cell surface G-protein-coupled receptors, named A_1, A_{2A}, A_{2B} and A_3. In particular, adenosine was found to exert its effects on cell proliferation, clone formation ability, UV resistance, and cell death mainly through the A_3 subtype, which is highly expressed in tumor cells. Furthermore, adenosine also plays a role in the promotion of angiogenesis. The aim of this thesis is to clarify the A_3 receptor role and the signal transduction pathways in two different human tumor cell lines, the colon cell line HT29 and the melanoma cell line A375.

The studies have been performed in hypoxia, present in most solid tumors, which regulates the levels of adenosine by inhibiting enzymes involved in the destruction of adenosine and simultaneously increasing the activity of enzymes charged with the generation of adenosine. In fact, adenosine accumulates to high levels in hypoxic tissue as a result of ATP breakdown suggesting a role in the extracellular response to hypoxia.

In the first study I analyzed the role of adenosine in hypoxic colon carcinoma cells, in particular its ability to regulate the expression of Hypoxia-Inducible Factor-1α (HIF-1α) and Vascular Endothelial Growth Factor (VEGF) through the A_3 receptor stimulation, and Interleukin-8 (IL-8) through the stimulation of A_{2B} receptor. HIF-1 is one of the master regulators that orchestrate the cellular responses to hypoxia. It is a heterodimer composed of an inducibly expressed HIF-1α subunit and a constitutively expressed HIF-1β subunit. VEGF (also known as VEGF-A, but commonly referred to simply as VEGF) plays an important role in angiogenesis. As its name suggests, VEGF stimulates vascular endothelial cell growth, survival, and proliferation and it has been shown to facilitate survival of existing vessels, contribute to vascular abnormalities (eg, tortuousness and hyperpermeability) that may impede effective delivery of antitumor compounds, and stimulate new vessel growth. IL-8 is a chemokine produced by macrophages and other cell types such as epithelial cells and has been shown recently to contribute to human cancer progression through its potential functions as a mitogenic, angiogenic, and motogenic factor. I specifically studied the role of adenosine treating

the cells with specific agonists and antagonists of the adenosine receptors. In particular, I evaluated the role of caffeine, which is a methylxantine antagonist of adenosine receptors, in the regulation of HIF-1α, VEGF and IL-8 expression induced by selective adenosine agonists and I provided the possible signaling pathways involved, which include Akt, MEK and p38 MAPK. It has been shown that HIF-1α overexpression, either as a result of intratumoral hypoxia or genetic alterations, activates the transcription of genes, the protein products of which contribute to the basement membrane invasion of colon cancer cells. So I analyzed the role of caffeine on the migration ability mediated by the adenosine receptor agonists in the tumor colon cancer cells and on the migration of Human Umbilical Vein Endothelial Cells (HUVECs).

I then evaluated the action of two chemotherapeutic drugs, etoposide and doxorubicin, in the treatment of the melanoma. The aggressive nature of human melanomas is related to several abnormalities in growth factors, cytokines, and their receptor expression. For example, metastatic melanoma cells constitutively secrete IL-8, whereas nonmetastatic cells produce low to negligible levels of IL-8. It has been shown that this cytokine is an important mediator of immunological and inflammatory reactions, which is produced by a variety of different cell types, including melanoma cells. In addition to IL-8, aggressive melanoma cells secrete VEGF, and the hypoxic induction of VEGF is mediated by HIF-1. I analyzed the modulation of IL-8 and the production of VEGF in the human melanoma cell line A375, and I showed the influence of the adenosinergic signaling on the chemotherapeutic drug effects providing also a possible signaling pathway involved.

CHAPTER 2

ADENOSINE METABOLISM IN HYPOXIA AND HYPOXIA INDUCIBLE FACTOR-1

2.1 Adenosine production and consumption

The major pathway for adenosine formation in most tissues is stepwise dephosphorylation of ATP. Adenosine accumulates in the heart and other tissues when oxygen demand exceeds oxygen supply, e.g., during exercise, vascular deficiencies, and other conditions associated with tissue hypoxia/ischemia and/or increased ATP turnover (Deussen et al.,1991; Imai et al., 1964, Sparks et al., 1986). An increase in ATP turnover in tissues with relatively high metabolic rates may increase AMP levels and subsequently also adenosine levels independent of hypoxia (Arch et al., 1978; Kroll et al., 1993; Mo et al., 2001; Wagner et al., 1994), i.e., tissues with high metabolic rates are expected to have high adenosine concentrations even under normoxic conditions, as discussed later.

At least four enzymes and a membrane carrier are involved in controlling the interstitial adenosine concentration, as shown in Fig.1. Adenosine is produced by dephosphorylation of AMP and hydrolysis of *S*-adenosylhomocysteine (SAH). The hydrolysis of SAH to adenosine (and homocysteine) by SAH hydrolase is thought to be a constitutive pathway that contributes marginally to adenosine production (Kroll et al., 1993; Wagner et al., 1994). Dephosphorylation of AMP is the major source of adenosine under hypoxic/ ischemic conditions: this reaction occurs intracellularly by cytosolic-5'-nucleotidase and extracellularly by cd73/ecto-5'-nucleotidase. The relative contribution of the cytosolic and ecto pathways for adenosine production has been the subject of much debate (Borst et al., 1991; Deussen et al., 1999; Headrick et al., 1992; Ledoux et al., 2003; Ala-Newby et al., 2003; Schutz et al., 1981); however, recent studies in mice show that targeted disruption of cd73/ecto-5'-nucleotidase can modulate basal coronary vascular tone as well as other adenosine-mediated events, suggesting an important role for adenosine produced extracellularly (Koszalka et al., 2004;). This latter finding is consistent with the notion that physiologically significant amounts of adenosine are produced extracellularly by cardiomyocytes (Deussen et al., 2000; Deussen et al., 1999) and skeletal muscle fibers (Lynge et al., 2001) and that both cell

types are a sink rather than a source for adenosine. Histochemical and/or functional studies have demonstrated the existence of ecto-5'-nucleotidase on many different cell types including cardiomyocytes (Rubio et al., 1973) skeletal muscle fibers (Hellsten et al., 1999), Muller cells of the retina (Lutty et al., 2000), astrocytes (Zimmermann H., 1996), various cell types in the kidney (Le Hir et al., 1993), and fibroblasts (Le Hir et al., 1993; Mlodzik et al., 1995; Schmid et al., 1994). Two enzymes can utilize adenosine and thus decrease its concentration. Adenosine is either deaminated to form inosine via adenosine deaminase or rephosphorylated into AMP via adenosine kinase, using ATP as the phosphate donor. The nucleoside transporter shown in Fig. 1 represents a bidirectional equilibrative nucleoside transporter (ENT1) that translocates adenosine down its concentration gradient by facilitated diffusion (Baldwin et al.,2004; Cass et al., 1999).

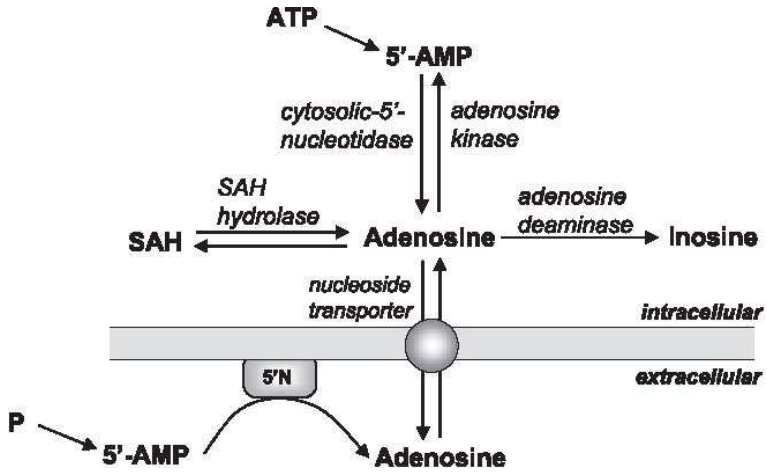

Fig. 1. Metabolic pathways for adenosine (Ado) production and consumption in intracellular and extracellular fluids. SAH, *S*-adenosylhomocysteine; 5'N, ecto-5'-nucleotidase.

Regulation of adenosine metabolism under hypoxic conditions

The adenosine concentration in interstitial fluids might be expected to increase when *1*) the activities of adenosine-producing enzymes (nucleotidases) are increased, *2*) the activities of adenosine-utilizing enzymes (adenosine kinase, adenosine deaminase) are decreased, *3*) the availability of the primary substrate for adenosine formation (AMP) is increased, and/or *4*) ENT is inhibited.

AMP hydrolysis via nucleotidase is the dominant pathway for adenosine production under normoxic conditions, and more than 90% of the adenosine produced under normoxic conditions is thought to be rephosphorylated to AMP via adenosine kinase

(this is called the salvage pathway) (Arch et al., 1978; Kroll et al., 1993; Wagner et al., 1994). Because of the large flux through this AMP-adenosine substrate cycle, small changes in the activities of adenosine kinase or nucleotidase can produce large changes in adenosine concentration. For example, Gu and associates (Gu et al., 2000) have shown that pharmacological inhibition of adenosine kinase can raise adenosine levels sufficiently to induce VEGF mRNA and protein expression in rat myocardial myoblasts. Modulation of adenosine deaminase activity, on the other hand, is expected to have a minimal effect on adenosine concentration under basal conditions because the Michaelis-Menten constant of the deaminase is far greater compared with that of the kinase (Arch et al., 1978).

Recent studies indicate that exposing cells to a hypoxic environment can increase the activity of nucleotidase and decrease the activity of adenosine kinase, thereby causing a net increase in the production of adenosine and hence an increase in the interstitial adenosine concentration (Linden et al., 2001). Decking and associates (Decking et al., 1997) perfused isolated guinea pig hearts with hypoxic perfusate and used a mathematical model to determine that hypoxia decreased adenosine kinase activity to 6% of basal levels. Although the mechanism by which hypoxia inhibits adenosine kinase activity is poorly understood, studies by Gorman et al. (Gorman et al., 1997) indicate that cytosolic levels of inorganic phosphate achieved under hypoxic conditions in the heart are capable of inhibiting adenosine kinase activity. Other studies (Ledoux et al., 2003) have shown that exposing aortic ECs to an anoxic environment (0% oxygen, 18 h) induced a twofold increase in cd73/ ecto-5'-nucleotidase activity and increased cell surface expression of the enzyme but had no effect on its synthesis. The hypoxic induction of cd73/ ecto-5'-nucleotidase activity can also occur in the ischemic heart (Minamino et al., 1996) and brain (Braun et al., 1997) of intact animals, possibly by way of a hypoxia inducible factor-1 (HIF-1)-dependent regulatory pathway (Synnestvedt et al., 2002). Hypoxia can also downregulate the gene expression of a dipyridamole-sensitive ENT (mENT1) in mouse cardiomyocytes and thereby decrease [^{3}H]adenosine uptake by cells (Chaudary et al., 2004). The hydrolysis of SAH to adenosine (and homocysteine) by SAH hydrolase is probably not upregulated under hypoxic conditions (Deussen et al., 1989; Kobayashi et al., 2000; Wagner et al., 1994).

Hypoxia not only regulates the activity of enzymes and transporters but may also increase the availability of the primary substrate for adenosine, AMP. Hellsten and associates (Hellsten et al., 1998) found that knee extensor exercise in humans increased

interstitial AMP levels by ~20-fold. Others (Mo et al., 2001) have confirmed that muscle contraction can increase interstitial AMP levels in perfused dog skeletal muscle; however, interstitial levels of AMP did not increase when resting muscles were perfused under hypoxic conditions. Using NMR spectroscopy, Pucar and associates (Pucar et al., 2004) showed that hypoxia induced a 2.5-fold increase in AMP levels in the Langendorff-perfused rat heart. Also, Kuzmin and associates (Kuzmin et al., 1998) found that ischemia increased interstitial ATP levels by ~10-fold in the Langendorff-perfused rat heart and that adenine nucleotides were sequentially dephosphorylated in the interstitial space by a chain of separate ectoenzymes. Therefore, it appears that hypoxia/ischemia can increase AMP levels in the interstitial fluid.

Hypoxic modulation of nucleoside transporters and enzymes of adenosine metabolism may require hours to days for full adaptation. Kobayashi and associates (Chaudary et al., 2004) found that prolonged exposure (but not acute exposure) of rat pheochromocytoma (PC12) cells to a hypoxic environment (5% oxygen, 48 h) caused the cells to shift toward an adenosine-producing phenotype. The adaptations consisted of decreased gene expression of the rENT1/nucleoside transporter, downregulation of adenosine-metabolizing enzymes (adenosine kinase, adenosine deaminase), and upregulation of adenosine-producing enzymes (cytosolic and cd73/ecto-5'-nucleotidase). It is likely that prolonged exposure to a hypoxic environment will prove necessary to determine the quantitative importance of adenosine in the angiogenesis process.

2.2 Hypoxia Inducible Factor-1

One of the main early cellular events evoked upon exposure to hypoxia is activation of HIF-1, a key heterodimeric transcription factor. In reduced oxygen conditions, HIF-1 binds to hypoxia-responsive elements (HREs) and induces transcription of various target genes involved in tumor angiogenesis, invasion, cell survival, and glucose metabolism. The concept of a transcription factor being activated in limiting oxygen conditions was put forth in 1992 to explain upregulation of erythropoietin (Epo), a hormone stimulating red blood cell production in response to hypoxia (Semenza and Wang, 1992). HIF-1α and HIF-1β were identified as proteins that contain a basic helix-loop-helix and a Per/ARNT/Sim (PAS) domain and were determined to be responsible for hypoxic induction of *Epo*. These subunits must associate to form the active HIF heterodimer responsible for transcriptional activation (Wang et al., 1995). HIF-1β is the aryl hydrocarbon receptor nuclear translocator (ARNT) and is constitutively expressed. ARNT2 and ARNT3 are highly homologous proteins to ARNT, and all three are implicated in forming dimers with the various HIF- subunits (Maynard and Ohh, 2004). HIF-1 has two closely related homologs, HIF-2 and HIF-3. HIF-2 (also known as endothelial PAS domain protein, or EPAS1) is 48% identical to HIF-1, is induced by hypoxia, and binds to HIF-1 to activate transcription of hypoxia-responsive genes (Tian et al., 1997). HIF-3 appears to be a dominant negative regulator of HIF, as it dimerizes with HIF-1β to generate a transcriptionally inactive heterodimer. Knockout mice homozygously deleted for HIF-1 exhibit embryonic lethality, dying at postcoitus day 10 with gross abnormalities in cardiac development and vasculature, underscoring the importance of HIF-1 in vascular development (Kline et al., 2002). Mice lacking HIF-2 die mid-gestation and show defects of cardiac development and reduced catecholamine levels (Tian et al., 1998). In normoxic conditions, HIF-1 is expressed ubiquitously at low levels in all organs, and HIF-2 is most abundantly expressed in the lung, followed by the heart, brain, liver, and various other organs. Despite their similarities in mediating transcriptional responses to hypoxia, HIF-1 and HIF-2 have distinct, nonredundant functions (reviewed in Semenza [2004]).

Regulation of HIF-1α protein stability under hypoxia

In response to changes in oxygen availability, mammalian cells launch a host of responses, most of which are mediated by HIF. Regulation of HIF by partial oxygen pressure is orchestrated by many molecular players that affect HIF-1/2 protein stability or the ability of these proteins to bind to cofactors essential for transcriptional activity. In normoxia, HIF subunits carrying an oxygen-dependent degradation (ODD) domain are highly labile proteins that are rapidly ubiquitinated and degraded by the proteasome (Crews, 1998). This ubiquitination is mediated by the von Hippel-Lindau protein (pVHL), the recognition component of an E3 ubiquitin ligase (Semenza, 2002). Mutations in the *VHL* gene result in the autosomal dominant von Hippel-Lindau syndrome that is characterized by the presence of highly vascularized tumors overexpressing vascular endothelial growth factor (VEGF) (Kaelin, 2002). The recognition of HIF-1/2 by pVHL is augmented by hydroxylation of two proline residues (P402 and P564) within the ODD domain by specific prolyl hydroxylases (PHD1, PHD2, PHD3) (Bruick and McKnight, 2001; Epstein et al., 2001). The PHDs are iron-dependent enzymes also requiring oxygen, 2-oxoglutarate, and ascorbate for activity. The catalytic activity of all three PHDs is reduced in hypoxia, with their respective rates of catalysis in normoxia being PHD2 PHD3 PHD1 (Tuckerman et al., 2004). In hypoxia, PHD1 and PHD3 are rapidly degraded by the proteasome pathway, which adds another layer of control to the system (Nakayama et al., 2004). On the contrary, PHD2 levels are upregulated by HIF-1 in hypoxic conditions and may be a mechanism to rapidly stop hypoxic signaling upon tissue reoxygenation (Metzen et al., 2004)**.** Overexpression of any of the three PHDs destabilizes HIF-1 protein in COS-1 cells (Tuckerman et al., 2004). On the contrary, short interfering RNA studies have demonstrated that specific silencing of only PHD2 and not PHD1 or PHD3 in a battery of immortalized human cell lines and primary cell cultures led to increased HIF stability, which suggests that PHD2 may be the only physiologically relevant hydroxylase involved in HIF regulation (Berra et al., 2003). This dilemma may be explained if the contribution of each isoform to HIF hydroxylation depends on its relative abundance in a given cell type and a given culture condition, and if all three function in a nonredundant fashion (Appelhoff et al., 2004). Clearly, transgenic and knockout studies currently ongoing will help verify this assumption. The arrest defective 1 protein (ARD1) is an acetyl-transferase that acetylates HIF-1 at Lys532

within the ODD domain. ARD1 stimulates HIF-1–pVHL association, ubiquitination, and subsequent proteasomal degradation (Jeong et al., 2002). Unlike HIF-1 hydroxylation, the acetylation reaction itself is not thought to be an oxygen-dependent process. However, the level of HIF acetylation is still influenced by hypoxia, as ARD1 mRNA levels are reduced in hypoxia (Jeong et al., 2002). Thus, ARD1-mediated acetylation adds to the regulation of HIF-1 protein stability in response to oxygenation.

Small ubiquitin-like modifier-1 (SUMO-1) is an 18-kDa protein that shares 18% identity with ubiquitin and uses an ubiquitin-like conjugation system to affect protein localization. In certain circumstances, sumoylation may counter the effects of ubiquitination (Seeler and Dejean, 2003). SUMO-1 has been shown to co-localize and interact with HIF-1 in response to hypoxia in neurons and cardiomyocytes (Shao et al., 2004). SUMO-1 induces sumoylation of HIF-1 at Lys391/Lys477, leading to its stabilization and increased transcriptional activity (Shao et al., 2004). Given that HIF-1 activation increases VEGF expression and that VEGF is a survival factor for neurons, this sumoylation may have a neuroprotective function in the CNS (Wang et al., 2004).

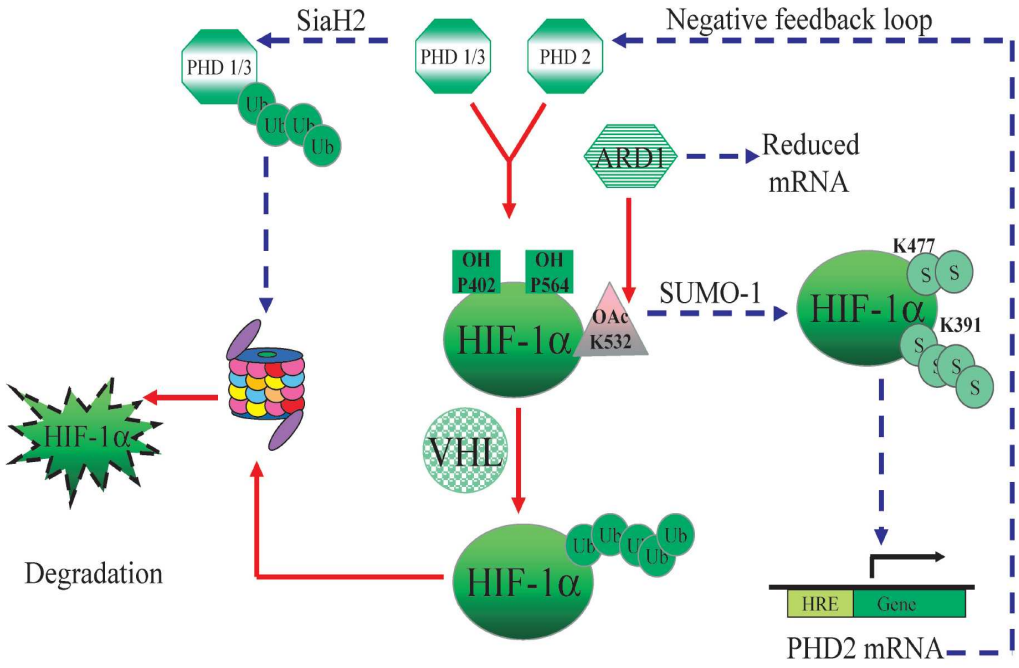

Figure 1. Factors affecting HIF-1 protein stability. PHD-mediated hydroxylations and ARD-mediated acetylation of specifi c residues within HIF-1 increase its affinity for pVHL, which leads to its ubiquitination (Ub) and degradation by the proteasomal pathway under normoxia (solid arrows). PHD1, 2, and 3 have a reduced catalytic activity in the absence of oxygen. Further, PHD1 and 3 and ARD have

reduced levels in hypoxia (dashed arrows), adding another level of control. SUMO-1-mediated sumoylation in hypoxia leads to HIF-1 stabilization (S) and activation, causing transactivation of specific downstream target genes. PHD2 is induced by HIF, which indicates a negative feedback loop.

Signaling pathways affecting HIF-1α regulation

Hypoxia-inducible factor can be activated by physiological or pathological activation of growth factor and cell adhesion pathways (Fig. 2). Growth-factor-induced activation of receptor tyrosine kinases (RTKs) leads to HIF1- stabilization and activation. Upon ligand binding, these receptors dimerize and autophosphorylate, which leads to their activation. Activated RTKs interact with p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI3K), which leads to its activation. PI3K is a lipid kinase that generates the signaling molecule phosphatidylinositol 3,4,5-triphosphate by phosphorylating its precursor phosphatidylinositol 4,5-biphosphate. Activated PI3K triggers a phosphorylation cascade that results in the phosphorylation/activation of AKT, a serine/ threonine kinase that promotes antiapoptotic and pro-survival responses of a cell (Newton, 2004). Activation of AKT has been shown to lead to an increase in HIF-1 protein translation by the AKT/FRAP/mTOR pathway (Fig. 2) (Laughner et al., 2001; Zhong et al., 2000). Inhibition of this pathway using LY294002, a selective inhibitor of PI3K, and with rapamycin, a selective inhibitor of mTOR, a downstream target of AKT, causes a reduction in HIF-1 amount and activity (Blancher et al., 2001).

Induction of HIF by growth factor receptors such as epidermal growth factor receptor (EGFR) or Her 2 (neu) is blocked by inhibitors of PI3K (LY294002 and wortmannin), which indicates the requirement of the PI3K pathway (Zhong et al., 2000). Activated RTKs also signal through the MAPK pathway, and phosphorylated p38 and extracellular-signal-regulated kinase 1/2 (ERK1/2) can further phosphorylate and activate HIF-1 (Wang et al., 2004b). Inhibition of ERK activity leads to inhibition of HIF activity without affecting HIF stabilization (Hur et al., 2001).

In addition to growth factor–mediated RTK activation, the PI3K/AKT pathway is also activated by extracellular matrix (ECM) adhesion mediated by integrins (Friedrich et al., 2004). Integrin ligation causes an activation of the integrin-linked kinase (ILK) leading to increased HIF-1, as well as increased VEGF production by the PI3K/AKT/FRAP/mTOR pathway (Tan et al., 2004). Increased activity of integrin-linked kinase has been reported in gliomas (Obara et al., 2004). Additionally, activation

of PI3K/AKT also leads to an increase in steady-state concentrations of heat shock proteins 90 and 70, both of which interact with and stabilize HIF-1 (Zhou et al., 2004).

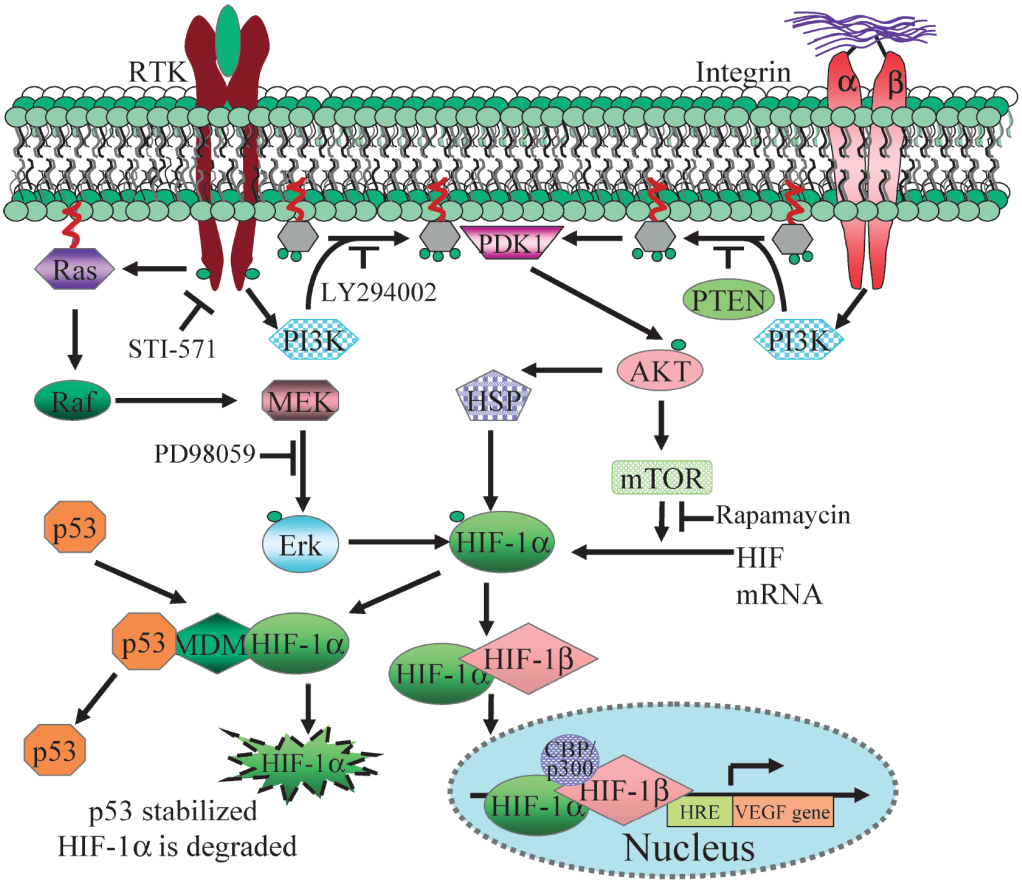

Figure 2. Molecular signals affecting HIF-1 regulation. Induction of Ras, PI3K, and AKT phosphorylation mediated by RTK activation or integrin ligation leads to increased HIF-1 by modulating its stability and increased translation by the PI3K/AKT/mTOR pathway. TP53 negatively modulates this process by inducing MDM2, which can ubiquitinate and lead to HIF-1 degradation by the proteasome pathway.

Hypoxic regulation of angiogenesis: Angiopoietin-2 and VEGF

Growth factors and hypoxia converge in the regulation of key angiogenic genes. The cellular expansion of tumors progressively distances cells from the vasculature, and thus from oxygen and nutrients. Tumor cells, like growing embryonic cells, send out signals that initiate the formation of new blood vessels. This adaptive process, termed angiogenesis, is a general feature of every tissue; however, it is often exacerbated in solid tumors. Thus, new tumor vessels showing structural malformations, chaotic blood flow and local regions of hypoxia might nonetheless prevail. Although many molecules and receptors have been characterized in this biological process, at least two factors seem critical for initiating vessel sprouting. These are VEGF-A and Ang-2 (Ferrara et al., 1996 e 2003), which are two receptor ligands expressed and secreted in response to hypoxia. VEGF-A is expressed in most cells, and attracts and guides sprouting neovessels into oxygen-depleted regions of the tumor mass (Carmeliet, 2003; Gerhardt, 2003). Endothelial cells situated at the tip of the sprouts sense and navigate through the environment using long filopodia that are rich in VEGF receptor-2 (VEGFR-2) (Gerhardt et al, 2003). Thus, migration of the tip cells is guided by a graded distribution of VEGF-A, particularly the long spliced forms that are retained in the extracellular matrix. Although in hypoxia the binding of HIF to the *vegf* promoter is a key determinant in its expression, two other major transcriptional controls are mediated through the Ras–ERK and PI(3)K–AKT pathways (Rak, et al., 2000; Pages et al., 2005) (Fig. 3). VEGF-A messenger RNA is upregulated by the ERK pathway through the phosphorylation of the transcription factor Sp1 and its recruitment to the proximal region of the *vegf* promoter (Milanini-Mongiat et al., 2002) (Fig. 3). This regulation is independent of hypoxic stress and reflects the intensity of growth-factor stimulation or oncogenic signals. Transcriptional activation also occurs through ERK-induced phosphorylation of HIF-1α (Richard et al.,1999) and the coactivator p300, which might improve the accessibility of RNA polymerase II to the *vegf* promoter. Other levels of regulation of VEGF-A occur, including the stabilization of the mRNA through the stress-activated kinase p38 (Pages et al., 2000), and translation by means of internal ribosome entry site (IRES) sequences present in 5´ non-coding regions of VEGF-A (Huez et al., 1998) and HIF-1α mRNAs (Lan et al., 2002), which are two important attributes for translation of VEGF-A under nutrient deprivation. This is another point of convergence between growth factors and hypoxia signaling at the level of translation.

As a ‘survival’ cytokine, VEGF-A is translated under conditions where the cell’s general translational machinery is turned off. The second molecule induced by hypoxia is Ang-2, a receptor ligand restricted to endothelial cells (Ferrara et al., 1996; Lang et al., 2002) that allows vessel remodelling by antagonizing the related molecule Ang-1. As shown in Fig. 3, Ang-1, through Tie-2 receptor tyrosine kinase signaling and platelet-derived growth factor-B (PDGF-B) action, induces pericyte recruitment (Lindblom et al., 2003) and maturation of blood capillaries. These capillary endothelial cells are rendered quiescent through the activation of the Notch pathway (Noseda et al., 2004), thus becoming unresponsive to VEGF-A action, unless Ang-2 is also secreted, leading to vessel destabilization. Ang-2 is a natural Ang-1 antagonist, which displaces Ang-1 from its receptor thus arresting Tie-2 signaling. Therefore Ang-2 secretion from Weibel–Palade bodies (Fiedler et al., 2004) is a critical, and perhaps limiting, step in angiogenesis permitting vessel remodelling upon VEGF-A action. It is remarkable that this angiogenic ‘couple’ VEGF-A and Ang-2 is expressed under hypoxic control when and where nutrients are needed. However, the precise mechanism of regulation of Ang-2 expression in hypoxia remains to be defined.

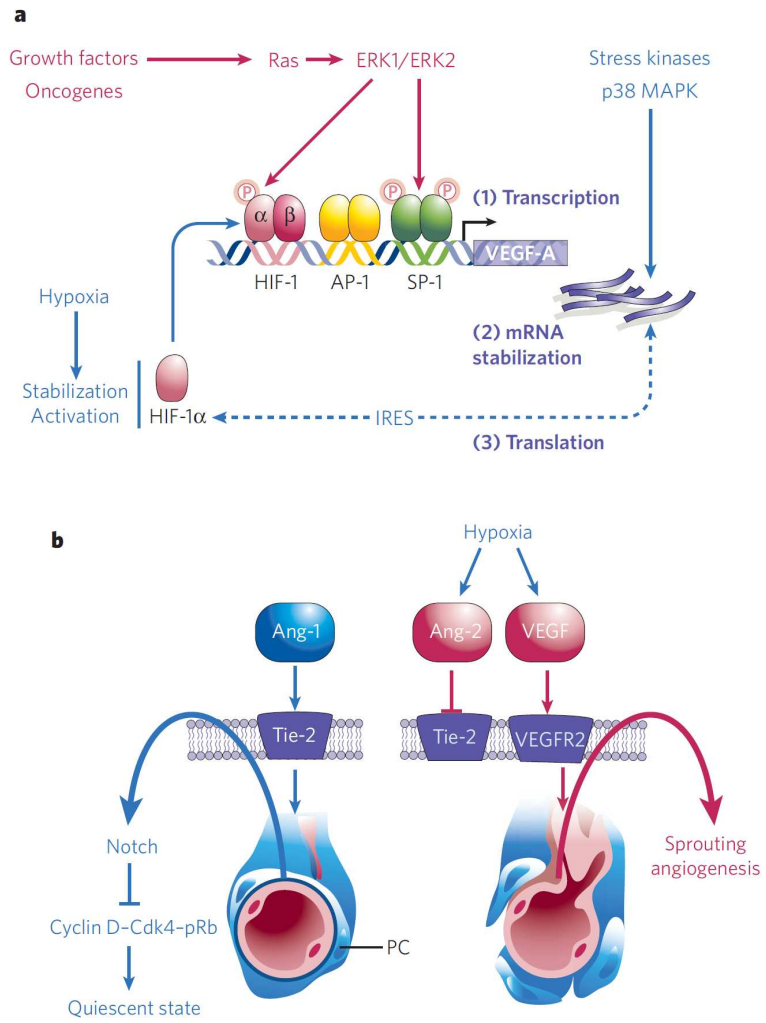

Figure 3. a, Control of vascular endothelial growth factor-A (VEGF-A) expression. VEGF-A expression is controlled at three levels: transcription, messenger RNA stability and translation. The Ras–MEK–extracellular signal-regulated kinase (ERK) pathway stimulates transcription through phosphorylation of the transcription factors Sp1 and hypoxia-inducible factor-1α (HIF-1α) subunit, and their recruitment to the *vegf* promoter. The transcription factor activator protein-1 (AP-1) might also modulate *vegf* transcription. HIF-1 is a heterodimer of a hypoxia-stabilized and activated α-subunit and an oxygen-insensitive β-subunit. VEGF-A mRNA is stabilized through the stress-activated kinase p38, and the translation of VEGF-A is ensured under hypoxic and nutrient-depleted conditions by means of internal ribosome entry site (IRES) sequences. Under these energy-reduced conditions, classic cap-dependent translation is inhibited. b, VEGF-A and angiopoietin-2 (Ang-2) are two angiogenic factors induced by hypoxia. Blood capillaries are maintained in a mature and dormant state through the recruitment of pericytes (PC) through platelet-derived growth factor-B (PDGF-B) and the signaling of the endothelial receptor Tie-2 upon Ang-1 binding. In addition, activation of the Notch pathway through cyclin D/Cdk4 and retinoblastoma protein (pRb) phosphorylation contributes to the quiescence of endothelial cells. Ang-2 is an antagonist ligand for Tie-2 in endothelial cells and, like VEGF-A, is induced under low oxygen conditions through the HIF. The initiation of sprouting angiogenesis requires the destabilization of capillaries. This action is mediated by Ang-2, thereby blocking Tie-2 signaling and allowing VEGF-A-induced cell migration and division. MAPK, mitogen-activated protein kinase.

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CHAPTER 3

A_3 ADENOSINE RECEPTOR AND REGULATION OF INTRACELLULAR PATHWAYS

3.1 The A_3 Receptor and the Mitogen-Activated Protein Kinases (MAPKs) Signal Transduction Cascade

GPCRs are critical players in converting extracellular stimuli into intracellular signals. Nowadays, as intracellular signaling is revealed as being an increasingly complex network, the ability of GPCRs to stimulate the regulatory pathways of the Mitogen activated kinases (MAPKs) illustrates their influence on cell growth and differentiation. The well-conserved and diverse protein family of MAPKs consists of three main groups: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 kinases. ERKs are mainly stimulated by growth factors, while JNKs and p38 MAPK are more responsive to cellular stress and cytokines. Following this first classification, other kinases have been included into the MAPK family based on structural similarity (Miyata and Nishida 1999). MAPKs modulate the activities of various proteins including other protein kinases and transcription factors. Practically all GPCRs are capable of activating one or more MAPKs (Luttrell 2008). The adenosine A_3 receptor has been shown to be no exception (Schulte and Fredholm 2003). There is considerable evidence for adenosine A_3 receptor-mediated effects on mitogenesis. Accordingly, the functional signaling of the adenosine A_3 receptor to MAPKs has been demonstrated in a multitude of different cellular models. The first example of A_3 receptor-mediated activation of ERK1/2 and the modulation of mitogenesis was described in human foetal astrocytes (Neary et al. 1998). This study made use of both an unselective adenosine receptor agonist (NECA) and a more selective agonist (IB-MECA) to demonstrate the selectivity of this effect towards the A_3 receptor. In addition, treatment with the inhibitor bisindolmalemide (Ro-318220) blocked this effect suggesting a role of PKC in this pathway. Subsequent and more detailed studies were performed in CHO cells stably expressing the adenosine A_3 receptor. One such study by Schulte and Fredholm demonstrated that physiological concentrations of adenosine (10–

100 nM) caused a transient increase in phosphorylation of ERK1/2 that peaked after 5 min in CHO cells transfected with any one of the four adenosine receptors (Schulte and Fredholm, 2000). Furthermore levels of adenosine reached during ischemia (3 mM) induce a more pronounced, but still transient, activation of ERK1/2. Thus, human A_3 adenosine receptors transfected into CHO cells are able to activate ERK1/2 at physiologically relevant concentrations of the endogenous agonist (Schulte and Fredholm 2000). It is perhaps useful at this point to highlight a potential caveat associated with some inhibitors of intracellular signaling when used to investigate the signal transduction pathways of adenosine receptors. Many such inhibitors, including genistein, chelerthrine and SQ22536 act at the ATP binding site of kinases or adenylate cyclase, respectively (Schulte and Fredholm 2002a). Perhaps not surprisingly then, these compounds were shown to have an affinity for A_1, A_{2A} and A_3 adenosine receptors at concentrations commonly used to examine cellular signaling. However, with the judicious use of inhibitors, A_3 receptor signaling to ERK1/2 in CHO cells was shown to be dependent on βγ release from PTX-sensitive G proteins, PI3K, Ras and MEK (Schulte and Fredholm 2002b). In the same study ERK1/2 phosphorylation was shown to be independent of Ca2+, PKC and c-SRC. Importantly, there are several examples of ERK1/2 phosphorylation mediated by endogenously expressed adenosine A_3 receptors. The agonist Cl-IB-MECA, by selectively stimulating the A_3 receptor in both primary mouse microglia cells and in the N13 microglia cell line, induces a biphasic phosphorylation of ERK1/2 (Hammarberg et al. 2003). In addition, functional A_3 receptors activating ERK1/2 have been also described in colon carcinoma and glioblastoma cells (Hammarberg et al. 2003; Merighi et al. 2006, 2007a). Interestingly, in the human melanoma A375 cell line it has been demonstrated that A_3 receptor stimulation was unable to activate ERK phosphorylation while the A_3 antagonists are able to improve MEKs activity (Merighi et al. 2002). Similar results were obtained in melanoma murine cells (Fishman et al. 2002). Furthermore, it has been demonstrated that stimulation of adenosine A_3 receptors inhibits A375 melanoma cell proliferation by the impairment of ERK kinase activation (Merighi et al. 2005a). Such a discrepancy may be due to the presence of different signaling pathways in different cell lines. As discussed later, in the case of the A375 melanoma cell line this result may be due to crosstalk between the PI3K/AKT pathway and the ERK1/2 pathway. MAPKs activation has been linked to the regulation of the adenosine A_3 receptor expressed in CHO cells. This study demonstrated that inhibition of agonist-mediated MAPK activation

prevented both homologous A_3 receptor desensitization and internalization by impairing phosphorylation. Furthermore, inhibition of MAPK by PD98059 prevented G protein-coupled receptor kinase (GRK2) translocation, suggesting that this kinase is a target for the A_3 receptor-mediated MAPK cascade. These results suggested that the MAPK cascade is involved in A_3 receptor regulation by a feedback mechanism that controls GRK2 activity and receptor phosphorylation. (Trincavelli et al. 2002).

Importantly, the activation of MAPKs have been implicated in ischemia/reperfusion injury. In particular it has been postulated that whereas ERK1/2 exerts a cytoprotective effect and is involved in cell proliferation, transformation and differentiation, p38 and JNK promote cell injury and death. Matot and co-workers observed an increase in phosphorylated JNK, p38, and ERK1/2 levels in lung tissue at the end of reperfusion compared with non-ischaemic control lung tissue. Interestingly, pretreatment with A_3 agonists upregulated phosphorylated ERK1/2 levels but did not modify phosphorylated JNK and p38 levels (Matot et al. 2006).

This pretreatment was associated with a marked improvement in lung injury and attenuation of apoptosis after reperfusion. Furthermore, ERK1/2 are also involved in cardiac hypertrophy and can play a protective role in ischaemic myocardium (Michel et al. 2001). Interestingly, A_3 receptor activation in rat cardiomyocytes has been demonstrated to increase ERK1/2 phosphorylation by involving Gi/o proteins, PKC and tyrosine kinase dependent and -independent pathways. It has been found that Cl-IB-MECA produced a biphasic effect on cAMP accumulation with a stimulatory action starting at a concentration of 3 nM. This activity was triggered through PLC/PKC and not via direct Gs coupling (Germack and Dickenson 2004). Besides ERK1/2, there is experimental evidence that adenosine A_3 receptors also activate p38 MAPKs in hCHO-A_3 cells (Hammarberg et al. 2004). Furthermore, it has been demonstrated that A_3 receptor stimulation is able to increase p38 phosphorylation in human hypoxic melanoma, glioblastoma and colon carcinoma cells (Merighi et al. 2005b, 2006, 2007a). In the current literature on A_3 receptor signaling, nothing has been reported on JNK activation by A_3 receptor stimulation.

3.2 The A_3 Receptor and the Phosphatidylinositol 3-Kinase/Protein Kinase B/Nuclear Factor-kB (PI3-K/AKT/NF-kB) Signal Transduction Cascade

A_3 receptors have been associated with the PI3K/Akt pathway (Merighi et al. 2003). Active Akt causes a variety of biological effects, including suppression of apoptosis by phosphorylation and inactivation of several targets along pro-apoptotic pathways (Vivanco and Sawyers 2002). In particular, activated Akt is able to phosphorylate a variety of downstream substrates, for example the pro-apoptotic molecule Bad, caspase-9, the forkhead family transcription factors, I-κ (a kinase that regulates the NF-kB transcription factor) and Raf.

There is evidence that A_3 adenosine receptor activation triggers phosphorylation of PKB/Akt, protecting rat basophilic leukemia 2H3 mast cells from apoptosis by a pathway involving the bg subunits of Gi and PI3K-b (Gao et al. 2001). More recently, it has been demonstrated that A_3 receptors trigger increases in Akt phosphorylation in rat cardiomyocytes via a Gi/Go-protein and tyrosine kinase-dependent pathway (Germack et al. 2004). In human melanoma A375 cells it has been shown that A_3 adenosine receptor stimulation results in PI3K-dependent phosphorylation of Akt. In particular, it has been demonstrated that serum-deprived A375 melanoma cells had no basal Akt phosphorylation whereas the A_3 receptor agonist Cl-IB-MECA treatment resulted in the phosphorylation of Akt at the Ser 573 phosphorylation site. Furthermore, it has been shown that the antiproliferative effect of Cl-IB-MECA is mediated by a PLC-PI3K-Akt signaling pathway (Merighi et al. 2005a). Resveratrol preconditions the heart through activation of adenosine A_3 receptors protecting the heart through a cAMP response element-binding (CREB)-dependent Bcl-2 pathway in addition to an Akt-Bcl-2 pathway (Das et al. 2005a, b).

In lipopolysaccharide (LPS)-treated BV2 microglial cells A_3 receptor activation suppresses tumor necrosis factor-α (TNF-α) production by inhibiting PI3K/Akt and NF-kB activation (Lee et al. 2006). Furthermore, it has been reported that in mouse RAW 264.7 cells the A_3 receptor inhibits LPS-stimulated TNF-α release by reducing calcium-dependent activation of NF-κB and ERK1/2 (Martin et al. 2006).

According to these results, it has been demonstrated that A_3 receptor agonists exert significant anti-rheumatic effects in different autoimmune arthritis models by suppression of TNF-α production (Baharav et al. 2005). The molecular mechanism involved in the inhibitory effect of IB-MECA on adjuvant-induced arthritis included

receptor down-regulation and deregulation of the PI3K-NF-κB signaling pathway (Fishman et al. 2006; Madi et al. 2007). On the contrary, it has been reported that activation of the A_3 receptor protects against ischemia/reperfusion injury in the heart through activation of NF-κB (Zhao and Kukreja 2002).

Solid tumors contain hypoxic cells that are resistant to chemotherapies such as with taxanes. Paclitaxel, the most widely studied taxane has been shown not to be highly active against newly diagnosed or recurrent glioblastoma multiforme – the most common subtype of malignant brain tumor. Interestingly, activation of PI3KAkt-pBad (a pro-apoptotic member of the Bcl-2 family) by A_3 receptor stimulation has been recently demonstrated in human glioblastoma multiforme cells. This signaling pathway is responsible for an adenosine-mediated inhibition of paclitaxel induced apoptosis in hypoxic conditions (Merighi et al. 2007b). Further studies indicate that A_3 receptor activation, by interfering with PKB/Akt, can decrease interleukin-12 production in human monocytes (Hasko et al. 1998; la Sala et al. 2005). It has been demonstrated that protein kinase A (PKA) and PKB/Akt phosphorylate and inactivate glycogen synthase kinase 3b (GSK-3b), a serine/threonine kinase acting as a key element in the Wnt signaling pathway (Fishman et al. 2002).

Activation of the A_3 receptor by the agonist IB-MECA is able to decrease the levels of PKA, a downstream effector of cAMP, and of the phosphorylated form of PKB/ Akt in melanoma and in hepatocellular carcinoma cells (Fishman et al. 2002; Bar-Yehuda et al. 2008). This implies the deregulation of the Wnt signaling pathway, generally active during embryogenesis and tumorigenesis to increase cell cycle progression and cell proliferation. Similar results were observed in synoviocytes from rheumatoid arthritis patients and in adjuvant-induced arthritis rats (Ochaion et al. 2008). In particular, it has been shown that a decrease in the expression levels of PKB/Akt, IκB kinase (IKK), I kappa B (IκB), NF-κB and tumor necrosis factor alpha (TNF-α) in a rat experimental model of adjuvant-induced arthritis (AIA). In addition, the expression levels of GSK-3β, β-catenin, and poly(ADP-ribose)polymerase (PARP), known to control the level and activity of NF-κB, were downregulated upon treatment with an A_3 receptor agonist (Ochaion et al. 2008).

3.3 Crosstalk between MAPK and PI3K/Akt signaling pathway and modulation by A_3 receptor

Crosstalk between the PI3K and the Raf/MEK/ERK pathways has been reported on multiple levels, with some research stating that PI3K activity is essential for induction of Raf/MEK/ERK activity (Vivanco and Sawyers 2002; Sebolt-Leopold and Herrera 2004). Additional studies suggest that the PI3K pathway enhances and/or synergizes with Raf/MEK/ERK signaling to provide a more robust signal through the lower components of the MAPK cascade (i.e. ERK). However, there is conflicting evidence that states that Akt is able to phosphorylate Raf, thereby efficiently abrogating Raf activity on downstream substrates (Rommel et al. 1999; Guan et al. 2000; Reusch et al. 2001; Moelling et al. 2002; Zimmermann and Moelling 1999).

In melanoma cells Akt phosphorylation mediated by the A_3 agonist Cl-IBMECA induced Raf phosphorylation at an inhibitory phosphorylation site on Ser 259. As a consequence, Cl-IB MECA inactivated Raf inducing a cross talk between ERK1/2 and Akt pathways in these cells (Merighi et al. 2005a). Ras-Raf-MEK-ERK pathway is normally activated by A_3 receptor stimulation as is the PI3K-Akt route. It is clear that these apparently separate routes should actually interact. A_3 receptor stimulation inhibits the proliferation of melanoma cells partly by a PLC-sensitive mechanism. Pretreatment of cells with a PLC-g inhibitor strongly abrogated the Cl-IB-MECA effect on cell proliferation and on ERK1/2 phosphorylation, suggesting a critical role for PLC-g in A_3 receptor signaling. Furthermore, pretreatment of A375 cells with a PI3K inhibitor and an Akt inhibitor impaired Cl-IB-MECA-induced inhibition of cell proliferation and the effects of A_3 receptor stimulation on Raf, MEK1/2 and ERK1/2 phosphorylation. These data suggest that the A_3 adenosine receptor signals through a pathway including PI3K-Akt. On the contrary, Ras was not activated. These results confirm that in A375 cells A_3 receptors decrease MEK1/2-ERK1/2 phosphorylation and cell proliferation via the inhibition of Raf, by a PI3K-Akt pathway without affecting Ras (Merighi et al. 2005a).

3.4 The A_3 Receptor and the Hypoxia-Inducible Factor 1 (HIF-1)

Over the last several years, HIF-1 has emerged as an attractive target for cancer therapy. It is a heterodimer composed of an inducibly expressed HIF-1α subunit and a constitutively expressed HIF-1β subunit. Overexpression of HIF-1α protein has been reported in several human cancers, where it has been positively associated with tumor

progression, treatment failure, and poor survival (Giaccia et al. 2003; Semenza 2003). HIF-1 is a potent activator of angiogenesis and invasion through its upregulation of target genes critical for these functions (Carmeliet et al. 1998; Kung et al. 2000; Ratcliffe et al. 2000). Such genes share the presence of hypoxia response elements (HRE), which contain binding sites for HIF-1 (Semenza 2003). Therefore, since HIF-1α expression and activity appear central to tumor growth and progression, HIF-1 inhibition is an attractive anticancer target (Semenza 2003). Knowledge of the mechanisms of action of all the actors in the hypoxic pathway is thus becoming a priority in identifying new agents capable of specifically targeting HIF-1. However, there are few choices that are currently available for direct and specific inhibition of HIF-1α. Much attention is being paid to develop new HIF-1-targeting agents. The success of these efforts will result in a new chemotherapeutic drug class which hopefully will improve the prognoses of many cancer patients. Thus far, no pharmaceutical has been identified that directly regulates the activity of a human transcription factor. Selection of the most appropriate point of therapeutic intervention to modulate HIF-1 activity is also an important factor in pharmaceutical development. In this respect, HIF-1 modulation by adenosine, increased in hypoxia (Blay et al. 1997), appears to be an attractive target for selective inhibition of the HIF-1 system in tumor hypoxic cells, without inhibition of any of the other essential HIF-1 pathways in normal cells. In particular, HIF-1 accumulation has been detected upon A_3 receptor stimulation in hypoxic melanoma, glioblastoma and colon carcinoma cells (Merighi et al. 2005b, 2006, 2007a). Furthermore, in tumor hypoxic cells, A_3 receptor activation increases vascular endothelial growth factor, VEGF, via the HIF-1 pathway revealing the functional relevance of A_3 receptor-mediated HIF-1 accumulation. The pathways involved are Akt, MEK and p38 MAPK, activated by the A_3 receptor which is able, through this signaling, to enhance HIF-1α and VEGF protein expression in tumor hypoxic cells (Merighi et al. 2005b, 2006, 2007a).

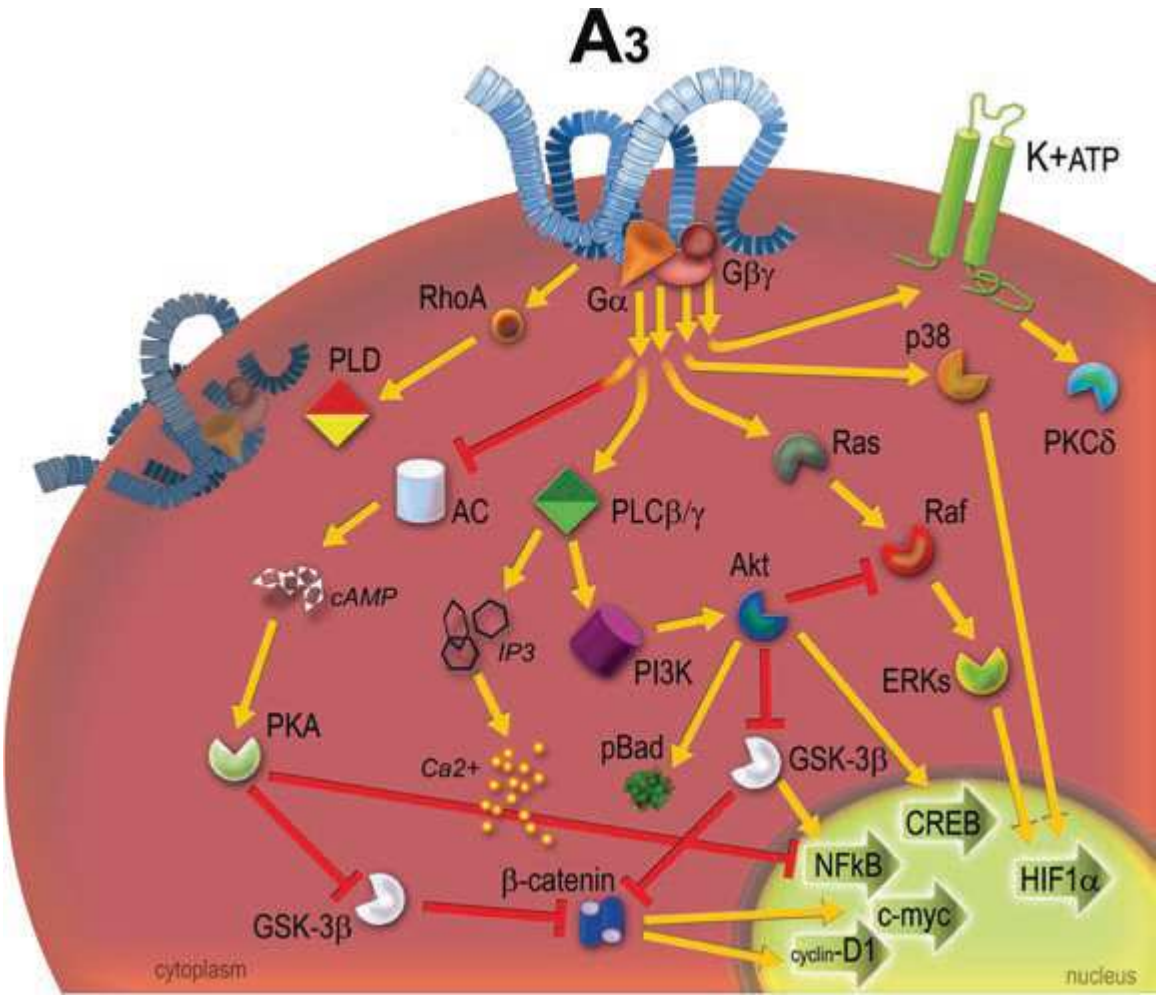

Fig. 1 Schematic representation of second messengers and intracellular signaling pathways mediated by A_3 receptor stimulation

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CHAPTER 4

A_3 ADENOSINE RECEPTOR AND COLON CANCER CELLS

Depending on the extracellular concentration, expression of different adenosine receptors subtypes and the signal transduction mechanisms activated following the binding of specific agonists, adenosine has been shown to modulate cell proliferation, differentiation and apoptosis in tumoral cells (Fishman et al., 2000; Merighi et al., 2002; Mujoomdar et al., 2003, 2004). A large body of literature attributed pro or anti mitogenic effects to A_1 and A_{2A} adenosine receptors (Merighi et al., 2003a). However, the development of potent A_3 agonists and selective antagonists revealed that the A_3 subtype plays a pivotal role in the adenosine-induced modulation of tumor cell proliferation (Bar-Yehuda et al., 2001; Merighi et al., 2005a). Indeed, contrasting results have been reported about the effects mediated through the A_3 receptors activation; it seems that it profoundly affects cell survival, by promoting cell protection or cell death depending upon the cell type and/or agonist concentration (Jacobson, 1998; Merighi et al., 2003a). In support of the A_3 receptor involvement in tumors, it has initially been shown that A_3 receptors appeared highly expressed on the cell surface of tumor cells (Gessi et al., 2001, 2002; Merighi et al., 2001; Suh et al., 2001) and recently it has been reported that the overexpression is confirmed also in human colon tumor tissues (Gessi et al., 2004; Madi et al., 2004). Colorectal cancer is the third leading cause of cancer deaths in the United States. Despite major advances in uncovering the basic biochemical and genetic alterations involved in the development and progression of colorectal cancers, treatment of this disease still relies predominantly on surgical resection. Moreover, patient prognosis is determined primarily by the stage of disease at the time of diagnosis (Hellmich et al., 2000). The effects of adenosine in epithelial colon cell proliferation have been investigated in the past with controversial results and without considering the presence of A_3 subtype (Lelievre et al., 1998a,b; Barry and Lind, 2000; Mujoomdar et al., 2003; Fishman et al., 2004). However, after the introduction of more selective ligands as new tools to identify adenosine receptors (Baraldi and Borea, 2000; Jacobson and Gao, 2006), several actions of adenosine should be reconsidered.

Recently, it has been demonstrated that A_3 receptors are overexpressed in colon cancer tissues obtained from patients undergoing surgery in comparison to normal mucosa (Gessi et al., 2004), in agreement to Madi et al. (2004). Our group investigated the presence of the A_3 adenosine receptor on human colon cancer cells to evaluate the functional effect of this receptor on colon cancer cell biology. However as adenosine receptors are often coexpressed on a single cell (Fredholm et al., 2001) it was important to investigate the presence of the other adenosine subtypes. This is relevant in view of the evaluation of adenosine-mediated effects, and to see whether colon cancer cells reflect a similar pattern of expression of human tumors. The presence of all adenosine receptors mRNAs in both tissues and colon cancer cells was detected, by means of real time RT-PCR studies, with the A_{2B} being the more express in comparison to A_1, A_{2A}, and A_3 subtypes. However as this result is not predictive of the presence of adenosine receptors in the membrane surface due to posttranscriptional events, to quantify exactly the density of A_1, A_{2A}, A_{2B}, and A_3 adenosine receptor protein, saturation studies by using selective antagonist radioligands were performed. The results showed that the density of A_1, A_{2A}, and A_{2B} was quite low if compared with that of the A_3 subtypes. Similar results were obtained in colon cells suggesting for the first time that the pattern of expression of adenosine receptors is very similar in colon cancer tissues and colon carcinoma cell lines and that the A_3 subtype is the most abundant adenosine receptor present in both. Due to the discrepance between mRNA and binding data, protein levels were also evaluated by Western blotting experiments obtaining analogous results. This supports the emerging evidence that mRNA expression patterns are necessary but are by themselves insufficient for the quantitative description of biological systems. This evidence includes discoveries of posttranscriptional mechanisms controlling the protein translation rate or the half-lives of specific proteins or mRNAs (Gygi et al., 1999; Audic and Hartley, 2004; Weinzierl et al., 2007). Starting from the observation that adenosine could be detected in the interstitial fluid surrounding a carcinoma (Blay et al., 1997), a growing body of literature indicates that, depending upon the experimental conditions, adenosine may be either toxic and inhibit cell growth or alternatively stimulate cellular proliferation (Merighi et al., 2003a). Therefore in order to ascertain the potential effects of this nucleoside on colon carcinoma, our group chose human colon cancer cell lines at different degrees of differentiation such as Caco2, DLD1 and HT29 showing a well, intermediate and low differentiated aspect, respectively. Interestingly, binding experiments revealed a differential expression of adenosine receptors in these cell lines

(A_1 and A_3 in Caco 2 cells; A_1, A_{2A}, and A_3 in DLD1 cells and A_1, A_{2A}, A_{2B}, and A_3 in HT29 cells) rending this panel of cells an interesting model to investigate the contribution of each adenosine subtype in cell growth. It has been demonstrated that adenosine induces a dose dependent stimulatory effect in Caco2, DLD1 and HT29 cells with an EC_{50} in the range 3– 12 μM.

Growing evidence suggest that ERK1/2 pathway plays an important role in the pathogenesis, progression and oncogenic behavior of human colorectal cancer (Fang and Richardson, 2005). Therefore our group evaluated the A_3-mediated stimulation of ERK/MAPK pathway in colon cancer cell lines. The involvement of ERK1/2 kinases on Cl-IB-MECA-induced cell proliferation was investigated by using a specific inhibitor of MEK, which is the upstream kinase for ERK1/2. Pretreatment of colon cancer cells with increasing concentrations of U0126 reduced the DNA synthesis induced by Cl-IB-MECA, as measured by thymidine incorporation and cell cycle analysis. ERK1/2 activation was also confirmed by immunoblot analysis revealing MAPK phosphorylation. This effect was rapid and transient as it returned almost to the control levels within 1 h of treatment, according to which found in transfected cells (Schulte and Fredholm, 2000, 2002).

In conclusion, in line with literature data reporting that adenosine may promote cancer cell proliferation (Mujoomdar et al., 2003, 2004), stimulate angiogenesis (Montesinos et al., 2004), HIF activation (Merighi et al., 2005b), and inhibit anti-tumor immune response (MacKenzie et al., 1994), current data suggest that endogenous adenosine in colon cancer cells behaves like a stimulator of tumor growth, through the involvement of the A_3 adenosine subtype and ERK1/2 phosphorylation and that in colon cancer cell lines exist a tonic stimulatory effect on cell proliferation that is mediated by A_3 receptor activation.

Recently it was reported that there could be a link between coffe consumption and reduced risk of colorectal cancer. The constituents of coffee might have genotoxic, mutagenic, or antimutagenic properties, any of which could influence colorectal cancer risk. For example, caffeine has been reported to inhibit chemical carcinogenesis and UVB light- induced carcinogenesis in animal models (Ramos et al., 2008). Coffee is also a major source of the chlorogenic acid that contributes to its antioxidant effect (Rodriguez et al., 2002). Intake of chlorogenic acid has been shown to reduce glucose concentrations in rats and intake of quinides, degradation products of chlorogenic acid, increases insulin sensitivity (Shearer et al., 2003). Chronic hyperinsulinemia and insulin

resistance are confirmed markers of high risk for some cancer sites (Renehan et al., 2008). Hypermethylation of DNA is a common characteristic in tumor cells and is a key epigenetic mechanism for silencing various genes, including those encoding the tumor suppressor proteins, DNA repair enzymes, and receptors. Gene-specific hypermethylation is known to be associated with inactivation of various pathways involved in the tumorigenic process, including cell cycle regulation, inflammatory and stress response and apoptosis. It has been demonstrated that caffeic acid, the main ingredient of coffee, inhibits DNA methylation in cultured MCF-7 and MAD-MB-231 human cancer cells (Vucic et al., 2008) .

Our group reported for the first time results about the in vitro effect of caffeine on hypoxic cancer cells.

4.1

“Caffeine Inhibits Adenosine-Induced Accumulation of Hypoxia-Inducible Factor-1, Vascular Endothelial Growth Factor, and Interleukin-8 Expression in Hypoxic Human Colon Cancer Cells.”

INTRODUCTION

Coffee and tea are the most commonly consumed beverages in the world (Fredholm, 1999). Results of epidemiological studies have not resolved whether coffee consumption is related to colorectal cancer risk. A report by the World Cancer Research Fund concluded that the available evidence was not sufficient to draw any firm conclusions about a decreased risk of colorectal cancer associated with coffee consumption (World Cancer Research Fund/American Institute for Cancer Research, 1997). However, some researchers contend that a link between high consumption of coffee and a low incidence of colorectal cancer has been firmly established (Ekbom, 1999; Woolcott et al., 2002). Coffee is a leading source of methylxanthines, such as caffeine. A cup of coffee contains approximately 100 mg of caffeine (Fredholm, 1999); thus, caffeine can be found in micromolar concentrations in the human circulation as a result of dietary intake or pharmacological use. Most solid tumors develop regions of low oxygen tension because of an imbalance in oxygen supply and consumption. Clinical and experimental evidence suggests that tumor hypoxia is associated with a more aggressive phenotype (Hockel and Vaupel, 2001). Hypoxic tumor cells are resistant to conventional chemotherapy and radiotherapy. It is therefore rational to target the hypoxic regions of tumors or disrupt events initiated by hypoxia (Melillo, 2004). Interleukin-8 (IL-8), originally discovered as a chemotactic factor for leukocytes, has been shown recently to contribute to human cancer progression through its potential functions as a mitogenic, angiogenic, and motogenic factor (Xie, 2001). Although it is constitutively detected in human cancer tissues and established cell lines, IL-8 expression is regulated by various tumor microenvironment factors, such as hypoxia, acidosis, nitric oxide, and cell density. Furthermore, hypoxia is a potent stimulator of vascular endothelial growth factor (VEGF) expression, a key proangiogenic factor, and this induction is believed to be mediated primarily through hypoxia- inducible factor-1 (HIF-1) (Maxwell et al., 1997). HIF-1 is one of the master regulators that orchestrate the cellular responses to hypoxia. It is a heterodimer composed of an inducibly expressed HIF-1α subunit and a constitutively expressed HIF-1β subunit. A growing body of evidence indicates that HIF-1 contributes to tumor progression and metastasis (Giaccia et al., 2003; Semenza, 2003). Immunohistochemical analyses have shown that HIF-1α is present in higher levels in human tumors than in normal tissues (Zhong et al., 1999). HIF-1 is a potent activator of angiogenesis and invasion through its up-regulation of target genes critical for these functions (Carmeliet et al., 1998; Kung et al., 2000;

Ratcliffe et al., 2000). Such genes share the presence of hypoxia response elements, which contain binding sites for HIF-1 (Semenza, 2003). Therefore, because HIF-1α expression and activity seem central to tumor growth and progression, HIF-1 inhibition becomes an appropriate anticancer target (Maxwell et al., 1997; Kung et al., 2000; Giaccia et al., 2003; Semenza, 2003). It is interesting that VEGF is overexpressed not only in advanced colon cancers but also in premalignant colonic adenomas (Wong, 1999). The factors that may contribute to this enhanced VEGF expression are not defined fully. Although the mechanism of the possible protective effect of coffee or its products is unclear, potential protective effects could include antagonistic effects of the adenosine receptors. In particular, the A_3 subtype is highly expressed in tumor cells (Gessi et al., 2001, 2002; Merighi et al., 2001) and is able to significantly up-regulate the expression of HIF-1 in hypoxic tumors (Merighi et al., 2005a, 2006), suggesting that A_3 receptor overexpression may be a good candidate as a tumor cell marker (Gessi et al., 2004; Madi et al., 2004). Adenosine also plays a role in the promotion of angiogenesis (Montesinos et al., 2004). Regulation of expression of VEGF through adenosine receptors has been demonstrated in different cell types (Feoktistov et al., 2002, 2003, 2004; Leibovich et al., 2002). The aim of this study is to determine whether caffeine may regulate HIF-1α, VEGF, and IL-8 in colon cancer cells during hypoxia.

MATERIALS AND METHODS

Cell Lines, Reagents and Antibodies

HT29 human tumor colon cells were obtained from American Type Culture Collection (Manassas, VA). Human umbilical vein endothelial cells (HUVEC), tissue culture media and growth supplements were obtained from Lonza Bioscience (Bergamo, Italy). Antiadenosine A_{2B} and antiadenosine A_3 receptor antibodies (pAb) were from Alpha Diagnostic (Milano, Italy). Human anti-HIF-1α and human anti-HIF1β antibodies (mAb) were obtained from BD Transduction Laboratories (Milan, Italy). Anti-human vascular endothelial growth factor (VEGF) antibody was developed in goat using recombinant human VEGF165 as immunogen. U0126 (inhibitor of MEK-1 and MEK-2), SB202190 (inhibitor of p38 MAP kinase), human anti-ACTIVE MAPK, and human anti-ERK1/2 antibodies (pAb) were from Promega (Milan, Italy). SH5 (inhibitor of Akt) was from Vinci-Biochem (Florence, Italy). Human phospho-p38 and human p38 MAP

kinase antibodies were from Cell Signaling Technology (Milan, Italy). P11w, a firefly luciferase reporter plasmid, comprising the 5-flanking 985 to 939 base pairs of the human VEGF gene that include an HIF-1-binding site, and p11m, the mutated version of p11w containing a nonfunctional HIF-1-binding site (Forsythe et al., 1996), were obtained from the American Type Culture Collection. BriteLite Ultra-High Sensitivity Luminescence Reporter Gene Assay System kit was obtained from PerkinElmer Life and Analytical Sciences (Milan, Italy). Fugene 6 transfection reagent was purchased from Roche Molecular Biochemicals (Milan, Italy). ZM 241385 and [^{3}H]ZM 241385 (specific activity, 17 Ci/mmol) were obtained from Tocris Cookson Ltd. (Bristol, UK). MRE 2029F20, MRE 3008F20, and B64 were synthesized by Dr. Pier Giovanni Baraldi (Department of Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy). [^{3}H]MRE 2029F20 (specific activity, 123 Ci/mmol) and [^{3}H]MRE 3008F20 (specific activity, 67 Ci/mmol) were obtained from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). [^{3}H]DPCPX (specific activity, 120 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Adenosine A_{2B} and A_3 receptor small interfering RNAs (siRNAs) were from Santa Cruz Biotechnology (Santa Cruz, CA). Unless otherwise noted, all other chemicals were purchased from Sigma (Milan, Italy).

Cell Culture

HT29 human tumor colon cells were maintained in RPMI 1640 medium containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) at 37°C in 5% CO_2/95% air. HUVEC used in this study were from passages 2 to 7.

Establishment of Hypoxic Culture Condition

For hypoxic conditions, cells were placed for the indicated times in a modular incubator chamber and flushed with a gas mixture containing 1% O_2, 5% CO_2, and balance N_2 (MiniGalaxy, RSBiotech, Irvine, Scotland). Maintenance of the desired O_2 concentration was constantly monitored during incubation using a microprocessor-based oxygen controller.

Caffeine Treatment of Cancer Cells

Exponentially growing cells (70–80% confluence) in complete medium were pretreated for 1h with different concentrations of caffeine, followed by continual incubation in normal culturing conditions or exposure to hypoxia (1% O_2) for indicated time intervals according to the purpose of the experiment.

Membrane Preparation

For membrane preparation, the culture medium was removed. The cells were washed with PBS and scraped off of T75 flasks in ice-cold hypotonic buffer (5 mM Tris HCl and 2mM EDTA, pH 7.4). The cell suspension was homogenized with a Polytron homogenizer (Kinematica, Basel, Switzerland), and the cell suspension was centrifuged for 10 min at 1000*g*. The supernatant was then centrifuged again for 30 min at 100,000*g*, and the membrane pellet was frozen at -80°C until the use in competition binding experiments.

Competition Binding Experiments at A_1, A_{2A}, A_{2B}, and A_3 Adenosine Receptors.

Binding of [^{3}H]DPCPX to A_1 receptors expressed in HT29 cells was performed for 120 min at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 1 nM [^{3}H]DPCPX, diluted membranes (100 μg of protein per assay), and caffeine. Nonspecific binding was determined in the presence of 1 μM DPCPX and was always ≤10% of the total binding. Binding of 1 nM [^{3}H]ZM 241385 to human A_{2A} expressed in HT29 membranes (100 μg of protein per assay) was performed using 50 mM Tris-HCl buffer, 10 mM $MgCl_2$ pH 7.4, and different concentrations of caffeine for an incubation time of 60 min at 4°C. Nonspecific binding was determined in the presence of 1 μM ZM 241385 and was approximately 20% of total binding. Competition experiments to human A_{2B} expressed in HT29 membranes were performed using 3 nM [^{3}H]MRE 2029F20 for an incubation time of 60 min at 4°C. Nonspecific binding was defined as binding in the presence of 1 μM MRE 2029F20 and was 25% of total binding. Binding of [^{3}H] MRE 3008F20 to human A_3 expressed in HT29 membranes was carried out in 50 mM Tris-HCl buffer, 10 mM $MgCl_2$, and 1 mM EDTA, pH 7.4, containing 1 nM [^{3}H] MRE 3008F20, membranes (100 μg of protein per assay), and caffeine for 120 min at 4°C. Nonspecific binding was defined as binding in the presence of 1μM MRE 3008F20 and was approximately 25 to 30% of total binding. Eight different concentrations of caffeine were studied.

Measurement of cAMP Levels

HT29 cells in exponential growth were exposed to drugs for 2 h. After the incubation, the HT29 cells were collected, washed three times in ice-cold PBS, lysed, and centrifuged. The supernatants were assayed for cAMP determination using an R&D cAMP assay kit following the manufacturer's instructions (Parameter kit; R&D Systems, Minneapolis, MN).

Conditioned Medium

To obtain conditioned medium from *N6*(3- iodobenzyl) 2-chloroadenosine-5'*N*-methyluronamide (Cl-IB-MECA)- treated HT29 human tumor colon cells, we plated 106 HT29 cells in a 10-cm diameter plate containing RPMI 1640 medium with 10% fetal bovine serum. After 24 h, the medium of these cells was replaced with fresh growth medium containing Cl-IB-MECA (0 or 100 nM). The plates were then incubated under normoxic or hypoxic conditions. After 1 day of incubation, conditioned medium was removed and centrifuged at 4000*g* for 20 min at 4°C through an Amicon Ultra-4 centrifugal filter (Millipore, Billerica, MA) to remove any trace of Cl-IB-MECA. The molecular mass cutoff of the filters was 5 kDa, and the molecular mass of Cl-IB-MECA is 0.544 kDa. The flow-through containing excess Cl-IB-MECA was discarded, and the retentate was collected. Furthermore, to exclude that Cl-IB-MECA itself may have an inhibitory effect on the migration assay, we treated HUVECs directly with Cl-IB-MECA 100 nM, which was insufficient to modulate HUVEC migration. The final filter retentate was concentrated 40-fold for use in the migration and proliferation assays.

JAM Test

This assay measures cell death by quantifying the amount of fragmented DNA. Target cells were labeled with 1 μCi/ml [^{3}H]thymidine for 20 h in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (2 mM). The cells were then washed and treated with new unlabeled medium containing adenosine analogues for 24 h. At the end of the incubation period, the cells were trypsinized and dispensed in four wells of a 96-well plate, filtered through Whatman GF/C glass fiber filters using a Micro-Mate 196 cell harvester (PerkinElmer Life Sciences). The filter-bound radioactivity was counted on Top Count Microplate Scintillation Counter (efficiency 57%) with Micro-Scint 20. The amount of apoptotic and necrotic cells, measured as the loss of radioactivity associated with the loss of

fragmented and degraded DNA, was detected by filtration and subsequent washing with a Micro-Mate 196 cell harvester followed by quantification with a Top Count Microplate Scintillation Counter. The percentage of cell death is expressed as 100 x $(dpm_{(U)} - dpm_{(T)})/dpm_{(U)}$, where $dpm_{(U)}$ represents the radioactivity of untreated cells and $dpm_{(T)}$ is the radioactivity of treated cells (Merighi et al., 2005b).

MTT Assay

The MTS assay was performed to determine colon cell viability and proliferation according to the manufacturer's protocol from the CellTiter 96 AQueous One Solution (Promega) cell proliferation assay, as described previously (Merighi et al., 2005b). Cells (10^5) were plated in 24-multiwell plates; 500 µl of complete medium was added to each well with different concentrations of caffeine. The cells were then incubated for 24 h. At the end of the incubation period, MTS solution was added to each well. The optical density of each well was read on a spectrophotometer at 492 nm. For each experiment, four individual wells of each drug concentration were prepared. Each experiment was repeated three times.

Migration Assay

Cell migration was performed with the Transwell system (Chemicon International, Temecula, CA), which allows cells to migrate through 8-µm pore size polycarbonate membrane. In brief, cells were trypsinized, washed, and resuspended in serum-free Dulbecco's modified Eagle's medium ($5x10^5$cells/ml). This suspension (300 µl) was added to the upper chamber of Transwells. The lower chamber was filled with 500 µl of conditioned medium. After the incubation (6–24 h), filters were removed, and cells remaining on the upper surface of the membrane (i.e., that had not migrated through the filter) were removed with a cotton swab. Then, membranes were washed with PBS, and cells present beneath the membrane were fixed with ice-cold methanol for 15 min and stained with the Cell Stain Solution (QCM Colorimetric Cell Migration Assay; Chemicon International). The stained insert was transferred to a well containing the extraction buffer. The dye mixture was transferred to a 96-well microtiter plate suitable for colorimetric measurement. Analysis was performed on three wells for each condition, and each experiment was repeated three times.

Western Blotting

Whole-cell lysates, prepared as described previously (Merighi et al., 2005b), were resolved on a 10% SDS gel and transferred onto the nitrocellulose membrane. Western blot analyses were performed as described previously (Merighi et al., 2005a) with anti-HIF-1α (1:250 dilution) and anti-HIF-1β antibodies (1:1000 dilution) in 5% nonfat dry milk in PBS/0.1% Tween 20 overnight at 4°C. Aliquots of total protein sample (50 µg) were analyzed using antibodies specific for phosphorylated (Thr183/Tyr185) or total p44/p42 MAPK (1:5000 dilution), phosphorylated (Thr180/Tyr182) or total p38 MAPK (1:1000 dilution), and for phosphorylated Akt (Ser473) (1:1000 dilution). The protein concentration was determined using BCA protein assay kit (Pierce, Rockford, IL).

Membranes were washed and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies against mouse and rabbit IgG (1:2000 dilution). Specific reactions were revealed with the Enhanced Chemiluminescence Western blotting detection reagent (GE Healthcare). The membranes were then stripped and reprobed with antitubulin antibodies (1:250) to ensure equal protein loading.

Densitometry analysis

The intensity of each band in immunoblot assay was quantify using molecular analyst/PC densitometry software (Bio-Rad Laboratories, Hercules, CA). Mean densitometry data from independent experiments were normalized to results in cells in the control. The data were presented as the mean ± S.E., and analyzed by the Student's test.

Treatment of Cells with siRNA

HT29 cells were plated in six-well plates and grown to 50-70% confluence before transfection. Transfection of siRNA was performed at a concentration of 100 nM using RNAiFect™ Transfection Kit (Qiagen, Valencia, CA). Cells were cultured in complete media, and at 48 total proteins were isolated for Western Blot analysis for A_{2B} and A_3 receptor protein. A nonspecific random control ribonucleotide sense strand (5'-ACU CUA UCU GCA CGC UGA CdTdT-3') and antisense strand (5'-dTdT UGA GAU AGA CGU GCG ACU G-3') were used under identical conditions (Merighi et al., 2005b).

Enzyme-Linked Immunosorbent Assay

The levels of VEGF and IL-8 protein secreted by the cells in the medium were determined by a VEGF and an IL-8 enzyme-linked immunosorbent assay kit (R&D Systems). In brief, subconfluent cells were changed into fresh medium in the presence of solvent or various concentrations of adenosine analogs in hypoxia. The medium was collected, and VEGF and IL-8 protein concentrations were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions. The results were normalized to the number of cells per plate. The data were presented as mean ±S.D. from three independent experiments.

Transient Transfection and Luciferase Reporter Assay

HT29 human tumor colon cells were prepared for transfection by seeding them into 24-well plates (30,000 cells/well) in 0.5 ml of standard growth medium. After an overnight culture, the cells were transfected with 100 ng of p11w or p11m. Transfections were performed with 1.2 µl of Fugene 6 per well. The cells were then treated with drugs or the solvent vehicle only, then incubated under hypoxic (1% O_2) or normoxic conditions. The cells were then prepared for the luciferase-reporter assay according to the manufacturer's instructions. In brief, the cells were lysed at ambient temperature for 2 min with 200 µl of 1X lysis buffer. The extracts were assayed for plasmids (p11w and p11m) and control (*Renilla reniformis*) luciferase activities with a PerkinElmer Life and Analytical Sciences luminometer. Samples were normalized for transfection efficiency based on the *R. reniformis* luciferase activity.

Statistical analysis

All values in the figures and text are expressed as mean ± S.E. (standard error) from three independent experiments except where indicated. Data sets were examined by analysis of variance (ANOVA) and Dunnet's test (when required). A P value less than 0.05 was considered statistically significant.

RESULTS

Caffeine Inhibits Adenosine-Induced HIF-1α Protein Accumulation in Human Colon Cancer Cells.

HIF-1α protein is undetectable in human HT29 colon cancer cells cultured under normoxic conditions, whereas it is present in hypoxia (Fig. 1A). Adenosine (10 and 100 μM) is able to increase HIF-1α protein accumulation in HT29 hypoxic colon cancer cells (Fig. 1A). The presence of adenosine receptors was recently investigated in HT29 cells, which express all four adenosine receptor subtypes. In particular, A_1 receptors are present with 32 ± 4 fmol/mg of protein, A_{2A} receptors with 49 ± 4 fmol/mg of protein, A_{2B} receptors with 52 ± 4 fmol/mg of protein, and A_3 receptors with 257 ± 22 fmol/mg of protein (Gessi et al., 2007). To evaluate whether A_3 receptors may have a functional role in HIF-1α protein expression under hypoxic conditions, we tested the effect of increasing concentrations (10–1000 nM) of the high-affinity A_3 receptor agonist Cl-IB-MECA (Table 1) (Merighi et al., 2005b). A_3 adenosine receptor stimulation promoted HIF-1α protein accumulation under hypoxic conditions, whereas it did not modify HIF-1β expression in normoxia or in hypoxia (Fig.1A). To confirm that A_3 receptors have a functional role in HIF-1α protein expression under hypoxic conditions, we tested the effect of the high-affinity and selective A_3 receptor antagonist MRE 3008F20 (Table 1) (Varani et al., 2000). MRE 3008F20 (0.1–10 nM) is able to decrease the induction of HIF-1α expression under hypoxic conditions obtained through Cl-IB-MECA 10 nM (Fig. 1B). These results indicate that adenosine increases HIF-1α protein expression via A_3 receptors. We next asked whether caffeine, an adenosine receptor antagonist (Fredholm et al., 1999), inhibits adenosine-induced HIF-1α protein expression in hypoxia. In HT29 cells, 10 μM caffeine was able to inhibit HIF-1α protein accumulation induced by 10 to 100 nM Cl-IB MECA (Fig. 1C). Furthermore, we observed that pretreatment of HT29 cells with 10 μM caffeine abrogated 10 and 100 μM adenosine-induced HIF-1α protein accumulation (Fig. 1D). To rule out the possibility of a cytotoxic effect on HIF-1α protein suppression by caffeine, cell viability assay using MTS was done. No obvious changes in cell viability were observed in HT29 cells after being challenged with different concentrations of caffeine (0.1–100 μM) under both normoxic and hypoxic conditions for 24 h (Fig. 1E), indicating that the inhibition of HIF-1α protein expression by caffeine was not ascribed to nonspecific tumor cell toxicity. To confirm these results, we analyzed the effect of caffeine on cell survival by the JAM test. HT29 cells, previously labeled with

[^{3}H]thymidine, were treated for 24 h with increasing concentrations of caffeine (0.1–100 μM). Caffeine did not induce cell death, as shown in Fig. 1F.

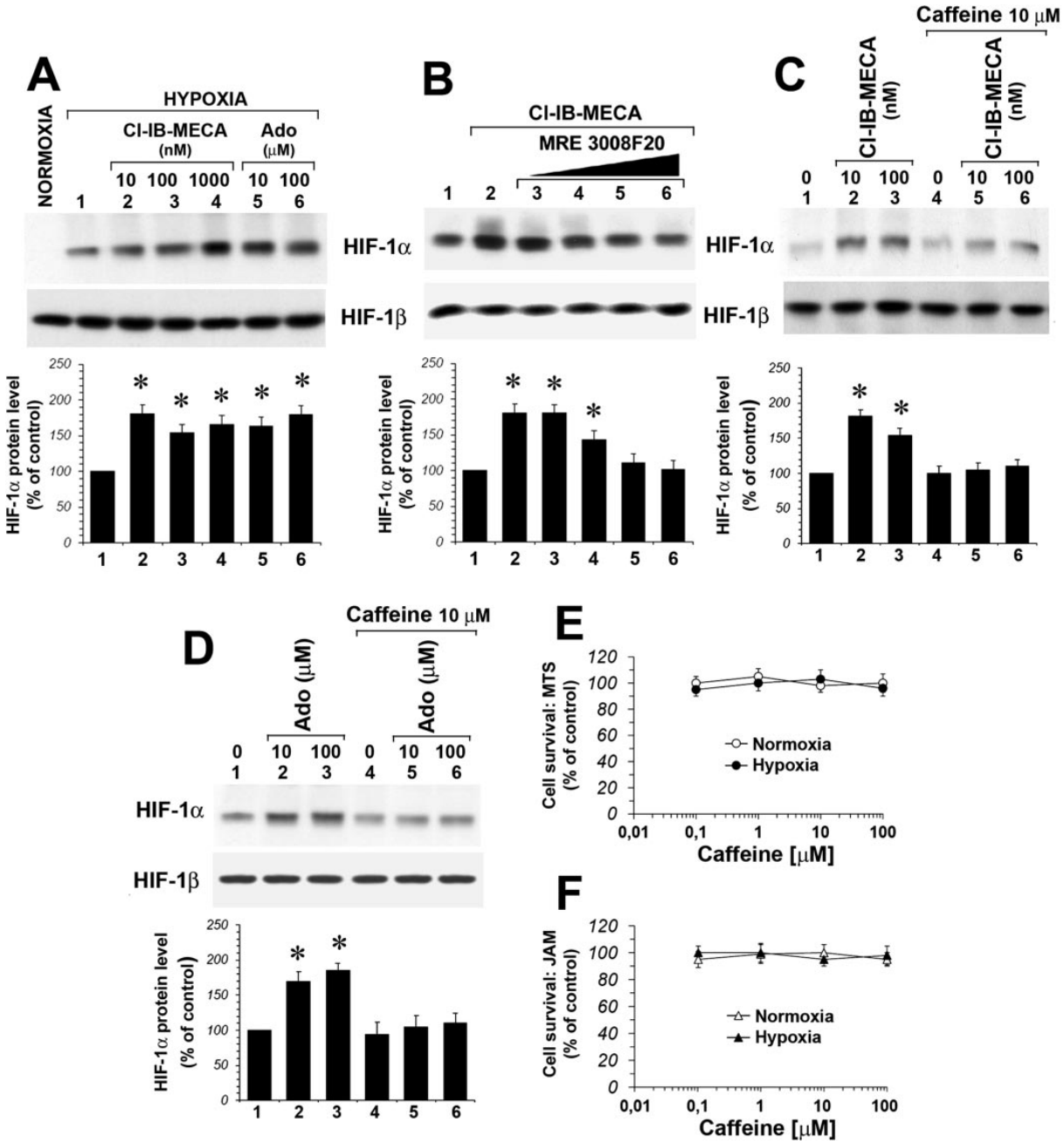

Fig. 1. Modulation of HIF-1α expression by adenosine. A, Western blot analysis for HIF-1α and HIF-1β levels of 35 μg of total protein lysates from HT29 cells treated in normoxia or in hypoxia (1% O_2, 4 h) without or with the selective A_3 agonist Cl-IB-MECA 10, 100, and 1000 nM, and adenosine 10 and 100 μM. B, effect of the selective A_3 antagonist MRE 3008F20. HT29 cells were treated in hypoxia (1% O_2, 4 h) without (lane 1) or with Cl-IB-MECA 10 nM (lanes 2–6) and MRE 3008F20 0.1 nM (lane 3), 1 nM (lane 4), 3 nM (lane 5), and 10 nM (lane 6). C, effect of caffeine on HIF-1α expression induced by Cl-IB-MECA. Western blot analysis for HIF-1α and HIF-1β levels. HT29 cells were treated in hypoxia (1% O_2, 4 h) without (lane 1) or with 10 nM Cl-IB-MECA (lanes 2 and 5), 100 nM Cl-IB-MECA (lanes 3 and 6), and 10 μM caffeine (lanes 4–6). D, effect of caffeine on HIF-1α expression induced by adenosine. Western blot analysis for HIF-1α and HIF-1β levels. HT29 cells were treated in hypoxia (1% O_2, 4 h) without (lane 1) or with 10 μM adenosine (lanes 2 and 5), 100 μM adenosine (lanes 3, 6), and 10 μM caffeine (lanes 4–6). The mean densitometry data from independent experiments (one of which is shown here) were normalized to the result obtained in hypoxic cells in the absence of drug treatment (control). Plots are mean ± S.E. values (n = 3). *, $P < 0.01$ compared with the control. E and F, HT29 cells were treated with increasing concentrations of caffeine (0.1–100 μM) for 24 h under both normoxic and hypoxic conditions, and cell viability was assayed by an MTS test (E) and a JAM test (F). In MTS, the cell growth is expressed as a percentage of the OD measured on untreated cells (control) assumed as 100% of cell viability. Ordinate reports means of four different OD quantifications with standard error

(vertical bar). In JAM test, percentage of cell survival is reported in ordinate with standard error (vertical bar). Values represent means (± S.E.M.) of four separate quantifications in the same experiment. During the experiment, cells treated with the solvent DMSO served as controls.

Caffeine Inhibits Adenosine-Induced Phosphorylation of Akt, ERK1/2, and p38 MAPK.

HT29 cells were cultured in the absence and in the presence of adenosine analogs for 0.5 to 4 h in hypoxia. We found that exposure to the A_3 receptor agonist Cl-IB-MECA (1–100 nM) and to the nonselective adenosine analog NECA (0.1–1 μM) (Table 1) resulted in a sustained increase in the phosphorylated p38 and in a transient increase in Akt and ERK1/2 phosphorylation levels in colon cells (Fig. 2A). The phosphorylation of p38 kinases occurs at early time points after A_3 receptor activation (Fig. 2A). Furthermore, 10 μM caffeine was able to block the increase in the phosphorylation of p38 kinase mediated by A_3 receptor stimulation in hypoxic HT29 cells (Fig. 2B). Similar results are reported for Akt and ERK1/2 phosphorylation in HT29 colon cancer cells (Fig. 2B). These data suggest that caffeine acts as an adenosine receptor antagonist.

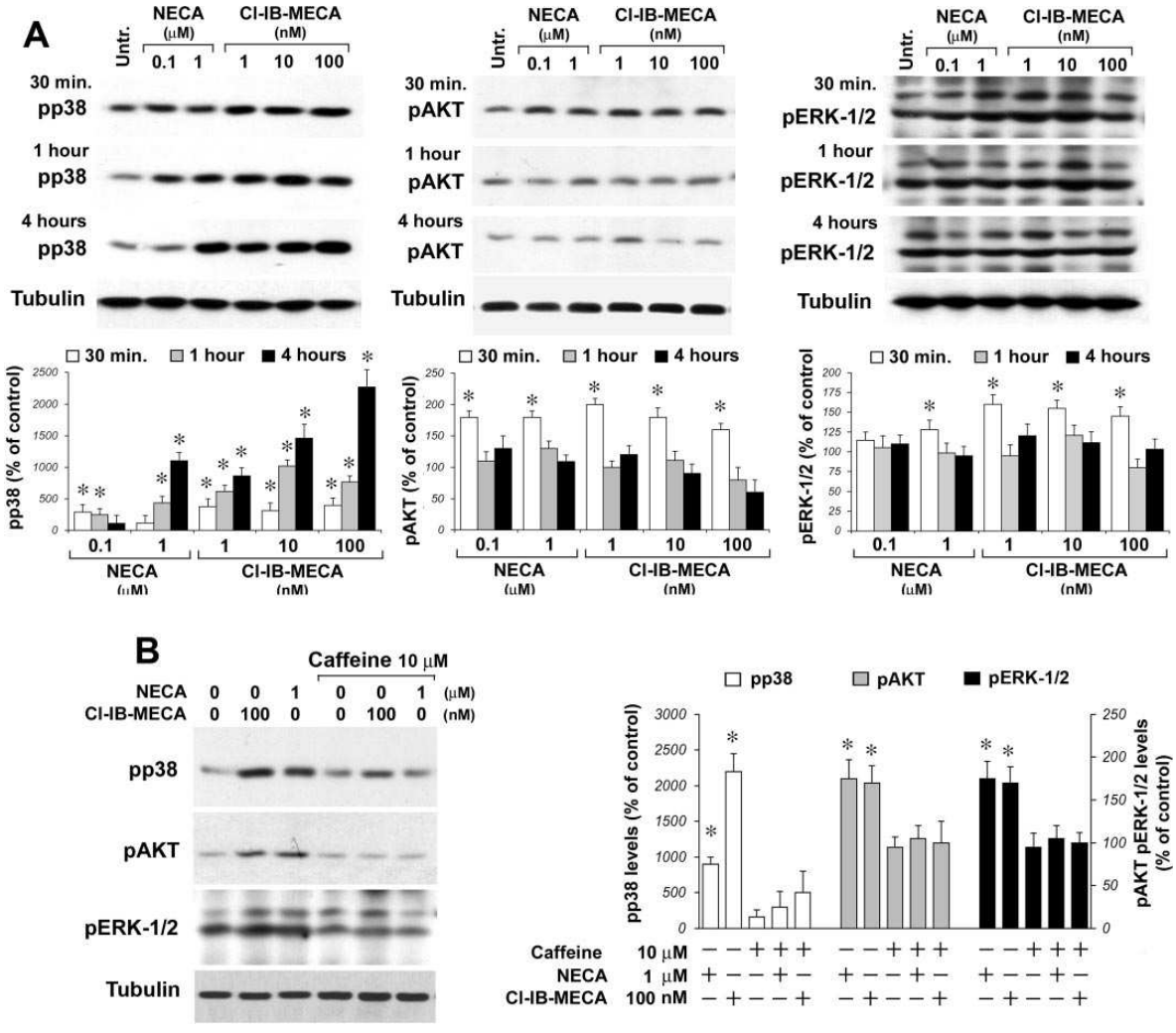

Fig. 2. p38, Akt, and ERK1/2 phosphorylation in hypoxic colon HT29 cancer cells. A, pp38, pAkt, and pERK1/2 MAPK phosphoprotein levels under the selective A_3 agonist Cl-IB-MECA and the adenosine receptor agonist NECA treatment in hypoxia (1% O_2): dose- and time-relation effect. The mean densitometry data from independent experiments were normalized to the results obtained in cells in the absence of Cl-IB-MECA or NECA (lane 0, Untreated). Plots are mean ±S.E. values (n =3); *, $P < 0.01$ compared with the control. B, effect of caffeine on pp38, pAkt, and pERK1/2 MAPK phosphoprotein levels under Cl-IB-MECA and NECA treatment in hypoxia. The mean densitometry data from independent experiments were normalized to the results obtained in cells in the absence of Cl-IB-MECA or NECA (lane 0, Untreated). Plots are mean ± S.E. values (n =3); *, $P < 0.01$ compared with the control.

The Site of Action of Caffeine.

To investigate whether caffeine interacts with signaling molecules downstream of adenosine receptors such as Akt, mitogen-activated protein kinases, or p38, we treated HT29 cells with caffeine (1–10μM) for 4 h in hypoxia, and then we evaluated the effects of caffeine treatment on the kinases under study. Figure 3A shows that caffeine, at these concentrations, did not interact with the signaling molecules investigated because the phosphorylation levels of Akt, ERK1/2, and p38 were unchanged after caffeine treatment. Furthermore, we demonstrated that SH5, an Akt inhibitor, SB202190, an inhibitor of p38 MAPK, and U0126, which is a potent inhibitor of MEK1/2, are selective at a concentration of 10 μM, as shown in Fig. 3A.

To consider whether caffeine-dependent alterations in cAMP levels could be influencing the results obtained, we evaluated potential cAMP modulations in colon cells treated with caffeine. HT29 cells were exposed to 2 h of hypoxia alone and in the presence of caffeine (1–10 μM). Hypoxia significantly increased cAMP levels from 10 ± 1 to 25 ± 2 pmol/106 cells. The incubation with caffeine in hypoxia did not modulate cAMP levels in these cells (Fig. 3B). As positive control, we show that the stimulation of adenylate cyclase with 1 to 10 μM forskolin increased cAMP levels up to 5-fold with respect to hypoxic control (Fig. 3B). To better address the site of action of caffeine in the inhibitory effects of adenosine-induced responses in hypoxic colon cancer cell cultures, we performed a series of competition binding assays to human adenosine receptors in HT29 cells. Table 2 reports the affinity values versus A_1, A_{2A}, A_{2B}, and A_3 adenosine receptor subtypes, expressed as the inhibitory binding constant (Ki) of caffeine. The results were obtained through [^{3}H]DPCPX, [^{3}H]ZM 241385, [^{3}H]MRE 2029F20, and [^{3}H]MRE 3008F20 competition binding experiments performed for A_1, A_{2A}, A_{2B}, and A_3 in HT29 membranes, respectively. We found that caffeine has affinity in the micromolar range versus all adenosine receptor subtypes, confirming that this antagonist interferes with ligand binding to purinergic receptors.

TABLE 1

Binding affinity of agonists and antagonists at A_1, A_{2A}, A_{2B}, and A_3 adenosine receptor subtypes
*K*i values are shown with S.E.M. or 95% confidence intervals in parentheses.

	A_1	A_{2A}	A_{2B}	A_3	References
NECA	14 (6.4–29)	20 (12–35)		6.2 (3.5–11)	Fredholm et al., 2001
NECA			260 ± 30		Varani et al., 2005
Cl-IB-MECA	115 (114–116)	2100 (1700–2500)	>100,000 (from a cAMP assay)	11 (9.4–13)	Fredholm et al., 2001
MRE 3008F20	1120 ±130	165 ± 18	1500 ± 165	0.9 ± 0.1	Varani et al., 2005
MRE 2029F20	200±25	>1000	3.2 ± 0.3	>1000	Varani et al., 2005
B64	708 (598–838)	495 (402–608)	34 (26–45)	3.7 (3.2–4.3)	Baraldi et al., 2002

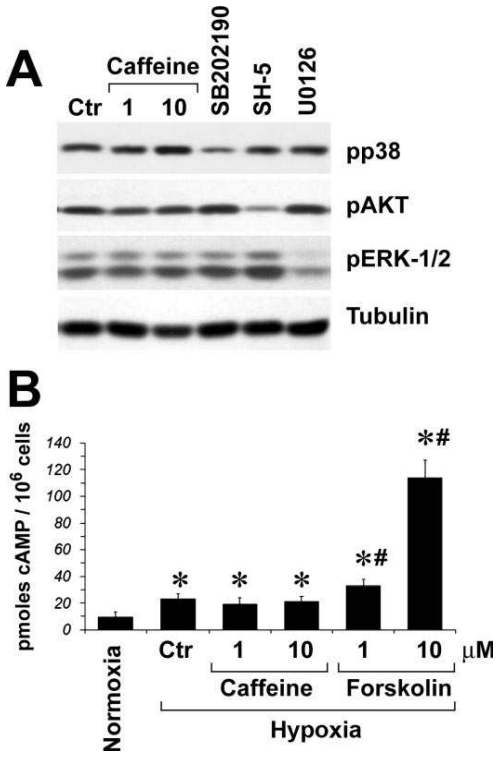

Fig. 3. Caffeine signaling in HT29 cells. A, pp38, pAkt, and pERK1/2 MAPK phosphoprotein levels under caffeine (1–10 µM) treatment in hypoxia (1% O_2, 4 h). The effect of SH5, an Akt inhibitor, SB202190, inhibitor of p38 MAPK, and U0126, inhibitor of MEK1/2, at the concentration of 10 µM is shown. Inhibitors were added to the cells 30 min before hypoxia. B, cAMP levels in normoxia and upon treatment of HT29 cells with caffeine (1–10 µM) or forskolin (1–10 µM), or no drug (Ctr) for 2 h in hypoxia. Results shown are mean ± S.E. values (n = 3); *, $P < 0.01$ compared with the control (normoxia); #, $P < 0.01$ compared with the control (untreated hypoxic cells).

Caffeine Inhibits Adenosine-Induced HIF-1α Protein Accumulation via Blocking of Akt, ERK1/2, and p38 MAPK Phosphorylation.

To determine whether Akt and MAPK pathways were required for HIF-1α protein increase induced by A3 receptor activation, HT29 cells were pretreated with SH5, SB202190, or U0126. The cells were then exposed to 100 nM Cl-IB-MECA for 4 h in

hypoxia. As shown in Fig. 4, SH5 (10 μM), SB202190 (10 μM), and U0126 (10 μM) were able to inhibit Cl-IB-MECA-induced increase of HIF-1α protein expression.

TABLE 2
Inhibition of [^{3}H]DPCPX, [^{3}H]ZM 241385, [3H]MRE 2029F20, and [3H]MRE 3008F20 binding by caffeine at A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors expressed in human HT29 cells, respectively.
Data are expressed as the mean ± S.E.M. *Ki* value represents the concentration of drug able to displace 50% of the radioligand.

	[^{3}H]DPCPX	[^{3}H]ZM2029F20	[^{3}H]MRE2029F20	[^{3}H]MRE3008F20
			μM	
Caffeine Ki	45 ±5	18 ±3	10 ±1	13 ± 2

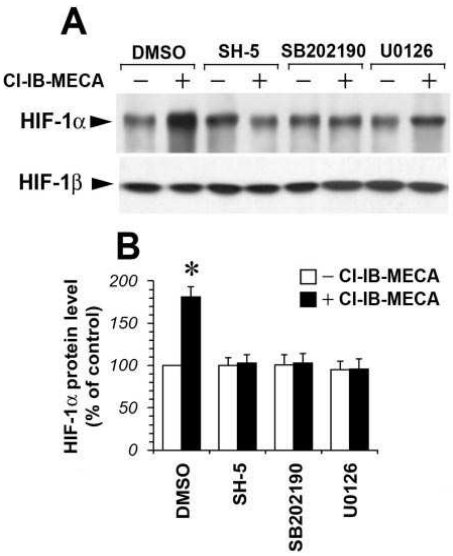

Fig. 4. Signaling pathway. A, HT29 cells were pretreated 30 min with or without SH5, an Akt inhibitor, SB202190, inhibitor of p38 MAPK, and U0126, inhibitor of MEK1/2, at the concentration of 10 μM and then exposed to the selective A_3 agonist Cl-IB-MECA 100 nM (+) for 4 h in hypoxia (1% O_2). The mean densitometry data from independent experiments (one of which is shown here) were normalized to the results obtained in hypoxic cells in the absence of Cl-IB-MECA (lane 1). Plots are mean ± S.E. values (n = 3); *, P < 0.01 compared with the control.

Caffeine Inhibits Adenosine-Induced VEGF Expression.

The effects of A_3 receptor stimulation through the agonist Cl-IB-MECA on secreted VEGF levels in HT29 colon cells were determined under hypoxic conditions. Cl-IBMECA (10 nM) increased VEGF levels after 48 h of hypoxia in HT29 cells (Fig. 5A). To determine the concentration of caffeine required to inhibit adenosine-regulated VEGF protein increase under hypoxia, HT29 cells were treated with caffeine. VEGF levels were analyzed after 48 h of hypoxia. Complete abrogation of VEGF accumulation induced by 10 nM Cl-IB-MECA was observed with 10 μM caffeine (Fig. 5A), at which HIF-1α accumulation induced by A_3 receptor stimulation was also inhibited (Fig. 1C). To define the adenosine receptor subtype involved, HT29 cells were treated with Cl- IB-MECA in combination with the A_{2B} antagonist MRE 2029F20 or with the A_3 receptor antagonist MRE 3008F20 (Table 1) (Varani et al., 2000). When used alone under hypoxic conditions, MRE 2029F20 and MRE 3008F20 had no effect

on VEGF protein levels analyzed after 48 h of hypoxia (Fig. 5A). Complete abrogation of VEGF accumulation induced by 10 nM Cl-IB-MECA was seen with MRE 3008F20 10 nM, whereas the antagonist MRE 2029F20 (10 nM) did not block the Cl-IB-MECA effect (Fig. 5A), pointing to a role for the A_3 receptor. To evaluate whether a different A_3 receptor antagonist with affinity also for A_{2B} receptors was able to modulate VEGF levels induced by Cl-IB-MECA, HT29 cells were treated with the A_{2B}-A_3 receptor antagonist B64 (compound 44 in Baraldi et al., 2002) (Table 1). When used alone under hypoxic conditions, the B64 compound had no effect on VEGF protein levels analyzed after 48 h of hypoxia (Fig. 5A). Complete abrogation of VEGF accumulation induced by 10 nM Cl-IB-MECA was seen at a concentration of 10 nM B64 adenosine receptor antagonist (Fig. 5A), indicating the involvement of the A_3 receptor.

To investigate whether the MAPK pathway was involved in the expression of A_3-induced VEGF protein, HT29 cells were cultured in hypoxia for 48 h after the addition of the MEK1/2 inhibitor U0126, the AKT inhibitor SH5, or the inhibitor of p38 MAPK, SB202190, 30 min before the treatment of 10 nM Cl-IB-MECA. U0126, SH5, and SB202190 (10 M) significantly inhibited the VEGF protein levels induced by 10 nM Cl-IB-MECA (Fig. 5A).

Caffeine Inhibits Adenosine-Induced IL-8 Expression.

Figure 5B shows that stimulation of adenosine receptors in HT29 cells with increasing concentrations of NECA (0.01–10 µM) for 24 h of hypoxia induces secretion of IL-8. The relatively low potency of NECA agrees with previous reports of A_{2B} receptor-mediated IL-8 production (Feoktistov et al., 2003). To better define the adenosine receptor subtype involved, HT29 cells were treated with 1 µM NECA in combination with the A_{2B} antagonist MRE 2029F20 or with the A_3 receptor antagonist MRE 3008F20 (Table 1) (Varani et al., 2000). When used alone under hypoxic conditions, MRE 2029F20 and MRE 3008F20 had no effect on IL-8 protein levels analyzed after 24 h of hypoxia (data not shown). Complete abrogation of IL-8 accumulation induced by 1 µM NECA was seen with 10 nM MRE 2029F20, whereas the antagonist MRE 3008F20 (10 nM) did not block the NECA effect (Fig. 5C), pointing to a role for the A_{2B} receptor. Furthermore, to evaluate whether a different A_{2B} receptor antagonist with affinity also for A_3 receptors was able to modulate IL-8 levels induced by NECA, HT29 cells were treated with the A_{2B}-A_3 receptor antagonist B64 (Table 1). Complete abrogation of IL-8 accumulation induced by 1 µM NECA was seen at a concentration of

10 nM B64 adenosine receptor antagonist (Fig. 5C), indicating the involvement of the A_{2B} receptor. Based on these results, we have chosen the incubation of 24 h in hypoxia with 1 µM NECA in further studies to analyze the effect of caffeine and the signaling pathways involved in adenosine-induced IL-8 production. Complete abrogation of IL-8 accumulation induced by 1 µM NECA was observed with 10 µM caffeine (Fig. 5C). Furthermore, we evaluated a potential role of Akt, ERK 1/2, and p38 MAP kinase in NECA-induced synthesis of IL-8. As shown in Fig. 5C, 10 µM SH5, 10 µM U0126, and 10 µM SB202190 completely blocked NECA-induced production of IL-8.

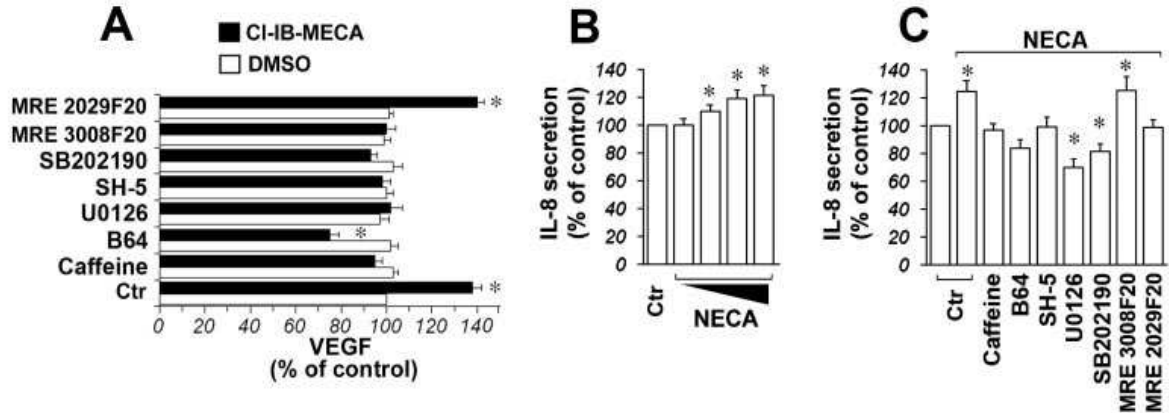

Fig. 5. Effect of adenosine receptor stimulation on VEGF and IL-8 expression in hypoxic (1% O_2) cells. A, VEGF release into culture media of HT29 cells cultured 48 h in the absence and in the presence of the selective A_3 agonist Cl-IB-MECA (10 nM), caffeine (10 µM), the A_{2B}-A_3 antagonist B64 (10 nM), U0126 (10 µM), SH5 (10 µM), SB 202190 (10 µM), the selective A_{2B} antagonist MRE 2029F20 (10 nM), and the selective A_3 antagonist MRE3008F20 (10 nM); the inhibitors were added 30 min before Cl-IB-MECA, and then the cells were exposed to hypoxia. Plots are mean ± S.E. values (n =3); *, $P < 0.01$ compared with the control (untreated hypoxic cells). B, effect of the adenosine receptor agonist NECA (0.01, 0.1, 1, and 10 µM) on IL-8 expression in hypoxic HT29 cells cultured 24 h. C, effect of 1 µM NECA on IL-8 expression in hypoxic HT29 cells cultured 24 h in the absence andin the presence of 10 µM caffeine, 10 nM B64, 10 µM SH5, 10 µM U0126, 10 µM SB 202190, 10 nM MRE 2029F29, and 10 nM MRE 3008F20. Plots are mean ± S.E. values (n = 3); *, $P < 0.01$ compared with the control (untreated hypoxic cells).

A_3 Receptors Modulate VEGF Promoter Activity.

HIF-1 is a transcription factor that mediates the effects of hypoxia on VEGF expression by binding to the hypoxia response element of the VEGF promoter. To examine whether adenosine interacts with the HIF-1 pathway to upregulate VEGF transcription, we used two luciferase reporters described previously. The p11w reporter is regulated by a fragment of the VEGF promoter that includes an HIF-1-binding site. The p11m reporter is identical except for a 3-base pair mutation that prevents HIF-1 binding (Forsythe et al., 1996). We transfected HT29 colon cells with these reporters and treated

the cells with adenosine for different times in hypoxia. As shown in Fig. 6A, hypoxia increased luciferase activity of the p11w reporter in HT29 cells in a time-dependent manner. The maximum increase in p11w reporter activity is present at 72 h of hypoxia. Hypoxia also stimulated activity of the p11m reporter but to a minor extent (Fig. 6A). Incubation of the cells for 48 h under hypoxic conditions with adenosine resulted in a dose-dependent increase in p11w reporter activity. As shown in Fig. 6B, increasing concentrations of adenosine (1–100 μM) up-regulated the p11w reporter up to 41% with respect to untreated hypoxic HT29 cells. In particular, the increase induced by 10 μM adenosine at 48 h of hypoxia is blocked by 1 to 10 μM caffeine (Fig. 6B).

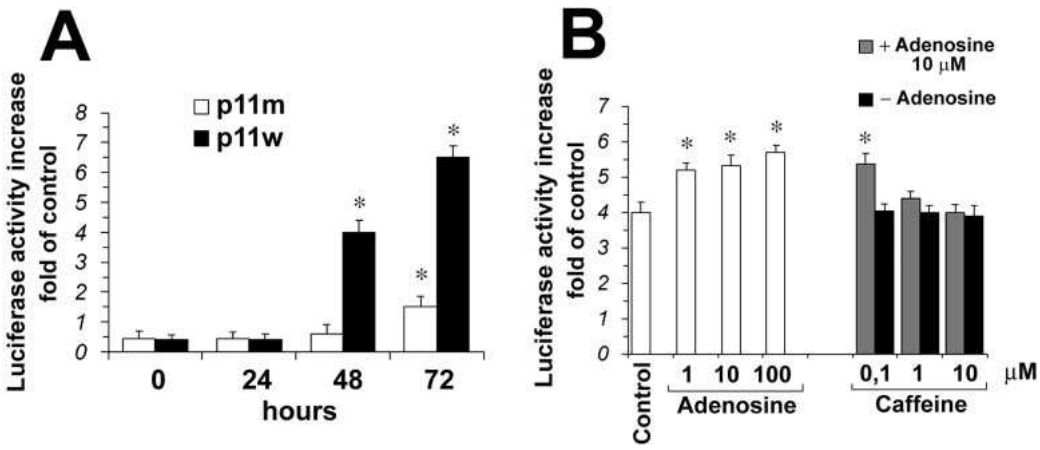

Fig. 6. Effect of hypoxia (1% O_2) and adenosine on HIF-1-dependent VEGF reporter activity. HT29 cells were transfected with plasmids encoding luciferase reporters driven by the VEGF promoter region containing a native HIF-1-binding element (p11w) or a mutated hypoxia response element unable to bind HIF-1 (p11m). A, transfected cells were incubated under hypoxia for 24, 48, and 72 h. *, $P < 0.01$ compared with the control (time 0 from the transfection). B, HT29 cells were transfected with p11w for 48 h under hypoxia with adenosine (1–100 μM). The effect of 10 μM adenosine in combination with caffeine (0.1–10 μM) is shown. Plots are mean ± S.E. values ($n = 3$); *, $P < 0.01$ compared with the control (48 h from the transfection with p11w in the absence of adenosine).

A_{2B} and A_3 Receptor Gene Silencing.

To demonstrate more conclusively a role for A_{2B} or A_3 receptors in the responses being measured, we tried to knock down A_{2B} and A_3 receptor expression in hypoxic HT29 colon cells using siRNA, leading to a transient knockdown of the A_{2B} and A_3 receptor gene. HT29 cells were transfected with nonspecific random control ribonucleotides or with small interfering RNAs that target A_{2B} ($siRNA_{A2B}$) or A_3 receptor mRNA ($siRNA_{A3}$) for degradation. After transfection, the cells were cultured for 48 h in complete media, and then total proteins were isolated for Western blot analysis of A_{2B} and A_3 receptor protein. As expected, A_{2B} and A_3 receptor protein expression were

strongly reduced in siRNA$_{A2B}$- and siRNA$_{A3}$-treated cells, respectively (Fig. 7A). To confirm the specificity of the siRNA$_{A3}$-mediated silencing of A_3 receptor, we investigated the expression of A_{2B} receptor protein in siRNA$_{A3}$-treated cells. (Fig. 7A). Figure 7A demonstrates that treatment of HT29 cells with siRNA$_{A3}$ reduced the expression of A_3 protein but had no effect on the expression of A_{2B} receptor. Similar results were obtained when HT29 cells transfected with siRNA$_{A2B}$ were analyzed for the expression of the A_3 receptor (Fig. 7A). Therefore, at 48 h from the siRNA$_{A3}$ transfection, HT29 cells were exposed to increasing concentrations of the A_3 adenosine receptor agonist Cl-IB-MECA (10–100 nM) for 4 h in hypoxia. We found that the inhibition of A_3 receptor expression is sufficient to block Cl-IB-MECA-induced HIF-1α accumulation (Fig. 7B). Furthermore, HT29 cells were transfected with siRNA$_{A3}$ and exposed to 10 nM Cl-IB-MECA to evaluate VEGF levels after 48 h of hypoxia. Complete abrogation of VEGF accumulation induced by Cl-IB-MECA 10 nM was observed when the A_3 receptor was knocked down in colon cells (Fig. 7C). Likewise, to confirm the role of A_{2B} receptors in the regulation of IL-8 expression, HT29 cells transfected with siRNA$_{A2B}$ were treated with 1 μM NECA and IL-8 protein levels were measured after 24 h of hypoxia. We found that the inhibition of A_{2B} receptor expression is sufficient to block NECA-induced IL-8 accumulation.

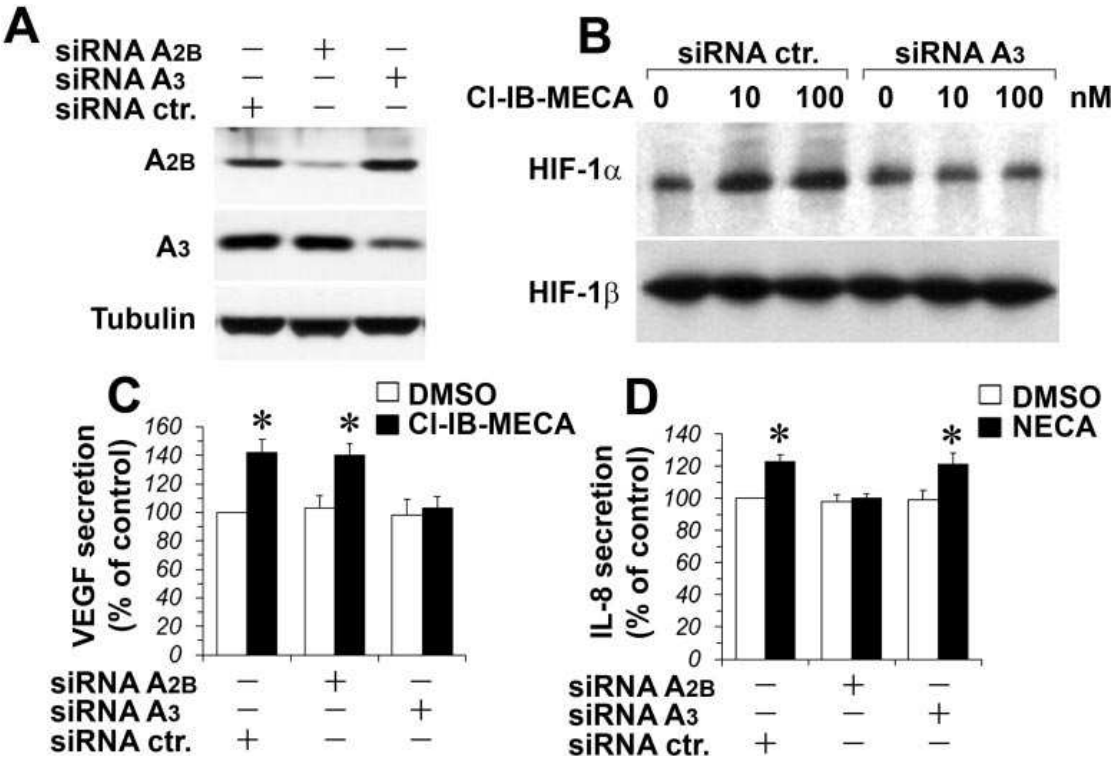

Fig. 7. A_{2B} and A_3 receptor expression silencing by siRNA transfection. A, Western blot analysis using an anti-A_{2B} and an anti-A_3 receptor polyclonal antibody of protein extracts from HT29 cells transfected with control (ctr)

ribonucleotides or with siRNA$_{A2B}$ or siRNA$_{A3}$ and cultured for 48 h. Tubulin shows equal protein loading. B, Western blot analysis using an anti-HIF-1α monoclonal antibody of protein extracts from HT29 cells transfected with control ribonucleotides or siRNA$_{A3}$ for 48 h and cultured with the selective A_3 agonist Cl-IB-MECA (0–100 nM) for 4 h in hypoxia (1% O_2). HIF-1β shows equal protein loading. C, VEGF release into culture media of HT29 cells transfected with control (ctr) ribonucleotides or with siRNAA_{2B} or siRNA$_{A3}$ and cultured 48 h in hypoxia (1% O_2) in the absence and in the presence of 10 nM Cl-IB-MECA. Plots are mean ± S.E. values (n = 3); *, $P < 0.01$ compared with the control (DMSO-treated siRNA-ctr transfected hypoxic cells). D, IL-8 release into culture media of HT29 cells transfected with control (ctr) ribonucleotides or with siRNA$_{A2B}$ or siRNA$_{A3}$ and cultured for 24 h in hypoxia (1% O_2) in the absence and in the presence of the adenosine receptor agonist NECA (1 μM). Plots are mean ± S.E. values (n = 3); *, $P < 0.01$ compared with the control (DMSO-treated siRNA-ctr transfected hypoxic cells).

Effect of Caffeine on Cell Migration of HT29 Cells.

Recent studies have shown the possible role of HIF-1 in the regulation of colon carcinoma cell invasion (Krishnamachary et al., 2003). To investigate whether caffeine can inhibit cancer cell migration, an in vitro cell migration assay was done. We examined whether hypoxic condition enhances cell migration of HT29 cells and whether caffeine can suppress tumor migration. Our results show that exposure to hypoxia for 6 to 24 h in the presence of Cl-IB-MECA 100 nM significantly stimulated migration of HT29 cells under serum-free conditions (Fig. 8A). The stimulatory effect of Cl-IB-MECA induced migration of HT29 cells was completely abrogated by pretreatment with 10 μM caffeine. These results indicated that caffeine suppressed the Cl-IB-MECA-stimulated migration of HT29 cells.

The Conditioned Medium of Colon Cancer Cells and the Migration of HUVECs.

To determine the functional importance of Cl-IB-MECA-induced increases in VEGF expression, we evaluated the effects of conditioned medium from Cl-IB-MECA-treated colon cells on the migration of HUVECs. Conditioned medium was obtained from the supernatants of colon cells treated with or without 100 nM Cl-IBMECA for 48 h in hypoxia. We prepared three batches of conditioned media for three independent HUVEC migration experiments. HUVECs were incubated for 6 h with endothelial basal medium or conditioned medium. The conditioned medium from Cl-IB-MECA-treated HT29 colon cells significantly enhanced HUVEC migration compared with the control conditioned medium from untreated colon cells (Fig. 8B). This effect was completely abrogated when conditioned medium from Cl-IB-MECA-stimulated colon cells was preincubated with anti-VEGF neutralizing antibodies, whereas 1 μg/ml nonspecific goat IgG failed to block the conditioned medium effect (Fig. 8B).

In contrast to its effects on migration, Cl-IB-MECA did not significantly modulate the compared with the untreated cells (data not shown). Likewise, the conditioned medium from Cl-IB-MECA-treated colon cells did not modulate the proliferation of HUVECs. Finally, we have shown that a commercial VEGF preparation enhances HUVEC migration, but this effect was abrogated when HUVECs were preincubated with the anti-VEGF neutralizing antibodies (Fig. 8C), whereas 1 µg/ml nonspecific goat IgG failed to lock the VEGF effect.

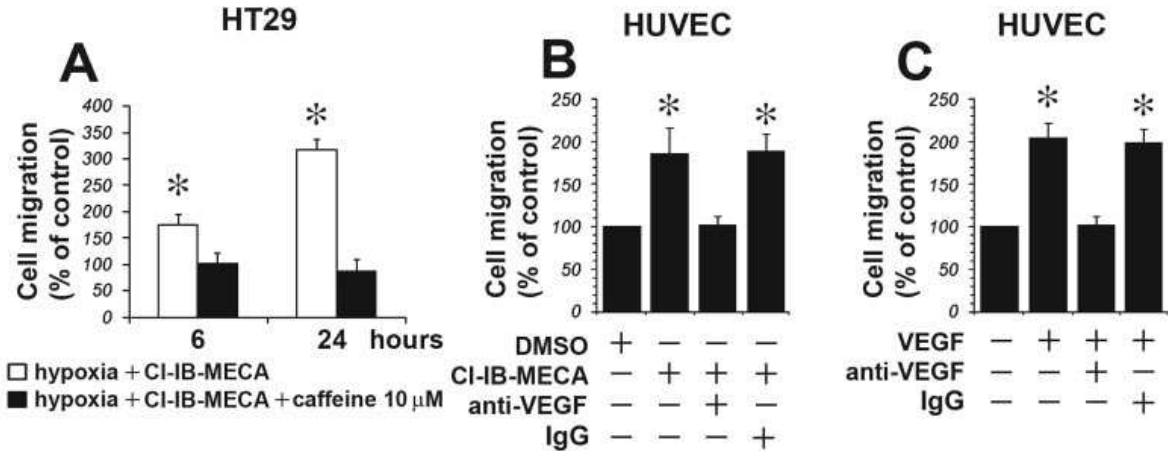

Fig. 8. Cell migration of HT29 and HUVECs. A, cell migration of HT29 cells. The cells were cultured for 6 and 24 h at 37°C under hypoxia in the presence of the selective A_3 agonist Cl-IB-MECA (100 nM). The effect of 10 M caffeine is shown. Plots are mean ± S.E. values (n = 3); *, P < 0.01 compared with the control (hypoxic untreated cells). B, cell migration of HUVECs incubated for 6 h without (DMSO) or with conditioned medium from Cl-IB-MECA-treated hypoxic HT29 cells (Cl-IB-MECA). The effect of treatment of conditioned medium from Cl-IB-MECA-treated HT29 cells with 1µg/ml anti-VEGF antibodies (anti-VEGF) is shown. Anti-human VEGF was developed in goat using recombinant human VEGF165 as immunogen. In this assay, 1 µg/ml anti-VEGF antibodies was incubated with conditioned medium from Cl-IB-MECA-treated HT29 cells for 1 h at 22°C. After the preincubation, HUVECs were added to the antigen-antibody mixture. The assay mixture was incubated at 37°C for 6 h. As negative control, 1 µg/ml nonspecific goat IgG (IgG) was used. The plots are mean ± S.E. values (n = 3); *, P < 0.01 compared with the control (DMSO-treated HT29 cells). C,cell migration of HUVECs incubated for 6 h without (+) or with VEGF 1 µg/ml. The effect of 1 µg/ml anti-VEGF antibodies (anti-VEGF) is shown. Plots are mean ± S.E. values (n = 3); *, P < 0.01 compared with the control (untreated cells).

DISCUSSION

Because substantial amounts of caffeine are ingested by people drinking coffee, tea, or caffeinated soft drinks, an understanding of the biological effects of caffeine is of considerable importance. The concentrations of caffeine used in this study (10 μM) may seem unphysiologically high. In fact, we want to emphasize that even higher concentrations are reached in coffee drinkers (Ekbom, 1999). To our knowledge, this is the first report examining the in vitro effect of caffeine on hypoxic cancer cells. Taken together, our data suggest three potential chemopreventive targets for caffeine: 1) HIF; 2) VEGF and IL-8; and 3) cell migration. In the current study, we have demonstrated that caffeine inhibits the up-regulation of HIF-1α, VEGF, and IL-8 expression induced by the adenosine receptor agonist Cl-IB-MECA in human colon cancer cells exposed to severe hypoxia. In particular, we have shown that HIF-1α and VEGF are increased through A_3 adenosine receptor stimulation, whereas the effects on IL-8 are mediated via the A_{2B} subtype. We have demonstrated previously that, in hypoxic glioblastoma cells, adenosine is able to increase the production of the proangiogenic factor VEGF (Merighi et al., 2006) through the A_3 receptor subtype. Furthermore, our results indicate that, in tumor colon hypoxic cells, adenosine increases VEGF promoter activity via the HIF-1 pathway and that caffeine is able to block this effect. It has been reported, in previous studies, that A_{2B} receptors stimulate IL-8 production in normoxic conditions (Zeng et al., 2003). In this study, we found that also in hypoxia, there is a modulation in IL-8 levels mediated by the adenosine receptor agonist NECA. These effects may seem rather modest and were examined only during concomitant hypoxia. However, the aim of this work was to study the effects of caffeine on HIF-1 protein accumulation and on VEGF and IL-8 expression in the human colon cancer cell line HT29 under hypoxic conditions. The signaling pathways involved are Akt, MEK, and p38 MAPK, having a key role in A_3 receptor ability to enhance HIF-1α and VEGF protein expression. Moreover, we have shown that Akt, ERK1/2, and p38 MAPK activities were required for the IL-8 expression increase induced by A_{2B} receptor activation. Although caffeine did not interact with signaling molecules downstream of adenosine receptor activation, such as Akt, mitogen-activated protein kinases, p38, or cAMP, we have demonstrated that it interferes with adenosine receptor binding as an antagonist with micromolar affinity. As a consequence, we suggest that caffeine may serve as an antagonist of adenosine receptor activities in hypoxic cells as a means to retard tumorigenesis in vivo.

In particular, it will be of interest to study paraxanthine in future studies. Paraxanthine is the main metabolite of caffeine in humans, and, at least in some receptor subtypes, it is as potent as the parent compound. As a consequence, when discussing the plasma concentrations of caffeine achieved clinically, one underestimates the amount of adenosine receptor antagonism, because plasma concentrations of paraxanthine can be just as high (Biaggioni et al., 1991). It has been shown that HIF-1α overexpression, either as a result of intratumoral hypoxia or genetic alterations, activates the transcription of genes, the protein products of which contribute to the basement membrane invasion of colon cancer cells. In the present study, we have shown that caffeine inhibited the stimulatory effects of the adenosine receptor agonist Cl-IB-MECA on the migration ability of hypoxic tumor colon cancer cells (Fig. 8), which could be attributed to its potent inhibitory effects on Cl-IB-MECA induced HIF-1α protein accumulation and VEGF expression. Even if these are only "in vitro" results that are in accordance with the in vitro observation that caffeine inhibits tumor cell motility (Lentini, 1998), they may be indicative of increased tumor migration in vivo. However, caffeine was not able even to prevent the effects produced by hypoxia alone. This implies that, under the conditions of the assays, not enough endogenous adenosine was generated to mediate the effects of hypoxia on markers of tumor growth. In our in vitro cell model, the effects demonstrated for caffeine are those related to adenosine receptor antagonism. Furthermore, to determine the functional importance of adenosine-induced increases in VEGF expression, we evaluated the effects of conditioned medium from Cl-IB-MECA treated colon cells on the migration of HUVECs. Our data indicate that the increased VEGF expression produced by Cl-IB-MECA-treated colon cancer cells stimulates migration of vascular endothelial cells. The finding that the Cl-IB-MECA-stimulated increase in VEGF was blocked by caffeine indicates that strategies aimed at blocking adenosine receptors will not only affect colon cell migration but also will affect surrounding vasculature dependent on tumor-derived VEGF. Although it is well-known that hypoxia stimulates VEGF levels, hypoxia coordinately stimulates IL-8 in tumor cells (Desbaillets et al., 1997), and in tumor xenografts, hypoxic areas of tumors coexpressed VEGF and IL-8. Targeting HIF-1α is an attractive strategy, with the potential for disrupting multiple pathways crucial for tumor growth. However, recent findings have investigated whether the inhibition of HIF-1 alone is sufficient to block tumor angiogenesis (Mizukami et al., 2005). In particular, it has been demonstrated that HIF-1α deficiency in cancer cells can inhibit proliferation and overall growth but not

angiogenesis. The new finding of these studies is that compensatory pathways can be activated to preserve the tumor angiogenic response. In particular, it has been demonstrated that in the absence of HIF-1, the proangiogenic cytokine IL-8 is induced in a compensatory manner to maintain tumor vascularity. The absence of HIF-1 can therefore stimulate IL-8 on a transcriptional level, and this is further enhanced in hypoxia. Our results provide evidence that an additional role of adenosine in colon tumor progression may be the enhancement of angiogenesis via up-regulation not only of VEGF, A_3-HIF-1- mediated, but also of IL-8, A_{2B}-mediated. It has been suggested that strategies that inhibit HIF-1α may be most effective when IL-8 is simultaneously targeted. Therefore, we suggest that an A_{2B}-A_3 receptor antagonist may be regarded as a target for the development of a new antitumor drug through its ability to inhibit HIF-1α, VEGF, and IL-8 in the context of tumor hypoxia, a common feature of most invasive cancers.

Although our studies have been performed using tumor cell lines, our finding that caffeine is able to prevent HIF-1α, VEGF, and IL-8 accumulation induced by adenosine receptor activation provides proof-of-principle that the application of small molecules such as caffeine might be used in chemotherapy to reduce morbidity and mortality associated with neoplastic disease. This possibility was especially compelling because high caffeine intake has been associated with decreased cancer mortality in human populations (Michels et al., 2005; Baker et al., 2006). In this context, further studies are needed to better investigate possible antitumor effects of caffeine and to clarify the involvement of adenosine in the development of tumors.

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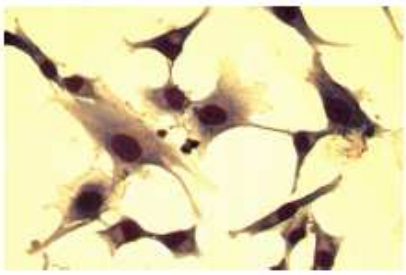
CHAPTER 5



A_3 ADENOSINE RECEPTOR AND MELANOMA

Malignant melanoma is a serious form of skin cancer. Unfortunately, the incidence of this disease appears to be increasing, such that currently about 1 in 100 persons in the United States can expect to develop this cancer in a lifetime (Ariza et al., 1999). Patients with metastases in the liver, brain or bone have a median survival of 3-4 months, whereas those with metastases to the skin, subcutaneous tissue, distant lymph nodes or the lungs have a better survival ranging from 12 to 15 months. For disseminated uveal melanoma the median survival is reported to be about 4 months (Mooy, 1996); notably, patients with no hepatic involvement (however, these are the minority) have a far better prognosis with a median survival of more than 1 year. Additional prognostic factors have been revealed by multivariate analysis (Eton, 1998).

It is well known that ultraviolet radiation is associated with cutaneous malignant melanoma (Landi et al., 2002) and excessive exposure to ultraviolet among Caucasians is the main etiologic factor implicated in the incidence of melanoma (MacKie, 1998). In view of these hallmarks, recent advances in the understanding of extracellular adenosine-mediated transmembrane signaling through adenosine receptors together with the availability of molecular tools to study adenosine receptors will allow more detailed and precise evaluation of the effects of extracellular adenosine under well controlled conditions and in the pathogenesis of tumors of the skin, such as basal cell carcinoma, squamous cell carcinoma, and melanoma. Our group performed a lot of works in these years focusing on the role of the adenosine receptors in the melanoma cell line A375.

In a first work it has been characterized the expression pattern of the adenosine receptors on the surface of human melanoma A375 cells and documented the ability of these receptors to signal after selective agonists binding (Merighi et al., 2001). A375 cells are an established human melanoma cell line that belongs to a relatively

undifferentiated and highly metastatic class of melanocytic cells with epithelioid shape and absence of pigmentation (Okazawa et al., 1998). Our group firstly analysed the expression of the human adenosine receptors by checking the production of the RNA of all the adenosine receptors known. By RT±PCR it has been detected the expression of the transcript of A_1, A_{2A}, A_{2B} and A_3 receptors. Furthermore, there have been found strong differences on RNA expression being A_1 and A_3 mRNAs less expressed than A_{2A} and A_{2B} mRNAs.

Then, it has been characterized A_1, A_{2A} and A_3 adenosine receptors on A375 membranes by using the selective radioligands [^{3}H]-DPCPX, [^{3}H]-SCH 58261 and [^{3}H]- MRE 3008F20. To investigate the kinetic behaviour, the binding was carried out under pseudo-first order conditions. Analysis of association and dissociation kinetic parameters produced equilibrium constants for A_1, A_{2A} and A_3 which are in good agreement with those obtained from the equilibrium saturation binding assays. In saturation experiments the antagonist [^{3}H]-DPCPX labelled a single saturable binding site with a good affinity but with a low receptor density (Bmax= 23±7 fmol mg^{-1} of protein) while the radiolabelled [^{3}H]-SCH 58261 identified a large number (Bmax= 220±7 fmol mg^{-1} of protein) of high-affinity binding sites in A375 membranes. At the same time, [^{3}H]-MRE 3008F20 labelled a single class of recognition sites with binding capacity (Bmax) of 291±50 fmol mg^{-1} protein. Interestingly, despite the ability to transcribe A_{2A} and A_3 adenosine receptor genes at different levels, A375 cells present on the membrane surface similar amounts of receptor protein. Thus, our group performed a more detailed pharmacological and biochemical characterization for A_1, A_{2A} and A_3 receptor subtypes with the aim of increasing the evidences in support of the conclusion that the [^{3}H]-DPCPX, [^{3}H]-SCH58261 and [^{3}H]-MRE 3008F20 binding sites in melanoma membranes are A_1, A_{2A} and A_3 receptors. This goal comes from the results of competition binding assays and functional studies. The agonist and antagonist affinities for [^{3}H]-DPCPX, [^{3}H]-SCH58261 and [^{3}H]-MRE 3008F20 binding sites are consistent with the identification of these sites as A_1, A_{2A} and A_3 (Gessi et al., 2000; Varani et al., 2000a). Thermodynamic studies were performed and the enthalpic ($\Delta H°$) and entropic ($\Delta S°$) contributions to the standard free energy ($\Delta G°$) of the binding equilibrium were determined. The linearity of the van't Hoff plot indicates that $\Delta Cp°$ values for the drug interaction are nearly zero, which means that $\Delta H°$ and $\Delta S°$ values are not significantly affected by temperature variations at least over the temperature range investigated (Borea et al., 1996). The linearity of van't Hoff plots in a limited

range of temperatures (4 ± 25°C) appears to be a common feature of practically all membrane receptor ligands so far studied from a thermodynamic point of view (Gilli et al., 1994). [^{3}H]-DPCPX, [^{3}H]-SCH 58261 and [^{3}H]-MRE 3008F20 binding to human A_1, A_{2A} and A_3 receptors is enthalpy- and entropy-driven. All these data indicate, for the first time, that adenosine receptors present on A375 melanoma cell line have a pharmacological and biochemical profile typical of the A_1, A_{2A}, A_{2B} and A_3 receptor subtype.

Because it has been reported that the activity of Akt or MAPK or both is elevated in many cancer cells and is known to play critically important roles in cellular proliferation, our group demonstrated in a second work focused on melanoma cells the regulation of the signaling pathways mediated by ERK1/2 and/or Akt by the stimulation of the A_3 receptor (Merighi et al., 2004). In order to investigate the functionality of A_3 receptors expressed in melanoma cells, it has been used the selective adenosine analogue Cl-IB-MECA.

It has been demonstrated that serum-deprived A375 melanoma cells had no basal Akt phosphorylation, whereas Cl-IB-MECA treatment resulted in the phosphorylation of Akt at the Ser^{473} phosphorylation site. Furthermore, Akt phosphorylation matched the phosphorylation of Raf at an inhibitory site (Ser^{259}). Serum-deprived A375 cells showed high basal levels of ERK1/2 phosphorylation. The high levels of ERK1/2 phosphorylation in unstimulated A375 cells may reflect a neurospecific characteristic, since ERK1/2 is not usually phosphorylated after long periods of serum deprivation in cells of muscular and adipose origin (Begum et al., 2000; Ruiz-Hidalgo et al., 2002). Interestingly, Cl-IB-MECA stimulation resulted in a time- and dose-dependent reduction in ERK1/2 phosphorylation. It is suggested that this mechanism may be peculiar for melanoma cells, having a misregulation of proliferative pathways, since A_3 receptors increased ERK1/2 phosphorylation in CHO-A_3 cells in a dose-dependent manner (Schulte et al., 2000) and induced a biphasic effect on the phosphorylation levels of ERK1/2 on microglia cells (Hammarberg et al., 2003). It has been shown that the Raf-MEK-ERK pathway can be inhibited by Akt in differentiated myotubes but not in their undifferentiated myoblast precursors. The authors suggested that regulation of a Raf/Akt interaction, underlying the ERK1/2 inhibition, might be mediated by stage-specific modification of these proteins or by stage-specific accessory proteins. This regulation might be intact in cells of neuronal origin, too. To this end, our group examined whether any cross-talk exists between ERK1/2 and Akt pathways in A375

melanoma cells. The classical MAPK cascade leads from the Ras kinases to the MAPK kinase MEK1/2. There is evidence that Akt is able to phosphorylate Raf, thereby efficiently abrogating Raf activity on downstream substrates (Guan et al., 2000; Reusch et al., 2001; Zimmermann et al., 1999). We studied the effects of A_3 receptor stimulation on the proliferation of melanoma cells in the presence of specific inhibitors of the PI3K and Akt signal transduction pathways. We could effectively block the Cl-IBMECA- induced reduction of ERK1/2 phosphorylation with an inhibitor of PI3K. Indeed, application of Cl-IB-MECA in combination with PI3K inhibition resulted in a clear increase of ERK1/2 phosphorylation when compared with P-ERK1/2 in the presence of Cl-IB-MECA alone. These data suggest that the Ras-Raf-MEK-ERK pathway is normally activated by A_3 receptor stimulation, as is the PI3K-Akt route. It is clear that these apparently separate routes should actually interact.

Pretreatment of A375 cells with a PI3K inhibitor and an Akt inhibitor impaired Cl-IB-MECA-induced inhibition of cell proliferation and the effects of A_3 receptor stimulation on Raf, MEK1/2, and ERK1/2 phosphorylation. These data suggest that the A_3 adenosine receptor signals through a pathway including PI3K-Akt. On the contrary, Ras was not activated, at least when measured with the pull-down assay. These results confirm the hypothesis of this study; in A375 cells, A_3 receptors decrease MEK1/2-ERK1/2 phosphorylation and cell proliferation via the inhibition of Raf by a PI3KAkt pathway without affecting Ras.

In a third work it has been demonstrated that A_3 receptor subtype mediates the adenosine effects on HIF-1α regulation in A375 melanoma cell line. The effects of adenosine on HIF-1α protein accumulation are not mediated by A_1, A_{2A}, or A_{2B} receptors. In support of this conclusion, DPCPX, SCH 58261, and MRE 2029F20, adenosine receptor antagonists for A_1, A_{2A}, and A_{2B} receptors, respectively, did not block the stimulatory effect of adenosine on HIF-1α protein increase. The conclusion that the effects of adenosine on HIF-1α accumulation are mediated through A_3 receptors is supported by the observation that the stimulatory effects of this nucleoside on HIF-1α protein are mimicked by the A_3 receptor agonist, Cl-IB-MECA, and inhibited by A_3 receptor antagonists. In particular, the potencies of these drugs are in agreement with their inhibitory equilibrium binding constant (Ki) observed in binding experiments for the adenosine A_3 receptor (Merighi et al., 2001) Furthermore, the inhibition of A_3 receptor expression at the mRNA and protein levels is sufficient to block A_3 receptor–induced HIF-1α protein accumulation. Therefore, our results indicate that the cell

surface A_3 adenosine receptor transduces extracellular hypoxic signals into the cell interior.

In the present study p44/p42 and p38 MAPKs are necessary to increase HIF-1α levels, and these kinases are included in the molecular signaling pathways generated by A_3 receptor stimulation. Based on these data, adenosine, through A_3 receptors, is able to increase the levels of HIF-1α through p44/p42 and p38 MAPK pathways. In the previous study on A375 melanoma cells, A_3 adenosine receptor stimulation decreased MAPK activity through the inhibition of Raf by a PI3K–Akt pathway (Merighi et al., 2005). However, the experimental conditions were quite different, being those experiments performed in normoxia and with high concentrations (micromolar) of the A_3 receptor agonist. Now, the concentration of the agonist is nanomolar and the cells are cultured in hypoxic conditions.

It is recognized that the inhibition of HIF-1 activity represents a novel therapeutic approach to cancer therapy, especially in combination with angiogenesis inhibitors, which would further increase intratumoral hypoxia and thus provide an even greater therapeutic window for use of an HIF inhibitor (Merighi et al., 2005; Giaccia et al., 2003). Recent studies indicate that pharmacologic inhibition of HIF-1α, and particularly of HIF-regulated genes important for cancer cell survival, may be more advantageous than HIF gene inactivation therapeutic approaches (Sitkovsky et al., 2004; Mabjeesh et al., 2003). In the previous work our group demonstrated the mechanisms of the antiproliferative action of A_3 receptor stimulation in A375 normoxic melanoma cells (Merighi et al., 2005). Now, given the ability of A_3 adenosine receptor antagonists to block HIF-1α and Ang-2 protein expression accumulation in hypoxia, these data may indicate a new approach for the treatment of cancer, based on the cooperation between hypoxic and adenosine signals, that ultimately may lead to the increase in HIF-1 mediated effects in cancer cells.

5.1

"A_{2B} and A_3 Adenosine Receptors Modulate Vascular Endothelial Growth Factor and Interleukin-8 Expression in Human Melanoma Cells Treated with Etoposide and Doxorubicin"

INTRODUCTION

The incidence and mortality of cutaneous melanoma are still on the rise [1]. Overall, melanoma accounts for 1% to 3% of all malignant tumors and is increasing in incidence by 6% to 7% each year. The prognosis of metastatic melanoma remains poor. Once the metastatic phase develops, it is almost always fatal [2]. Different therapeutic approaches for metastatic melanoma have been evaluated, including chemotherapy and biologic therapies, both as single treatments and in combination [3]. To date, however, none have had a significant impact on survival. Systemic chemotherapy is still considered the mainstay of treatment of stage IV melanoma and is used largely with palliative intent [3]. Numerous chemotherapeutic agents have shown some activity in the treatment of malignant melanoma with dacarbazine (DTIC) being the most widely used [4]. DTIC is a nonclassical alkylating agent, generally considered the most active agent for treating malignant melanoma [4]. However, response rates for single-agent DTIC are disappointing [5,6].

A major obstacle to a successful treatment of metastatic melanoma is its notorious resistance to chemotherapy [7]. Chemoresistance is widely explored in cancer research, and many mechanisms have been described by which a tumor can evade cell killing in a variety of malignancies [8]. However, the mechanisms of chemoresistance of malignant melanoma are not established.

The aggressive nature of human melanomas is related to several abnormalities in growth factors, cytokines, and their receptor expression. For example, metastatic melanoma cells constitutively secrete the cytokine interleukin-8 (IL-8), whereas nonmetastatic cells produce low to negligible levels of IL-8 [9–11]. In fact, IL-8, originally discovered as a chemotactic factor for leukocytes, may play an important role in the progression of human melanomas [10]. Serum levels of IL-8 are elevated in patients with malignant melanoma [12], and several studies have demonstrated that the expression levels of this interleukin correlate with disease progression in human melanomas in vivo [12–16]. In addition to IL-8, aggressive melanoma cells secrete vascular endothelial growth factor (VEGF), which promotes angiogenesis and metastasis of human cancerous cells [17]. Cytotoxic therapy, including radiotherapy, and other stress conditions such as hypoxia are known to induce IL-8 and VEGF release by tumor cells [18,19]. In particular, hypoxic induction of VEGF is mediated by the transcription factor hypoxia-inducible factor 1 (HIF-1), which plays a key role in regulating the adaptation of tumors to hypoxia [20]. HIF-1 is a heterodimer composed

of an inducibly expressed HIF-1α subunit and a constitutively expressed HIF-1β subunit. A growing body of evidence indicates that HIF-1 contributes to tumor progression and metastasis [20,21]. HIF-1 is a potent activator of angiogenesis and invasion through its up-regulation of target genes critical for these functions [20]. Therefore, because HIF-1α expression and activity seem central to tumor growth and progression, HIF-1 inhibition becomes an appropriate anticancer target [20].

Adenosine is a ubiquitous mediator implicated in numerous inflammatory processes [22]. Accumulating evidence suggests that adenosine-mediated pathways are involved in cutaneous inflammation and epithelial cell stress responses. Most adenosine effects are mediated by its interaction with four seven-transmembrane G protein–coupled receptor, namely, A_1, A_{2A}, A_{2B}, and A_3 [23]. Recently, it has been reported that epithelial cells release adenosine in response to various stimuli, including adenosine receptor agonists [24]. Moreover, we have demonstrated that, in addition to producing adenosine, melanoma cell lines also express functional adenosine receptors [25,26]. In particular, activation of A_{2B} receptor leads to the production and release of calcium, VEGF, and IL-8 [27–29], whereas A_3 receptor leads to the production and release of calcium, VEGF, and angiopoietin-2 [30–35]. Recently, it has been demonstrated that A_3 receptor induces a prosurvival signal in tumor cells [36]. Furthermore, A_3 receptor stimulation increases the levels of HIF-1α in hypoxic tumor cells [28,31,33]. Here, we investigate whether two chemotherapeutic drugs, etoposide (VP-16) and doxorubicin, modulate IL-8 and VEGF production in human melanoma A375 cells. In particular, because adenosine is able to modulate HIF-1, VEGF, and IL-8 in cancer cells, we analyze the influence of the adenosinergic signaling on the chemotherapeutic drug effects in human melanoma cells. We found, for the first time, that A2B receptor blockade can modulate IL-8 production, whereas blocking A_3 receptors, it is possible to further decrease VEGF reduction due to VP-16 and doxorubicin in melanoma cells.We thus conclude that adenosine receptor modulation may be useful with chemotherapeutic drugs for the treatment of malignant melanoma.

MATERIALS AND METHODS

Cell Lines, Reagents, and Antibodies

A375 human melanoma cells were obtained from American Tissue Culture Collection, LGC Standards s.r.l., Milano, Italy. Tissue culture media and growth supplements were

obtained from Cambrex (Bergamo, Italy). Anti–adenosine A_{2B} and anti–adenosine A_3 receptor polyclonal antibodies (pAbs) were from AlphaDiagnostic (DBA,Milano, Italy). Human anti–HIF-1α and human anti–HIF-1β monoclonal antibodies (mAbs) were obtained from Transduction Laboratories (Milano, Italy). U0126 (inhibitor of MEK-1 and MEK-2), SB 202190 (inhibitor of p38mitogen-activated protein kinase (MAPK)), human anti-ACTIVE MAPK and human anti–extracellular signal–regulated kinase 1/2 (ERK 1/2) pAbs were from Promega (Milano, Italy). SH-5 (inhibitor of Akt) was from Vinci-Biochem (Florence, Italy). [3H]-Thymidine was from Perkin-Elmer Life and Analytical Sciences (Milano, Italy). Anti– human phospho-p38, anti–human p38 MAP Kinase, anti–human phospho-Akt and anti–human Akt antibodies were from Cell Signaling Technology (Milano, Italy). 4-(2-[7-Amino-2-[furyl][1,2,4,] triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol was obtained from Tocris Cookson Ltd (Bristol, UK). N-Benzo[1,3]dioxol-5-yl- 2-[5-(2,6-dioxo-1, 3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)- 1-methyl-1H-pyrazol-3-yloxy]-acetamide (MRE 2029F20) and 5N-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2-(2-furyl)-pyrazolo- [4,3e]1,2,4-triazolo[1,5c] pyrimidine (MRE 3008F20) were synthesized by Prof. Pier Giovanni Baraldi (Department of Pharmaceutical Sciences, University of Ferrara, Italy) [37]. Adenosine A_{2B} and A_3 receptors and HIF-1α small interfering RNA (siRNA) were from Santa Cruz Biotechnology, D.B.A. ITALIA s.r.l., Milano, Italy. RNAiFect Transfection Kit was from Qiagen (Milano, Italy). Unless otherwise noted, all other chemicals were purchased from Sigma (Milano, Italy).

Cell Culture

A375 human melanoma cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) at 37°C in 5% CO2/95% air.

Establishment of Hypoxic Culture Condition

For hypoxic conditions, cells were placed for the indicated times in a modular incubator chamber and flushed with a gas mixture containing 1% O_2, 5% CO_2, and balance N_2 (MiniGalaxy; RSBiotech, Irvine, Scotland). Maintenance of the desired O_2 concentration (1%) was constantly monitored during incubation using a microprocessor-based oxygen controller. The cells gassed under hypoxic conditions can reach the 1% oxygen concentration in ~ 90 minutes [38].

Cytotoxic Treatment of Cancer Cells

Exponentially growing cells (70%-80% confluence) in complete medium were treated with different concentrations of cytotoxic drugs, followed by exposure to hypoxia (1% O2) for different indicated time intervals.

MTS Assay

The MTS assay was performed to determine cell viability and proliferation according to the manufacturer's protocol from the CellTiter 96 Aqueous One Solution Cell Proliferation Assay as previously described [32]. Briefly, 105 cells were plated in 24-multiwell plates; 500 μl of complete medium was added to each well with different concentrations of cytotoxic drugs. The cells were then incubated for 24 hours. At the end of the incubation period, MTS solution was added to each well. The optical density of each well was read on a spectrophotometer at 492 nm. For each experiment, four individual wells of each drug concentration were prepared. Each experiment was repeated three times.

[^{3}H]-Thymidine Incorporation: Cell Proliferation Test

Cells were seeded in fresh medium with 1 μCi/ml of [^{3}H]-thymidine in Dulbecco's modified Eagle medium containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). After 24 hours of labeling, cells were trypsinized, dispensed in four wells of a 96-well plate, and filtered through Whatman GF/C glass-fiber filters using a Micro-Mate 196 Cell Harvester (Perkin Elmer Life Sciences,Milano, Italy). The filter-bound radioactivity was counted on Top Count Microplate Scintillation Counter (efficiency, 57%) with Micro-Scint 20 (Perkin Elmer Life Sciences).

Flow Cytometry Analysis

A375 adherent cells were trypsinized, mixed with floating cells, washed with PBS, and permeabilized in 70% (vol/vol) ethanol-PBS solution at 4°C for at least 24 hours. The cells were washed with PBS, and the DNA was stained with a PBS solution, containing 20 μg/ml propidium iodide and 100 μg/ml RNAse, at room temperature for 30 minutes. Cells were analyzed with an EPICS XL flow cytometer (Beckman Coulter,Miami, FL), and the content of DNA was evaluated by the EXPO-32 program (Becton Dickinson

Italia Spa,Milano, Italy). Cell distribution among cell cycle phases and the percentage of apoptotic cells were evaluated as previously described [30]. Briefly, the cell cycle distribution is shown as the percentage of cells containing 2n (G0/G1 phases), 4n (G2 and M phases), and 4n > x > 2n DNA amount (S phase) judged by propidium iodide staining. The apoptotic population is the percentage of cells with DNA content lower than 2n.

Western Blot Analysis

Whole-cell lysates, prepared as described previously [32], were resolved on a 10% SDS gel and transferred onto the nitrocellulose membrane. Western blot analyses were performed as previously described [31] with anti–HIF-1α (1:250 dilution) and anti–HIF-1β antibodies (1:1000 dilution) in 5% nonfat dry milk in PBS/0.1% Tween-20 overnight at 4°C. Aliquots of total protein sample (50 μg) were analyzed using antibodies specific for phosphorylated (Thr183/Tyr185) or total p44/p42 MAPK (1:5000 dilution), phosphorylated (Thr180/Tyr182) or total p38 MAPK (1:1000 dilution), and for phosphorylated or total Akt (Ser473; 1:1000 dilution). The protein concentration was determined using BCA protein assay kit (Pierce, TEMA ricerca S.r.l., Bologna, Italy). Membranes were washed and incubated for 1 hour at room temperature with peroxidase-conjugated secondary antibodies against mouse and rabbit immunoglobulinG (1:2000 dilution). Specific reactions were revealed with the Enhanced Chemiluminescence Western Blotting Detection Reagent (Amersham, Corp, Arlington Heights, IL). The membranes were then stripped and reprobed with antitubulin antibodies (1:250) to ensure equal protein loading.

Densitometry Analysis

The intensity of each band in the immunoblot assay was quantified using molecular analyst/PC densitometry software (Bio-Rad, Milano, Italy). Mean densitometry data from independent experiments were normalized to results in cells in the control. Data were presented as the mean ± SE.

Treatment of Cells with siRNA

A375 cells were plated in six-well plates and grown to 50% to 70% confluence before transfection. Transfection of siRNAwas performed at a concentration of 100nMusing RNAiFect Transfection Kit. Cells were cultured in complete medium, and at 48 hours,

total proteins were isolated forWestern blot analysis of A_{2B}, A_3, and HIF-1α protein. A nonspecific random control siRNA was used under identical conditions [32].

Enzyme-Linked Immunosorbent Assay

The levels of VEGF and IL-8 protein secreted by the cells in the medium were determined by a VEGF and an IL-8 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, DBA, Milano, Italy). In brief, subconfluent cells were changed into fresh medium in the presence of solvent or various concentrations of drugs in hypoxia. The medium was collected, and VEGF and IL-8 protein concentrations were measured by ELISA according to the manufacturer's instructions. The results were normalized to the number of cells per plate. Data were presented as mean ± SD from three independent experiments.

Statistical Analysis

All values in the figures and text are expressed as mean ± SE of n observations (with $n \geq 3$). Data sets were examined by Student's t test or by the analysis of variance (ANOVA) and Dunnett test (when required). $P < .05$ was considered statistically significant.

RESULTS

Cytotoxic Activity of Chemotherapeutic Drugs

All the experiments were performed in hypoxic conditions at 1% O_2. An assessment of growth effects in A375 cells subjected to VP-16 and doxorubicin for 24 hours was performed by using the MTS assay that measured viable cell mass. As shown in Figure 1A, A375 melanoma cells were sensitive to the growth-inhibitory effects of both the DNA damaging agents tested. A375 cells were treated with VP-16 and doxorubicin (0.01-100 μM). The cell viability was reduced up to 40 ± 4% and 30 ± 4% for VP-16 and doxorubicin, respectively (control set at 100%, n = 4). A number of different mechanisms, such as 1) impaired DNA synthesis, 2) perturbations in cell cycle progression, and 3) induction of apoptosis, could contribute to the reduction in viable cell mass seen after treatment with cytotoxic agents. DNA synthesis was markedly inhibited in melanoma cells treated with increasing concentrations of chemotherapeutics (0.01-10 μM). This inhibition was dose-dependent as determined by tritiated thymidine incorporation after 24 hours of treatment (Figure 1B). The cytometry investigation showed a clear arrest in the G2/M and S cell cycle phases ofmelanoma cells treated with VP-16, 1 and 10 μM, respectively, to control cells. In particular, low concentration of VP-16 arrested cells in the G2/M phase, whereas higher doses of chemotherapeutic drug were needed to accumulate cell cultures in the S phase (Figure 1C). Similar results were obtained when melanoma cells were treated for 24 hours with doxorubicin 1 to 10 mM (Figure 1C). Furthermore, to evaluate the induction of apoptosis by chemotherapeutic drugs, A375 cells were incubated with or without VP-16 and doxorubicin (1-10 μM) for 24 hours. As shown in Figure 1D, both the chemotherapeutic drugs tested were able to significantly induce apoptosis.

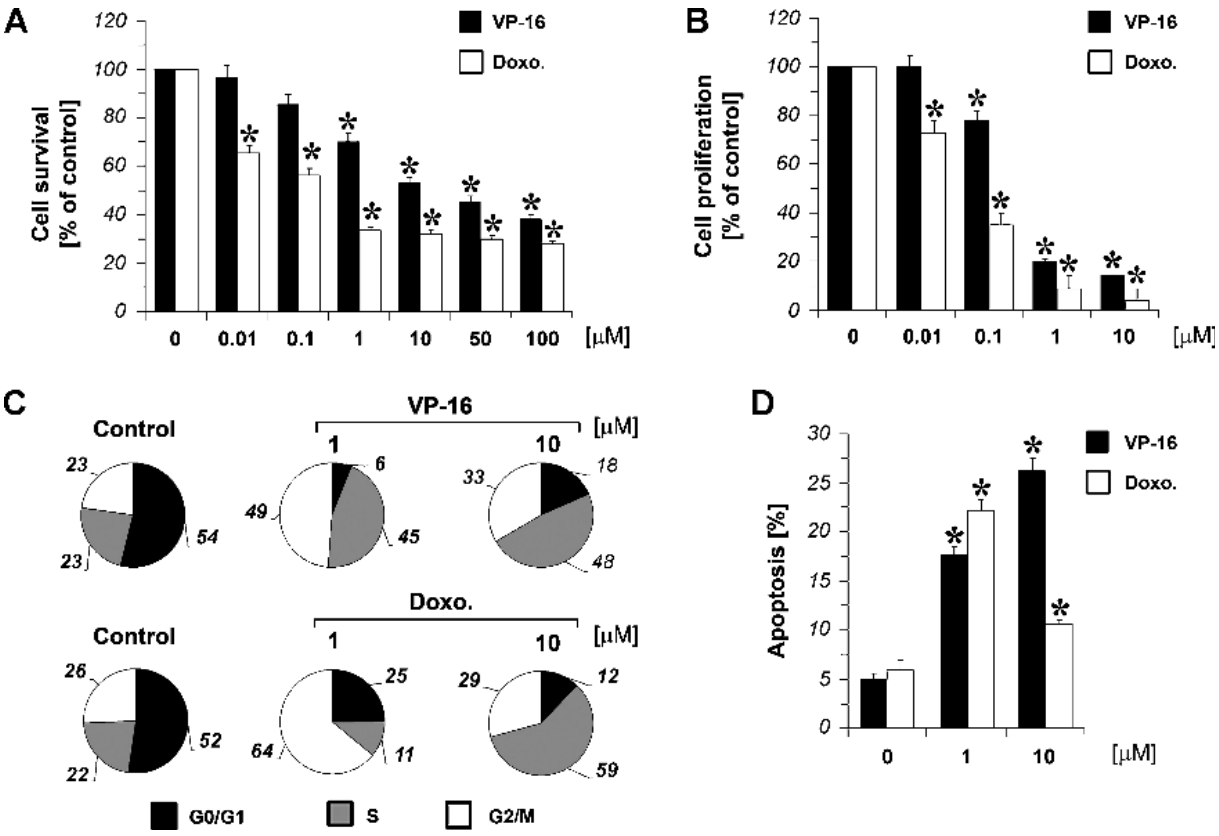


Figure 1. Effect of chemotherapeutic drugs. (A) A375 cellswere treated with increasing concentrations (0.01-100 μM) of VP-16 or doxorubicin (Doxo.) for 24 hours under hypoxic conditions, and cell viability was assayed by an MTS test. MTS: the cell growth is expressed as a percentage of the optical density measured on untreated cells (control, 100%). Ordinate reports means of four different optical density quantifications with SE (vertical bar). During the experiment, cells treated with the solvent DMSO served as controls. (B) Antiproliferative activity measured by [^{3}H]-thymidine incorporation assay. A375 cells were treated with VP-16 or doxorubicin (Doxo.) at the indicated concentrations. [^{3}H]-Thymidine incorporation is reported as percentage of DNA-labeled recovered on drug vehicle–treated cells. Ordinate reports means of four different [^{3}H]- thymidine incorporation experiments with standard error (vertical bar). (C) DNA content analysis of A375 cells by flow cytometry. Figures show percentage cell number of cells in different cell cycle phases. Two representative experiments of A375 cells treated with VP-16, doxorubicin (Doxo.), and DMSO (control) are reported. (D) Apoptosis of A375 cells, by flow cytometry, after treatment with VP-16 or doxorubicin (Doxo.) at the indicated concentrations (percentage of subdiploid cells). *P<.01 compared with the control (untreated cells); analysis was by ANOVA followed by Dunnett test.

Modulation of IL-8 by VP-16 and Doxorubicin

A375 cells were incubated withVP-16 and doxorubicin (0.1-10 μM), then IL-8 protein content was measured. As shown in Figure 2A, VP-16 10 μM and doxorubicin 0.1 to 10 μM significantly increased the levels of IL-8 in A375 cells after 24 hours of treatment. To determine whether Akt and MAPK pathways were required for IL-8 increase induced by VP-16 and doxorubicin, A375 cells were pretreated for 30 minutes with SH-5, an Akt inhibitor, with SB 202190 and U0126, which are potent inhibitors of p38 MAPK and MEK1/2, respectively. Cells were then exposed to VP-16 10 μM and doxorubicin 1 μM for 24 hours. As shown in Figure 2, B and C, SB 202190, U0126, and SH-5 1 μM were able to completely inhibit VP-16–induced increase of IL-8 protein expression, whereas they inhibited only partially the effect of doxorubicin. Furthermore, to evaluate a potential role for adenosine receptors in the increase of IL-8 induced by VP-16 and doxorubicin, A375 cells were treated with the chemotherapeutic drugs in combination with 1 μM of adenosine receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; for A_1), SCH 58261 (for A_{2A}), MRE 2029F20 (for A_{2B}), and MRE 3008F20 (for A_3). The results indicate that only the A_{2B} receptor antagonist MRE 2029F20 was able to significantly reduce IL-8 protein levels in hypoxic A375 cells. Furthermore, as expected, MRE 2029F20 partially blocked the increase in IL-8 induced by VP-16 and doxorubicin (Figure 2, B and C), suggesting that the A_{2B} receptor induced a signal able to increase IL-8 protein. To confirm this finding, we stimulated A_{2B} adenosine receptors in A375 cells with increasing concentrations of 5′-N-ethylcarboxamide-adenosine (NECA; 1-100 μM) for 24 hours. This drug induced the secretion of IL-8 in a dose-dependent manner (Figure 2D). The relatively low potency of NECA agrees with previous reports of A_{2B} receptor–mediated IL-8 production [28,39].

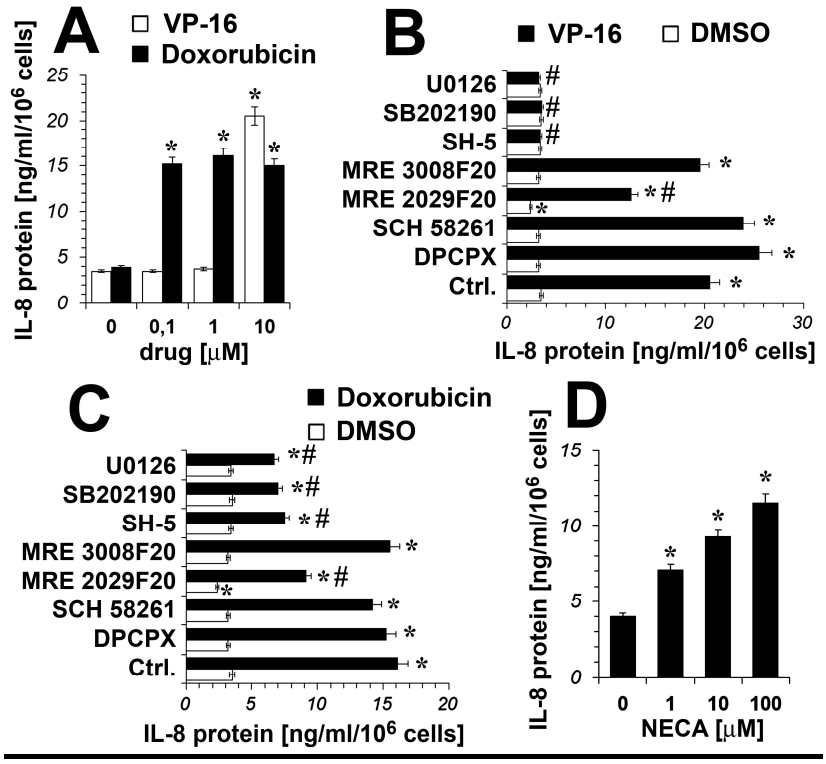


Figure 2. Effect of chemotherapeutic drugs on IL-8 expression in hypoxic (1%O_2) A375 cells. (A) IL-8 release into the culture medium of A375 cells cultured for 24 hours in the absence (0) and in the presence of increasing concentrations of VP-16 and doxorubicin. *P < .01 compared with the control (DMSO-treated hypoxic cells); analysis was by ANOVA followed by Dunnett test. (B and C) IL-8 release into the culture medium of A375 cells cultured for 24 hours in the absence and in the presence of 1 μM of U0126, SB 202190, SH-5, the A_3 antagonist MRE 3008F20, the A_{2B} antagonist MRE 2029F20, the A_{2A} antagonist SCH 58261, and the A1 antagonist DPCPX 1 μM alone (DMSO) or in the presence of the chemotherapeutic drug VP-16 5 μM (VP-16); B) or in the presence of the chemotherapeutic drug doxorubicin 1 μM (C); the inhibitors and the antagonists were added 30 minutes before the chemotherapeutic drug, then the cells were exposed to hypoxia. Ctrl. Indicates control and represents DMSO in empty bar and VP-16 or doxorubicin alone (filled bar) in panels B and C, respectively. Plots are mean± SE values (n=3). *P<.05 compared with the control (DMSO-treated hypoxic cells). #P<.05 compared with the control (VP-16–treated cells in panel B; doxorubicin-treated cells in panel C); analysis was by ANOVA followed by Dunnett test. (D) Effect of the adenosine receptor agonist NECA (1, 10, and 100 μM) on IL-8 expression in hypoxic A375 cells after 24 hours of treatment. *P < .01 compared with the control (0; untreated hypoxic cells). Analysis was by ANOVA followed by Dunnett test.

VP-16 and Doxorubicin Modulate Akt, p44/p42, and p38 MAPK Signaling Pathways

A375 cells were cultured in the absence and in the presence of VP-16 and doxorubicin for 6 hours. We found that exposure of melanoma cells to VP-16 10 μM and doxorubicin 1 μM resulted in the increase of p38, Akt, and ERK1/2 phosphorylation levels (Figure 3, A and B). Furthermore, we observed that the A_{2B} receptor antagonist

MRE 2029F20 1 μM was able to attenuate the increase in p38, Akt, and ERK1/2 phosphorylation levels induced by VP-16 and doxorubicin. In particular, we found that the A_{2B} receptor antagonist MRE 2029F20 1 μM, when used alone, reduces p38, Akt, and ERK1/2 phosphorylation basal levels in A375 cells (Figure 3, A and B).

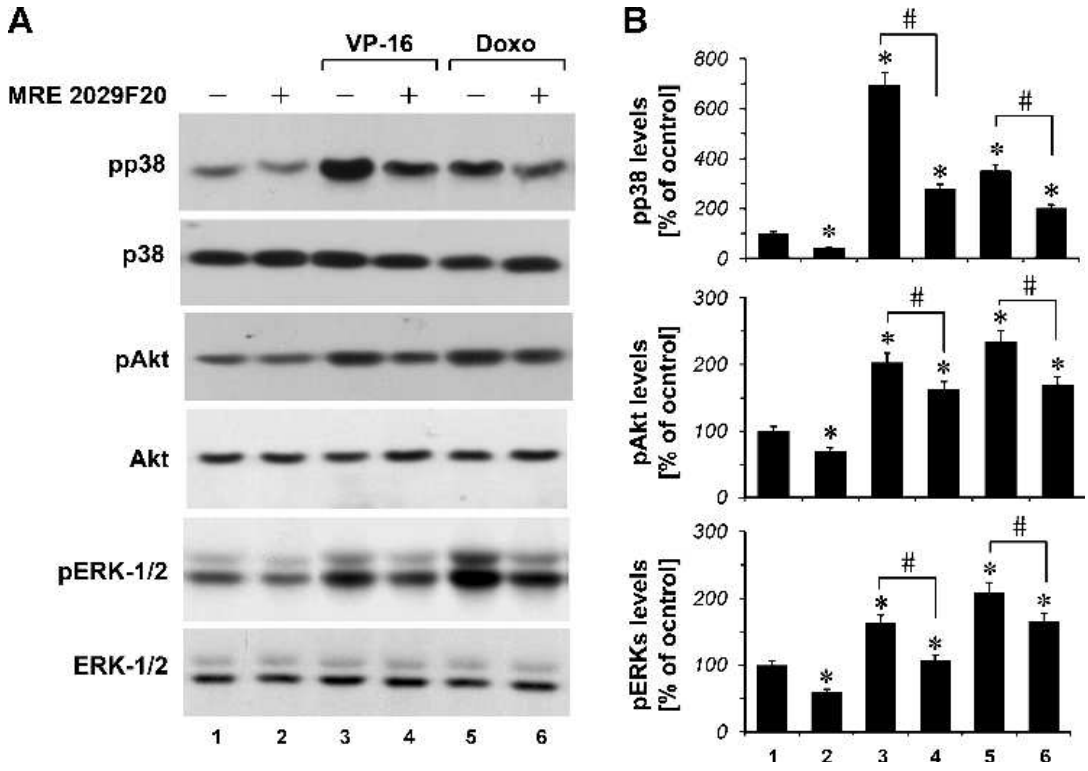


Figure 3. p38, Akt, and ERK1/2 phosphorylation in hypoxic (1% O_2) A375 cells. (A) pp38, phospho-Akt, and pERK1/2 MAPK phosphoprotein levels under VP-16 10 μM and doxorubicin (Doxo) 1 μM treatment in the absence and in the presence of the A_{2B} adenosine receptor antagonist MRE 2029F20 1 μM. (B) Densitometric data, means from three independent experiments, were normalized to the results obtained in cells in the absence of drugs (control). Plots are mean ± SE values (n = 3). *P < .01 compared with the control. Analysis was by ANOVA followed by Dunnett test. #P < .01 compared with cells treated with the chemotherapeutic alone; t test.

Modulation of VEGF by VP-16 and Doxorubicin

To investigate VEGF expression, we incubated A375 cells with VP-16 (5 μM) and doxorubicin (1 μM) and determined VEGF protein release. As shown in Figure 4A, VP-16 and doxorubicin significantly decreased the levels of VEGF after 24 hours of treatment. To determine whether Akt and MAPK pathways were required for VEGF decrease induced by VP-16 and doxorubicin, A375 cells were pretreated with 1 μM SH-5, SB 202190, or U0126. Cells were then exposed to VP-16 5 μM and doxorubicin 1 μM for 24 hours. As shown in Figure 4, A and B, differently from what we have

observed for IL-8- secretion, SB 202190, U0126, and SH-5 were not able to block VP-16– and doxorubicin-induced decrease of VEGF protein expression. To evaluate a potential role for adenosine receptors in themodulation of VEGF protein levels by VP-16 and doxorubicin, A375 cells were treated with the chemotherapeutic drugs in combination with 1 μM DPCPX, SCH58261, MRE 2029F20 and MRE 3008F20. The results indicate that the A_3 receptor antagonist MRE 3008F20 was able to further impair VEGF production already decreased byVP-16 and doxorubicin in A375 cells (Figure 4, A and B). Moreover, the A_3 receptor antagonist MRE 3008F20, also when used alone, was able to significantly reduce VEGF protein in A375 cells. Furthermore, we studied the effect of MRE 3008F20 1 μM in combination with different concentrations of VP-16 (1, 5, and 10 μM) and doxorubicin (0.1, 0.5, and 1 μM) onVEGF production.We found that MRE 3008F20 was able to further reduce VEGF levels already decreased by VP-16 1 to 5 μM and by doxorubicin 0.5 to 1 μM (Figure 4C).

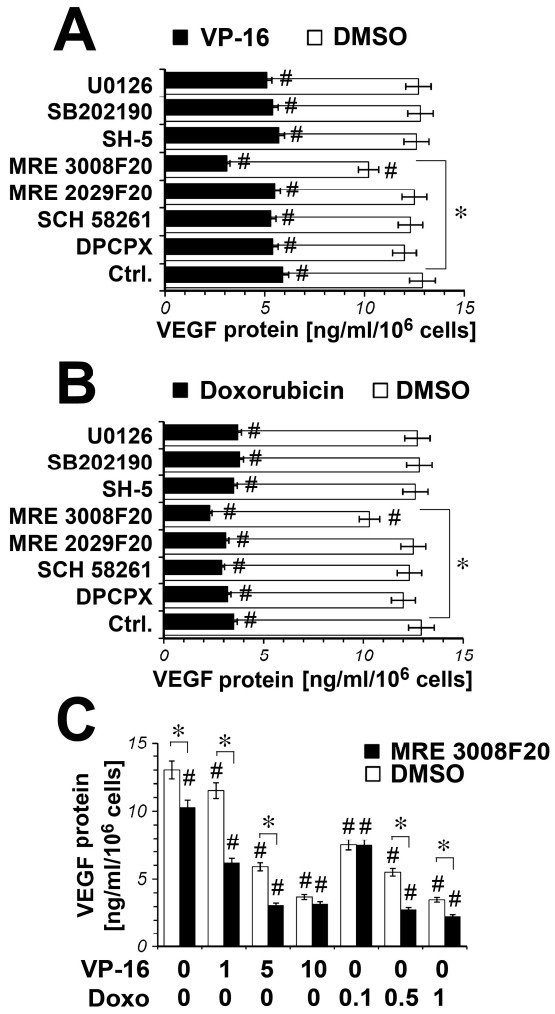


Figure 4. Effect of chemotherapeutic drugs on VEGF expression in hypoxic (1% O_2) A375 cells. (A) VEGF release into the culturemedium of A375 cells cultured for 48 hours in the absence and in the presence (1 μM) of U0126, SB 202190, SH-5, the A_3 antagonist MRE 3008F20, the A_{2B} antagonist MRE 2029F20, the A_{2A} antagonist SCH 58261

and the A1 antagonist DPCPX alone (DMSO) or in the presence of the chemotherapeutic drug VP-16 5 μM (VP-16; A) or in the presence of the chemotherapeutic drug doxorubicin 1 μM (B); the inhibitors and the antagonists were added 30 minutes before the chemotherapeutic drug, then the cellswere exposed to hypoxia. Plots aremean± SE values (n = 3). *P < .05 MRE 3008F20 plus chemotherapeutic drug–treated cells versus chemotherapeutic drug–treated cells: black filled bar indicates Ctrl.; analysis was by t test. #P < .05 compared with the control (DMSO-treated cells: empty bar indicates Ctrl.); analysis was by ANOVA followed by Dunnett test. (C) VEGF release into the culture medium of A375 cells cultured for 48 hours in the presence of increasing concentrations of the chemotherapeutic drug VP-16 (1-5-10 μM) and doxorubicin (0.1-0.5-1 μM) in the absence and in the presence of the A_3 antagonist MRE 3008F20 1 μM, which was added 30 minutes before the chemotherapeutic drug. Then the cells were exposed to hypoxia. *P < .05: MRE 3008F20-treated cells versus DMSO-treated cells; analysis was by t test. # P<.05 compared with the control (DMSO-treated hypoxic cells: empty bar indicates Ctrl.); analysis was by ANOVA followed by Dunnett test.

VP-16 and Doxorubicin Modulate the Expression of HIF-1α Protein

The levels of HIF-1α and HIF-1β protein in hypoxic A375 cells on drug treatment were investigated by Western blot analysis (Figure 5). HIF-1α protein expression was increased in a time-dependent manner [31]. In particular, HIF-1α protein expression was detected after 4 hours of exposure to hypoxia, and VP-16 10 μM and doxorubicin 1 μM strongly inhibited HIF-1α protein expression (lanes 2 and 3). The observed down-regulation of HIF-1α protein expression by the chemotherapeutic drugs was specific because no alterations were detected in the levels of the HIF-1β protein. Because MRE 3008F20, when used alone, was able to significantly reduce HIF-1α protein in A375 cells (Figure 5, lane 4), we have evaluated whether the A_3 receptor antagonist MRE 3008F20 was able to modulate HIF-1α protein when used in combination with the chemotherapeutic drugs. A375 cells were treated for 4 hours in hypoxia with VP-16 10 μM or doxorubicin 1 μM alone and in combination with MRE 3008F20 1 μM. As shown in Figure 5 (lanes 5 and 6), we found that MRE 3008F20 further increased the effect of chemotherapeutic drugs by further decreasing HIF-1α protein accumulation.

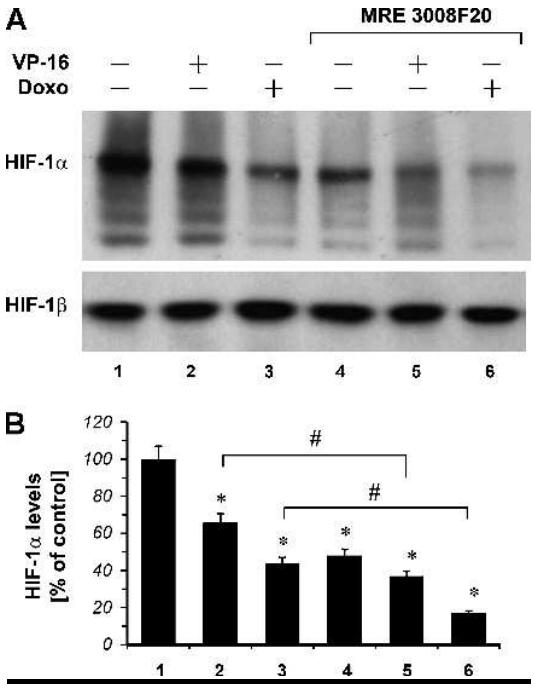


Figure 5. (A) Western blot analysis using an anti–HIF-1α mAb of protein extracts from A375 hypoxic cells (1%O2) under VP-16 10 μM and doxorubicin (Doxo) 1 μM treatment (4 hours) in the absence and in the presence of the A_3 adenosine receptor antagonist MRE 3008F20 1 μM. HIF-1β shows equal protein loading. (B) The mean densitometric data from three independent experiments were normalized to the results obtained in cells in the absence of drugs (control). Plots are mean ± SE values (n = 3). *P < .01 compared with control without chemotherapeutic treatment; analysis was by ANOVA followed by Dunnett test. $^{\#}$P < .01 compared with the cells exposed to chemotherapeutic alone (2 vs 5 and 3 vs 6 for VP-16 and doxorubicin, respectively). Analysis was by t test.

Role of HIF-1α

To investigate a possible role for the HIF-1α subunit in the chemotherapeutic-induced VEGF inhibition, we have performed a series of experiments in the presence of $siRNA_{HIF\text{-}1\alpha}$. HIF-1α protein level was reduced with siRNA at 72 hours after $siRNA_{HIF\text{-}1\alpha}$ transfection (Figure 6, A and B, lane 4 vs lane 1). The results show that when HIF-1α protein level was reduced by $siRNA_{HIF\text{-}1\alpha}$ transfection, VP-16 10 μM and doxorubicin 1 μM further decreased HIF-1α protein content in A375 cells. Furthermore, $siRNA_{HIF\text{-}1\alpha}$ transfection was able to decrease VEGF protein levels (Figure 6C). In particular, when HIF-1α protein level was reduced by $siRNA_{HIF\text{-}1\alpha}$ transfection, VP-16 1 μM and doxorubicin 0.5 μM decreased VEGF secretion levels to a major extent (Figure 6C).

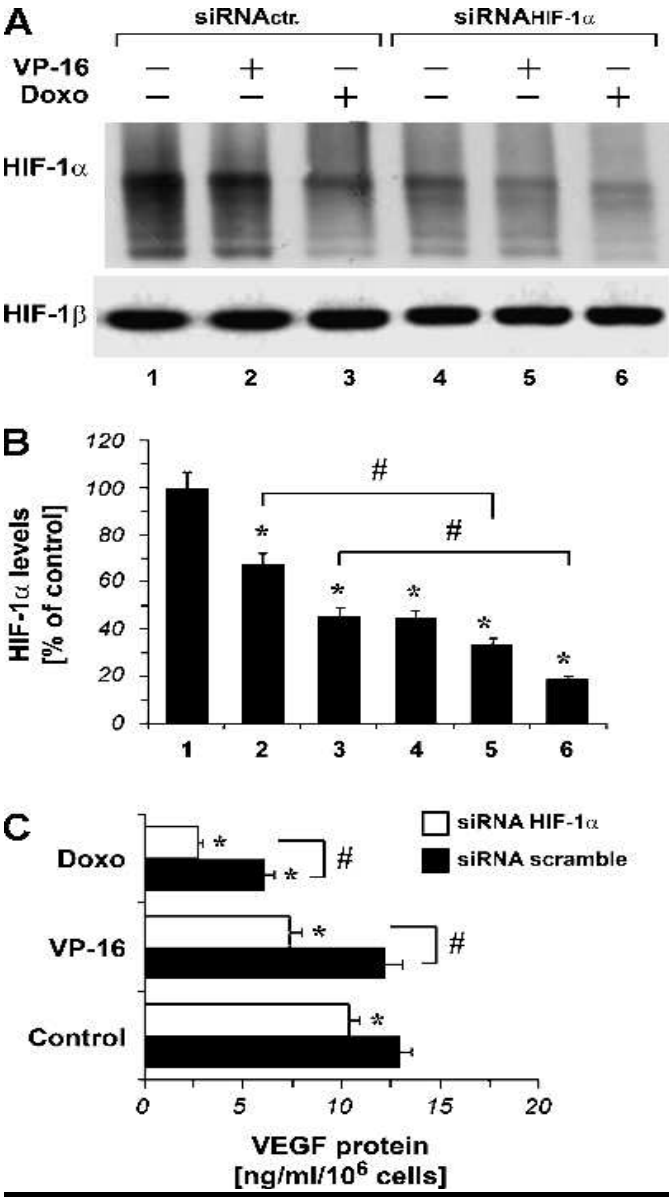


Figure 6. (A) Western blot analysis using an anti–HIF-1α mAb of protein extracts from A375 cells transfected with scramble ribonucleotides (siRNActr.) or $siRNA_{HIF\text{-}1\alpha}$ for 72 hours and cultured with VP-16 10 μM and doxorubicin (Doxo) 1 μM for 4 hours. HIF-1β shows equal protein loading. (B) The means of densitometry data from independent experiments were normalized to the results obtained in cells transfected with siRNActr. Plots are mean ± SE values (n = 3). *P < .01 compared with the siRNActr. without chemotherapeutic drug treatment. Analysis was by ANOVA followed by Dunnett test. #P < .01 cells treated with $siRNA_{HIF\text{-}1\alpha}$ compared with cells treated with siRNActr (2 vs 5 for VP-16; 3 vs 6 for doxorubicin). Analysis was by t test. (C) VEGF release into the culture medium of A375 cells transfected with scramble siRNA or with $siRNA_{HIF\text{-}1\alpha}$ for 48 hours and then cultured for 24 hours in hypoxia in the absence (Control) and in the presence of VP-16 1 μMand doxorubicin (Doxo) 0.5 μM. Plots are mean ± SE values (n = 3). *P < .01 compared with the Control siRNA- scramble–transfected cells (black filled bar); analysis was by ANOVA followed by Dunnett test. #P < .01 $siRNA_{HIF\text{-}1\alpha}$ versus siRNA scramble; analysis by t test.

A_{2B} and A_3 Receptors Gene Silencing

To demonstrate more conclusively a role for A_{2B} or A_3 receptors in the responses being measured, we tried to knock down A_{2B} and A_3 receptors expression in hypoxic A375 melanoma cells using siRNA, leading to a transient knockdown of the A_{2B} and A_3 receptor genes. A375 cells were transfected with nonspecific random control ribonucleotides (siRNA scramble) or with siRNA that target A_{2B} ($siRNAA_{2B}$) or A_3 receptor messenger RNA ($siRNA_{A3}$) for degradation. After transfection, the cells were cultured for 48 hours in complete medium, and then total proteins were isolated for Western blot analysis of A_{2B} and A_3 receptor protein expressions. As expected, A_{2B} and A_3 receptors protein expressions were strongly reduced in $siRNA_{A2B}$- and $siRNA_{A3}$-

treated cells, respectively (Figure 7A). To confirm the specificity of the siRNA$_{A3}$-mediated silencing of A_3 receptor, we investigated the expression of A_{2B} receptor protein in siRNA$_{A3}$-treated cells (Figure 7A). Figure 7A demonstrates that treatment of A375 cells with siRNA$_{A3}$ reduced the expression of A_3 protein but had no effect on the expression of A_{2B} receptor. Similar results were obtained when A375 cells transfected with siRNAA$_{2B}$ were analyzed for the expression of the A_3 receptor. Therefore, at 48 hours from the siRNA$_{A2B}$ and siRNA$_{A3}$ transfection, A375 cells were exposed to VP-16 10 μM and doxorubicin 1 μM for 24 hours in hypoxia. Then, IL-8 protein levels were measured. We found that the inhibition of A_{2B} receptor expression was able to reduce chemotherapeutic-induced IL-8 accumulation, whereas the inhibition of A_3 receptor expression with siRNA$_{A3}$ did not modify chemotherapeutic-induced IL-8 accumulation (Figure 7B). In particular, the silencing of the A_{2B} receptor by using siRNA$_{A2B}$ alone was able to significantly reduce basal IL-8 protein secretion in hypoxic A375 cells (Figure 7B). Furthermore, A375 cells were transfected with siRNA$_{A2B}$ and siRNA$_{A3}$ and exposed to VP-16 5 μMand doxorubicin 1 μM for 24 hours in hypoxia to evaluate VEGF levels. No effect in VEGF inhibition induced by the chemotherapeutic drugs was observed after the inhibition of A_{2B} receptor expression. On the contrary, the inhibition of A_3 receptor expression potentiates the reduction of VEGF secretion induced by the chemotherapeutic drugs (Figure 7C). In particular, the silencing of the A_3 receptor by using siRNA$_{A3}$ alone was able to significantly reduce VEGF protein in A375 cells (Figure 7C).

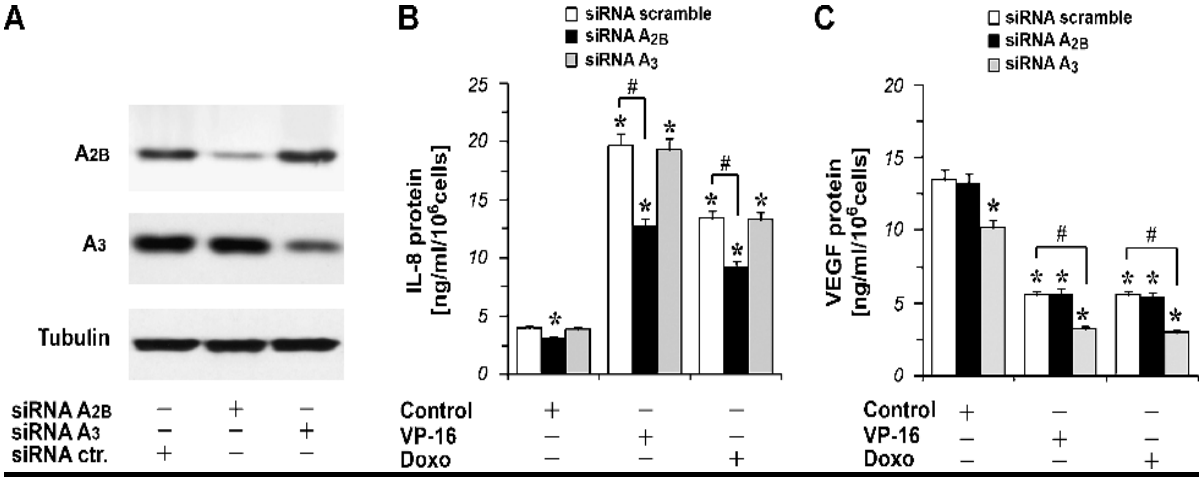


Figure 7. A_{2B} and A_3 receptor expression silencing by siRNA transfection in hypoxic A375 cells (1% O_2). (A) Western blot analysis using an anti-A_{2B} and an anti-A_3 receptor pAb of protein extracts from A375 cells transfected with scramble (control siRNA) ribonucleotides or with siRNA$_{A2B}$ or siRNA$_{A3}$ and cultured for 48 hours. Tubulin shows equal protein loading. (B) IL-8 release into the culture medium of A375 cells transfected for 48 hours with scramble ribonucleotides or with siRNA$_{A2B}$ or siRNA$_{A3}$ and then cultured for 24 hours in hypoxia (1% O_2) in the

absence (Control) and in the presence of VP-16 10 μM and doxorubicin (Doxo) 1 μM. Plots are mean ± SE values (n=3). *P < .01 compared with the control siRNA-scramble transfected cells without chemotherapeutic drug treatment; analysis was by ANOVA followed by Dunnett test. #P < .01 $siRNA_{A2B}$-transfected cells versus siRNA-scramble–transfected cells; analysis was by t test. (C) VEGF release into the culture medium of A375 cells transfected for 48 hours with scramble ribonucleotides or with $siRNAA_{2B}$ or $siRNA_{A3}$ and then cultured for 24 hours in hypoxia in the absence (Control) and in the presence of VP-16 5 μM and doxorubicin (Doxo) 1 μM. Plots are mean ± SE values (n = 3). *P < .01 compared with control siRNA-scramble–transfected cells without chemotherapeutic drug treatment. Analysis was by ANOVA followed by Dunnett test. #P < .01 $siRNA_{A3}$ transfected cells versus siRNA-scramble–transfected cells exposed to VP-16 or doxorubicin; analysis was by t test.

DISCUSSION

New treatments are urgently needed for the therapy for metastatic melanoma, and much effort is being devoted to the development of genetic and immune therapies, but the widespread availability of these remains a distant prospect. In the meantime, chemotherapy will remain the treatment of choice, and strategies to overcome resistance offer a more immediate possibility for improving the lot of these patients. This study was undertaken to examine whether two chemotherapeutic drugs, VP-16 and doxorubicin, modulate IL-8 and VEGF production in human melanoma A375 cells. In particular, because adenosine was able to modulate HIF-1, VEGF, and IL-8 in cancer cells, we analyzed the influence of the adenosinergic signaling on the chemotherapeutic drug effects in human melanoma cells. The aims of this study were as follows:

1. to investigate the effect of two drugs used in chemotherapy on cell vitality and on cytokine release induced in melanoma cells under hypoxic conditions;
2. to define a molecular signaling of the cancer cell response to these drugs;
3. to investigate the putative role of the adenosinergic system in these processes.

We demonstrated that human melanoma cells produce IL-8 and VEGF. In particular, we found that treatment of melanoma cells with the DNA-damaging drugs VP-16 and doxorubicin resulted in the upregulation of the proangiogenic cytokine IL-8 (Figure 2). These results are in accord with data indicating that in addition to their known cytotoxic effects, chemotherapeutic agents can trigger cytokine production in a variety of cell types in vitro [40,41].Moreover, our data indicate that the DNA-damaging drugs VP-16 and doxorubicin inhibit VEGF expression (Figure 4) through the inhibition of HIF-1 (Figure 5). A further objective of these studies was to assess whether the adenosinergic signaling, through its adenosine receptor subtypes, could modulate cytokine production

induced by chemotherapeutic agents. Using the human A375 melanoma cell line that expresses each of the four adenosine receptor subtypes [25], these studies demonstrated that the A_{2B} receptor blockade can modulate IL-8 production, whereas blocking A_3 receptors, it is possible to further decrease VEGF reduction because of VP-16 and doxorubicin. In this work, we have demonstrated that the inhibition of the A_{2B} receptor results in the reduction of IL-8 production, whereas inhibition of A_3 results in the reduction of VEGF. According to these results, it has been previously demonstrated that stimulation of A_{2B} adenosine receptors increased synthesis and secretion of IL-8, whereas A_3 receptors are responsible of the increase of VEGF [27,28].We hypothesize that the different effects of A_{2B} and A_3 adenosine receptors on the synthesis of angiogenic factors may imply their coupling to different G proteins. The mechanism of how A_{2B} adenosine receptor could decrease chemotherapy-induced cytokine production was also examined and was found to be dependent on the activation of MAPK. The current findings describe a putative mechanism by which this G protein–coupled receptor can decrease the cytokine-producing effects of chemotherapeutic agents in human melanoma.

Hypoxic cancer cells are resistant to chemotherapeutic treatment, leading to the selection of cells with a more malignant phenotype. HIF-1 has been shown to be responsible for an adaptive response of cells to hypoxia. If VP-16 would influence its activity under hypoxia, this could lead to changes in cell survival. To investigate this possibility, we measured HIF-1α protein level. The results indicate that hypoxia did increase HIF-1α protein level in melanoma cells, and this effect was influenced by VP-16 (Figure 5). Dacarbazine remains the reference standard treatment of metastatic melanoma, but only a minority of patients obtains long-lasting responses [7]. Polychemotherapy regimens have been reported to produce various response rates. Treatment with VP-16 is more common in lung cancer, leukemia, and testicular tumors and has been used in polychemotherapy regimens combined with cisplatin for the treatment of melanoma brain metastases, but the response rates remain less than 13% [42]. The significance of tumor cell–derived cytokine production in the therapeutic effectiveness or adverse effect profile of chemotherapeutic agents is unclear. However, significant levels of cytokines, including IL-8, tumor necrosis factor α, and others, can be found in patients undergoing chemotherapy for a variety of tumors [43,44]. It has been reported that overproduction of chemokines is a potential mechanism for melanoma cells to evade cell death and become resistant to chemotherapy. Strategies to

inhibit IL-8 signaling may sensitize hypoxic tumor cells to conventional treatments. These data may have a significant clinical relevance, justifying the combination of conventional chemotherapy with anti–IL-8 and/or anti-VEGF modalities, such as A_{2B} or A_3 adenosine receptor antagonists, for the treatment of malignant melanoma.

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THE DATA OF THE PRESENT THESIS ARE REPORTED ALSO IN THE FOLLOWING PUBLICATIONS:

Merighi S., Benini A., Mirandola P., Gessi S., Varani K., **Simioni C.**, Leung E., Maclennan S., Baraldi PG., Borea PA.

"CAFFEINE INHIBITS ADENOSINE-INDUCED ACCUMULATION OF HYPOXIA INDUCIBLE FACTOR-1α, VASCULAR ENDOTHELIAL GROWTH FACTOR AND INTERLEUKIN-8 EXPRESSION IN HYPOXIC HUMAN COLON CANCER CELLS".
Mol Pharmacol. 2007 Aug;72(2): 395-406

Merighi S., **Simioni C.**, Gessi S., Varani K., Mirandola P., Tabrizi MA., Baraldi PG., Borea PA.

"A_{2B} AND A_3 ADENOSINE RECEPTORS MODULATE VASCULAR ENDOTHELIAL GROWTH FACTOR AND INTERLEUKIN-8 EXPRESSION IN HUMAN MELANOMA CELLS TREATED WITH ETOPOSIDE AND DOXORUBICIN"
Neoplasia. 2009 Oct;11(10): 1064-73

LIST OF PUBLICATIONS

Merighi S., Benini A., Mirandola P., Gessi S., Varani K., **Simioni C.**, Leung E., Maclennan S., Baraldi PG., Borea PA

"CAFFEINE INHIBITS ADENOSINE-INDUCED ACCUMULATION OF HYPOXIA INDUCIBLE FACTOR-1α, VASCULAR ENDOTHELIAL GROWTH FACTOR AND INTERLEUKIN-8 EXPRESSION IN HYPOXIC HUMAN COLON CANCER CELLS".
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Merighi S., **Simioni C.**, Gessi S., Varani K., Borea PA.

"BINDING THERMODYNAMICS AT THE HUMAN CANNABINOIDS CB_1 AND CB_2 RECEPTORS"
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MEETINGS

Simioni C., Merighi S., Benini A., Mirandola P., Gessi S., Varani K., Leung E., Maclennan S., Baraldi PG., Borea PA. Hypoxia inhibits paclitaxel-induced apoptosis through adenosine-mediated phosphorylation of bad in glioblastoma cells. 33° Congresso Nazionale della Società Italiana di Farmacologia. Cagliari (Italy) 6/06/07-9/06/07

Merighi S., Benini A., Gessi S., Varani K., **Simioni C**., Mirandola P., Leung E., MacLennan S., Baraldi PG., Borea PA. Caffeine inhibits adenosine-induced accumulation of Hypoxia-Inducible Factor-1alpha, Vascular Endothelial Growth Factor, and Interleukin-8 expression in hypoxic human colon cancer cells. XI Seminario per dottorandi in Farmacologia e Scienze Affini. Certosa di Pontignano (Siena, Italy) 25/09/07

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