Chapter 16
Agonists and Antagonists: Molecular Mechanisms and Therapeutic Applications

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16.1 Cancer Cell Growth Is Driven by Cell Proliferation and Lack of Apoptosis

The growth and expansion of cells is determined on cell ability to go through DNA synthesis and cell division and on the other hand on apoptotic pathways, leading to cell death. Alterations in normal cell function may cause a disruption of normal cell growth and apoptosis, subsequently resulting in carcinogenesis (Jacobson et al. 1997; Danial and Korsmeyer 2004). Understanding of molecular mechanisms underlying the cell proliferation and death machinery is of great importance for improving the efficacy of targeted therapeutics and overcoming resistance to chemotherapeutic agents. Despite the clinical applications of cell cycle inhibitors, mostly chemotherapeutic agents, there is still an urgent need to develop novel drugs that can specifically target multiple sites and pathways of the cell cycle and apoptosis, while avoiding drug induced cytotoxicity.

During the last decade, adenosine was recognized as a cell signaling molecule which binds to specific cell surface receptors and modulate intracellular signaling, resulting in the regulation of physiological processes. Adenosine binds and activates 4 receptor subtypes, A_1, A_{2A}, A_{2B} and the A_3 (Stiles 1990; Linden 1991). Since most body cells express different adenosine receptors and their activation may lead to opposing effects, selective synthetic agonists were developed that induce specific effects on particular cell types.

In this chapter, we will summarize the current knowledge about the A_3AR target, known to be highly expressed in tumor cells. The development of novel A_3AR agonists and antagonists that specifically target the receptor, initiating signal transduction pathways leading to tumor cell cycle arrest and apoptosis will be presented.

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16.2 Over-Expression of A$_3$AR Is a Characteristic of Tumor Cells

Broad range of tumor cell lines express A$_3$AR mRNA and protein however, it is quite difficult to compare it to the expression of normal proliferating cells. Nevertheless, a side by side comparison can be made in human samples comparing tumor versus normal tissue. In tissues derived from patients with colon and breast carcinoma, higher A$_3$AR mRNA and protein expression levels are found in the malignant vs. adjacent non-neoplastic tissue or normal tissue. Additional analysis revealed that the lymph node metastasis expressed even more A$_3$AR mRNA than the primary tumor tissue, demonstrating that the A$_3$AR is directly correlated to the degree of malignancy (Madi et al. 2004; Fishman et al. 2007). Gessi et al. also showed that A$_3$AR expression level was linearly increased in colon tissue samples while progressing from polyp to adenoma, carcinoma and metastatic colon carcinoma (Gessi et al. 2004). Interestingly, the high A$_3$AR expression level in the tumor tissues was associated with elevated NF-κB levels (Bar-Yehuda et al. 2008). The latter is known to act as one of the A$_3$AR gene transcription factors and its up-regulation in tumor tissues may account for the high A$_3$AR expression levels (Madi et al. 2007). High A$_3$AR mRNA/protein expression levels were also demonstrated in other solid tumor types such as melanoma, pancreatic and small cell lung carcinoma, as well as hepatocellular carcinoma (HCC) (Madi et al. 2004; Fishman et al. 2007). Recent findings show that the A$_3$AR is over-expressed in the PBMCs of patients with colon cancer and HCC (Gessi et al. 2004; Bar-Yehuda et al. 2008). Therefore, it may be suggested that receptor expression in the PBMCs reflects receptor status in the remote tumor organ.

Bioinformatics analysis revealed a 2.3-fold increase in the expression of A$_3$AR 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$_3$AR was abundant in brain, kidney, lung, germ cells, placenta, and retina but brain, lung, and pancreatic tumors expressed more A$_3$AR 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$_3$AR was 1.6-fold higher in all of the cancer tissues compared with normal tissues (Madi et al. 2004).

## 16.3 Tumors Respond to A$_3$AR Agonists by Cell Cycle Arrest and Apoptosis

The findings showing up-regulation of the A$_3$AR in tumor cells prompted studies which led to the utilization of this receptor as a therapeutic target to combat the growth and development of malignant cells. The anti-cancer effect of synthetic A$_3$AR agonists was explored and the molecular mechanism involved with tumor cell growth inhibition was followed up.

The development and growth of leukemia, lymphoma, melanoma, colon, lung, breast and prostate carcinoma was suppressed *in vitro* upon treatment with IB-MECA, Cl-IB-MECA and thio-Cl-IB-MECA (Bar-Yehuda et al. 2005; Chung et al. 2006; Fishman et al. 2001, 2002a, b, 2003; Fishman and Bar-Yehuda 2003; Kim et al. 2008; Lee et al. 2005; Madi et al. 2003; Merighi et al. 2005a,b; Merimsky et al. 2003; Ohana et al. 2001, 2003; Panjehpour and Karami-Tehrani 2004, 2007). *In vivo* studies in syngeneic murine models showed that IB-MECA suppressed tumor growth inhibition in B16-F10 melanoma, CT-26 colon carcinoma and in an orthotopic model of N1S1 hepatocellular carcinoma (Bar-Yehuda 2008; Fishman et al. 2001, 2002a, 2003; Harish et al. 2003; Madi et al. 2003; Merimsky et al. 2003; Ohana et al. 2001, 2003). Marked tumor growth inhibition was also observed in xenograft mouse models of HCT-116 colon carcinoma and PC-3 prostate carcinoma (Bar-Yehuda et al. 2005; Fishman et al. 2003, 2004; Ohana et al. 2003). The anti-cancer effect was counteracted both *in vitro* and *in vivo* by the selective A$_3$AR antagonist, MRS1523, demonstrating that tumor growth inhibition is mediated via the A$_3$AR (Fishman et al. 2003, 2004; Madi et al. 2003; Merighi et al. 2005; Ohana et al. 2003; Panjehpour and Karami-Tehrani 2004, 2007). Interestingly, in a xenograft model and in cell culture studies of breast carcinoma cells, IB-MECA and thio-Cl-IB-MECA suppressed tumor growth, however the effect was not A$_3$AR mediated due to lack of receptor expression (Chung et al. 2006; Lu et al. 2003).

Mechanistically, the agonists induced down-regulation of cyclin D1 and c-Myc resulting in cell cycle arrest in the G0/G1 phase (Bar-Yehuda et al. 2005; Fishman et al. 2000, 2002a, 2003, 2004; Harish et al. 2003; Kim et al. 2008, Lee et al. 2005; Madi et al. 2003, 2004). Interestingly, two signaling pathways related to Wnt and NF-κB are known to control the expression levels of cyclin D1 and c-Myc (Joyce et al. 2001; Karim et al .2004; Smalley and Dale 1999). Upon treatment with the agonists, cAMP levels are decreased followed by down-regulation of PKA and subsequent decrease in PKB/Akt (known to be phosphorylated by PKA) (Chan et al. 1999; Olah and Stiles 2000; Murga et al. 2000). Modulation of the Wnt and the NF-κB pathways take place down-stream to PKA and PKB/Akt kinases (Hino et al. 2005; Kim and Chung 2002; Li et al. 2006; Vermeulen et al. 2003).

A key protein in the Wnt signaling pathway is glycogen synthase kinase-3β (GSK-3β), is phosphorylated by PKA and PKB/Akt. In quiescent cells, GSK-3β suppresses mammalian cell proliferation and survival by phosphorylating the cytoplasmic protein, β-catenin, which in its phosphorylated form is sorted to ubiquitination. In tumor cells the Wnt signaling pathway is highly activated and GSK-3β fails to phosphorylate β-catenin. The latter then accumulates in the cytoplasm and subsequently translocates to the nucleus where it associates with LEF/TCF to induce transcription of cyclin D1 and c-myc (Ferkey and Kimelman 2000; Novak and Dedhar 1999). PKB/Akt is also known to control the NF-κB signaling pathway by phosphorylating down-stream IKK, which subsequently phosphorylates IκB, thereby releasing NF-κB from its complex. Similar to β-catenin, NF-κB translocates to the nucleus, where among other genes, it induces the transcription of c-Myc and cyclin D1 (Joyce et al. 2001; Madrid et al. 2001; Perkins 2007).

De-regulation of these two pathways was observed in melanoma, colon, prostate, lung, breast and hepatocellular carcinoma *in vitro* and more importantly in tumor lesions derived from agonists' treated animals (Bar-Yehuda et al. 2008; Chung et al. 2006; Fishman et al. 2002a, 2003, 2007; Madi et al. 2004). A point to note is that Cl-IB-MECA induced apoptosis of hepatocellular carcinoma *in vivo*, evidenced by immuno-histochemistry TUNEL staining and by up-regulation of the anti-apoptotic genes caspase-3, Bad and Bax. This tumor type was the most responsive to the treatment of Cl-IB-MECA among all other tumors (Bar-Yehuda et al. 2008) (Scheme 16.1).
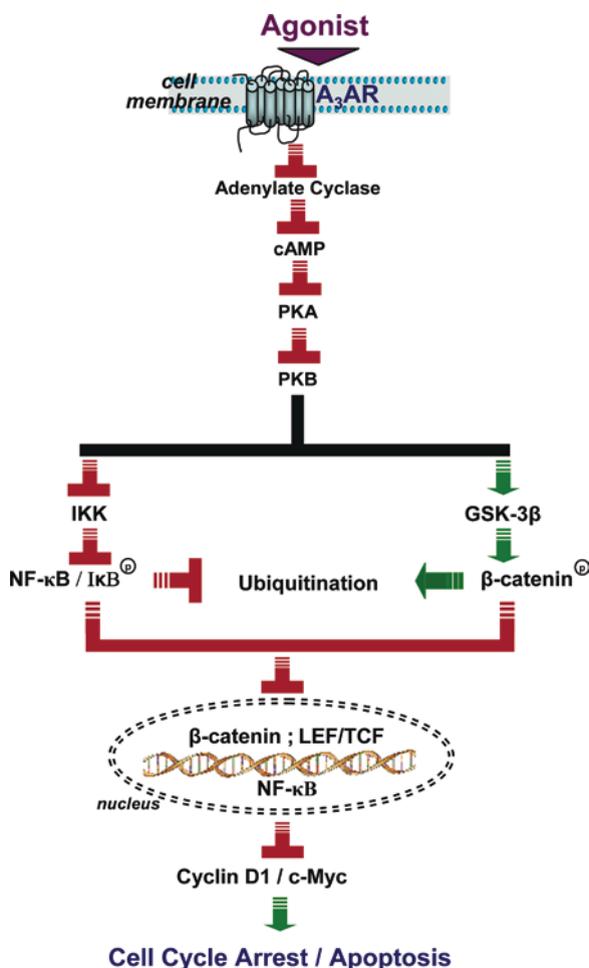
Scheme 16.1 Anti-cancer effect of A_3AR agonists – De-regulation of the NF-κB and the Wnt signaling pathways, resulting in cell cycle arrest and apopotosis of cancer cells

An indirect immunomodulatory effect was also observed in the syngeneic CT-26 colon carcinoma and B16-F10 melanoma tumor bearing mice, treated with IB-MECA and Cl-IB-MECA, respectively. The drug agonists induced up-regulation of Interleukin-12 and increased the activity of NK cells (Fishman et al. 2004; Ohana et al. 2003).

A_3AR fate upon treatment with IB-MECA or Cl-IB-MECA was extensively studied. *In vitro* studies revealed that internalization/recycling events that play an important role in turning on/off receptor-mediated signal transduction pathways, take place. In melanoma cells, A_3AR was rapidly internalized to the cytosol and "sorted" to the endosomes for recycling and to the lysosomes for degradation. Receptor distribution in the lysosomes was consistent with the down-regulation of receptor protein expression and was followed by mRNA and protein re-synthesis (Madi et al. 2003). In addition, in melanoma, prostate cancer and HCC tumor lesions derived from IB-MECA or Cl-IB-MECA-treated mice, A_3AR was down-regulated shortly after treatment. In the tumor lesions derived from prostate carcinoma bearing animals, A_3AR was tested also after 18 h and results indicate that receptor was fully expressed (Fishman et al. 2003, 2004; Madi et al. 2003; Ohana et al. 2003). This analysis was carried out after chronic treatment with the agonist, demonstrating that no tachyphylaxis occurred and the target is valid.

It is well established that NF-κB and the upstream kinase PKB/Akt are highly expressed in chemo-resistance tumor cells and play a major role in hampering the apoptotic pathway (Fahy et al. 2004; Wang and Cassidy 2003). Since CF101 has been shown to down-regulate PKB/Akt and NF-κB protein expression levels, it was assumed that its combination with chemotherapy will enhance the anti-tumor effect of the cytotoxic drug. IB-MECA was found to enhance the anti-cancer effect of cyclophosphamide and 5-fluorouracil (5-FU) in experimental animal models of melanoma and colon carcinoma, respectively (Bar-Yehuda 2005; Fishman et al. 2007; Ohana et al. 2003). Downregulation of PKB/Akt, NF-κB, cyclin D1, and up-regulation of caspase-3 protein expression level were observed in cells and tumor lesions upon treatment with a combination of IB-MECA and 5-FU (Bar-Yehuda 2005). Moreover, in mice treated with the combined therapy, myelotoxicity was prevented as was evidenced by normal white blood cell and neutrophil counts. These results support the notion that IB-MECA potentiates the cytotoxic effect of 5-FU, thus preventing drug resistance (Bar-Yehuda et al. 2002; Fishman et al. 2000, 2002b, 2003; Merimsky et al. 2003). The myeloprotective effect of IB-MECA grants the molecule an added value and suggests its development as a supportive treatment to chemotherapy.

16.4   Hypoxia

The ability to maintain O_2 homeostasis is essential to the survival of all invertebrate and vertebrate species. It is appreciated that all nucleated cells in the human body sense O_2 availability (hypoxia) that is either acute or chronic in duration

(Semenza 2000). As in other physiological systems, adaptive responses to acute changes in O_2 concentration (lasting from seconds or less to minutes) principally occur as a result of alterations (e.g., involving phosphorylation or redox state) of preexisting proteins, whereas chronic changes in O_2 concentration (lasting from minutes to hours or more) principally occur as a result of alterations in gene expression. Not only is O_2 homeostasis essential for survival, but also hypoxia plays an important role in the pathogenesis of major causes of mortality, including cancer, cerebral and myocardial ischemia, and chronic heart and lung diseases (Semenza 1999; Michiels 2004; Maxwell 2002; Hockel and Vaupel 2001).

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; Vaupel 2008). 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).

Growing evidence from experimental and clinical studies points to the fundamental, pathophysiologic role of hypoxia in solid tumors. In order to survive, humans have to be able to extract oxygen from the atmosphere and make it available to their cells where it is utilised for essential metabolic processes. Hypoxia describes the situation when the body is not receiving quite as much oxygen as it requires. Hypoxia is the result of an imbalance between the supply and consumption of oxygen. It is defined as a partial lack of oxygen. If steps are not taken to increase the supply of oxygen, it switches to anoxia, which is the complete absence of oxygen. Clinical investigations carried out over the last 15 years have clearly shown that the prevalence of hypoxic tissue areas is a characteristic pathophysiological property of solid tumors. As the oxygen concentration decreases with increasing distance from the capillary, both cell proliferation rates and drug concentration decrease. These two factors lead to resistance to anticancer drugs; firstly, because the majority of anticancer drugs are only effective against rapidly proliferating cells; and secondly, because chemotherapy drugs have to reach the tumour cells from the blood vessels (Bertout et al. 2008).

16.5   Adenosine in Hypoxia

One of the major features of solid tumors and even small deposits of tumor tissue is deficiency in the level of oxygen, because of an inadequate vascular supply. The adenosine elevation in response to hypoxia is not exclusive to tumor tissues, but, in this context, the adenosine elevation is localized to the tumor microenvironment, since the surrounding tissue is normally oxygenated. Adenosine is generated mainly by two enzymatic systems: intra- or extracellularly localized 5′-nucleotidases and cytoplasmic S-adenosylhomocysteine hydrolase. The processes of adenosine elimination in the cell involve reactions catalyzed by adenosine deaminase and adenosine kinase (Shryock and Belardinelli 1997) yielding inosine or 5′-AMP,

respectively. The reaction of phosphorylation predominates when adenosine occurs at a low physiological concentration (<1 μM) whereas adenosine deaminase is activated at higher concentrations of the substrate (>10 μM).

The accumulation of adenosine in hypoxia is at least partially explained by hypoxia-mediated regulation of enzymes that are involved in adenosine metabolism: (i) adenosine kinase (Decking et al. 1997) and (ii) 5′-nucleotidase (Headrick and Willis 1989; Kobayashi et al. 2000; Thompson et al. 2004). In particular, adenosine can be generated extracellularly through the hydrolysis of released nucleotides by ecto-5′-nucleotidases (Dunwiddie et al. 1997) or can be produced in the cytosol and transported to the extracellular space (Higgins et al. 1994).

1. Adenosine kinase, which rephosphorylates adenosine to convert it to AMP, was shown to be inhibited by hypoxia (Decking et al. 1997). In addition, extracellular adenosine concentrations may be further potentiated by preventing reutilization through hypoxic inhibition of adenosine deaminase (Sitkovsky et al. 2004; Kobayashi et al. 2000). In particular, first, it has been demonstrated that intracellular adenosine levels due to inhibition of adenosine kinase-dependent metabolism of adenosine to AMP would decrease the transcellular adenosine gradient, thereby decreasing flux through bidirectional equilibrative nucleoside transporters and, thus, elevating extracellular adenosine levels during hypoxia (Decking et al. 1997). Second, transcriptional repression of equilibrative nucleoside transporters decreases overall equilibrative adenosine transport capacities, thereby decreasing intracellularly directed adenosine transport (Eltzschig et al. 2005). It is reasonable that both mechanisms could function during hypoxia and, from this perspective, contribute synergistically to elevate extracellular adenosine during hypoxia and to increase the levels of free adenosine in the tumor extracellular fluid (Blay et al. 1997). In particular, it has been shown, using microdialysis of tumors growing *in vivo*, that adenosine concentrations in the tumor interstitial fluid are 20–30-fold higher than in the adjacent connective tissue. The concentration of adenosine measured in tumor extracellular fluid is of 1–10 μM (Blay et al. 1997).

2. The accumulation of adenosine in hypoxic tissues can also be explained by the hypoxia-mediated upregulation of 5′-nucleotidase activity, an enzyme that converts AMP to adenosine, which results in the accumulation of extracellular adenosine (Sitkovsky et al. 2004).

The levels of extracellular adenosine could increase step-wise up to micromolar levels as the outcome of the transport and/or diffusion of intracellular adenosine, formed from the large pools of intracellular ATP in hypoxic conditions (Sitkovsky et al. 2005, 2008). Hypoxia can upregulate an adenine nucleotide-metabolizing ecto-enzyme cascade comprising ecto-ATP apyrase (CD39) and CD73 (Synnestvedt et al. 2002).

An alternative potential source of extracellular adenosine is the adenosine-3′,5′-cyclic adenosine monophosphate (cAMP), which after being released from cells can be subsequently converted to adenosine (Brundege et al. 1997).

Furthermore, hypoxia increases extracellular adenosine half-life (Eltzschig et al. 2005).

In conclusion, hypoxia appears to induce a program which shifts the cellular phenotype toward an increase in intracellular adenosine.

16.6 Tumor Cells in Hypoxia: Hypoxia-Inducible Factor-1, HIF-1

A major goal in developing new cancer chemotherapeutics is to identify and target biological processes that differ between normal and malignant cells. Ideally, therapeutics should be directed against pathways that have limited redundancy and that are required for development by a broad range of tumors. To survive under hypoxic conditions, tumor cells run numerous adaptive mechanisms, such as glycolysis, glucose uptake, and survival factor up-regulation (Hockel and Vaupel 2001). Hypoxic adaptation involves gene induction via which up-regulates $\approx$60 genes by binding to 5′-RCGTG-3′ sequences in hypoxia response elements (Semenza 2002). Cancer biologists are therefore becoming increasingly interested in the hypoxia-inducible factor (HIF) transcriptional system. This follows the recognition that HIF is upregulated across a broad range of cancers and is involved in key aspects of tumor biology such as angiogenesis, invasion and altered energy metabolism. Hypoxia-inducible factor (HIF)-1 is a transcription factor that functions as a master regulator of oxygen homeostasis (Semenza 2003, 2008).

HIF-1 is a heterodimer composed of an inducibly-expressed HIF-1α subunit and a constitutively-expressed HIF-1β subunit (Epstein et al. 2001). HIF-1α and HIF-1β mRNAs are constantly expressed under normoxic and hypoxic conditions (Wiener et al. 1996).

The unique feature of HIF-1 is the regulation of HIF-1α expression: it increases as the cellular O_2 concentration is decreased (Minchenko et al. 2002; Semenza 2000). During normoxia, HIF-1α is rapidly degraded by the ubiquitin proteasome system, whereas exposure to hypoxic conditions prevents its degradation (Minchenko et al. 2002). The enzymatic hydroxylation of proline 564 of HIF-1α controls the turnover of the protein by tagging it for interaction with the von Hippel Lindau (VHL) protein (Ivan et al. 2001; Jaakkola et al. 2001; Yu et al. 2001). When cells are hypoxic, the proline residue is not hydroxylated and HIF-1α protein accumulates. The VHL protein forms a multiprotein complex that acts as the ubiquitin ligase that targets HIF-1α for degradation. The effect of hypoxia on Pro-564 hydroxilation can be mimicked by transition metals like cobalt, iron chelators and by inhibitors of the prolyl hydroxylase enzymes (Ivan et al. 2001; Jaakkola et al. 2001).

HIF-1α expression and activity are also regulated by phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signal transduction pathways (Zhong et al. 2000; Semenza 2002).

A growing body of evidence indicates that HIF-1 contributes to tumor progression and metastasis (Hopfl et al. 2004; Welsh and Powis 2003; Maynard and Ohh 2007). Immunohistochemical analyses have shown that HIF-1α is present in higher levels in human tumors than in normal tissues (Zhong et al. 1999). In particular, the levels of HIF-1 activity in cells are correlated with tumorigenicity and angiogenesis in nude mice (Carmeliet et al. 1998). Tumor cells lacking HIF-1 expression are markedly impaired in their growth and vascularization (Jiang et al. 1997; Maxwell et al. 1997; Ryan et al. 1998; Kung et al. 2000). Therefore, since HIF-1α expression

and activity appear central to tumor growth and progression, HIF-1 inhibition becomes an appropriate anticancer target (Semenza 2003, 2007; Kung et al. 2000; Ratcliffe et al. 2000).

16.7   HIF-1 and the A_3 Receptor

Hypoxia creates conditions that, on one hand, are conducive to the accumulation of extracellular adenosine and, on the other hand, stabilize hypoxia-inducible factors, such as HIF-1α (Linden 2001; Sitkovsky et al. 2004; Fredholm 2003; Hockel and Vaupel 2001; Minchenko et al. 2002; Semenza 2000). HIF-1, the most important factor involved in the cellular response to hypoxia, has been extensively studied this last decade. However, despite the substantial number of investigations into HIF-1, many secrets about its function remain to be revealed.

The actions of adenosine are most prominent in tissues where oxygen demand is high and there is reduction in oxygen tension, that is within solid tumors. In particular, it is recognized that significant levels of adenosine are present in the extracellular fluid of solid tumors (Blay et al. 1997), suggesting a role for this nucleoside in tumor growth. Adenosine mediates its effects through interaction with four adenosine receptor subtypes. Interestingly, it has been demonstrated that the A_3 adenosine receptors are overexpressed in several cancer cell lines as well as in cancer tissues in comparison to normal mucosa (Merighi et al. 2001; Gessi et al. 2001, 2004, 2007). In cancer, the rapid growth of solid tumor frequently results in poor vascularization which creates substantial regions of hypoxia and ischemia that are conducive to adenine nucleotide breakdown, responsible for the adenosine release.

Recent studies support a prominent role for the A_3 receptor as a mechanism to amplify HIF-1 signaling under hypoxic conditions.

It has been specifically focused on responses to chronic hypoxia that involve adenosine-induced changes in the transcription regulator HIF-1 expression. In particular, it has been investigated the correlation between adenosine receptor stimulation and/or blockade and HIF-1α expression modulation in hypoxia. It has been demonstrated that adenosine is able to increase HIF-1α protein expression in response to hypoxia in a dose- and time-dependent manner in human melanoma, glioblastoma and tumor colon cells (Merighi et al. 2005, 2006, 2007). These results indicate that the cell surface A_3 adenosine receptor transduces extracellular hypoxic signals into the cell interior.

In many types of cancers, the HIF-1 pathway is not only activated by hypoxia, it is also induced by a wide range of growth-promoting stimuli and oncogenic pathways. Increased HIF-1α protein synthesis through the activation of Akt or MAPKinase pathways is a common theme accounting for the up-regulation. To evaluate how A_3 receptor accumulates HIF-1α in hypoxia, it has been investigated the signaling pathway generated by A_3 receptor stimulation. It has been found that p44/p42 and p38 MAPKinase activity is required for the HIF-1α expression increase induced by A_3 receptor activation in melanoma and glioblastoma cells and p44/p42, p38 MAPKinase and pAkt activity in colon carcinoma cells (for review see Gessi et al. 2008) (Fig. 16.1).
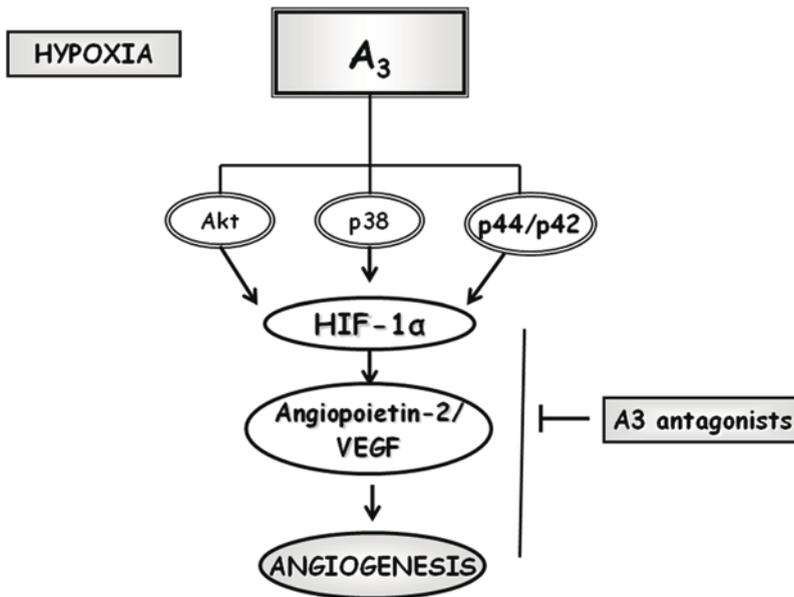
Fig. 16.1 Intracellular mechanism of A_3-mentioned protumoral effect and anticancer effect of A_3 antagonists

16.8 A_3 Receptor and the Angiogenic Response

Angiogenesis is a complex process that involves multiple gene products expressed by different cell types (Conway et al. 2001).

Blockade of angiogenesis is useful in tumor therapy (Ferrara and Kerbel 2005; Menakuru et al. 2008). Over the last decade, the most extensively examined proangiogenic molecule has been VEGF, a secreted protein that, through activation of tyrosine kinase receptors, promotes key events in angiogenesis that include increases in vascular permeability, and stimulation of endothelial cell proliferation and migration (Ferrara 2003).

There is now strong evidence that adenosine, in addition to controlling oxygen delivery acutely by regulating vascular tone, serves a long-term role by enhancing vascular growth in areas with reduced oxygen tension (Adair 2005). Thus, adenosine in physiologically relevant concentrations can stimulate migration and proliferation of endothelial cells. Interestingly, adenosine acting through its receptors also regulates endothelial cell function and promotes angiogenesis. As with VEGF, adenosine-promoted angiogenesis may be considered beneficial in contexts such as wound healing and myocardial ischemia, or detrimental in disease states such as cancer or in

retinopathy of prematurity (Adair 2005). Stimulation of angiogenesis may be achieved by stimulating A_{2A} and/or A_{2B} receptors and recently also A_1 in a variety of cells including endothelial, smooth muscle cells, bronchial epithelial cells, macrophages, monocytes through both HIF-independent and HIF-dependent pathways (Feoktistov et al. 2002, 2004; Montesinos et al. 2004; Allen-Gipson et al. 2006; Clark et al. 2007; for review see Auchampach 2007). However there is evidence for a role of the A_3 receptor and HIF-1 in angiogenesis when cancer cells are studied (Merighi et al. 2005, 2006, 2007). In particular, adenosine, released during tissue injury, ischemia and tumor growth, is able to promote angiogenesis by stimulation of angiopoietin-2 secretion via A_3 receptors (Feoktistov et al. 2003; Merighi et al. 2005). It has been proposed that the effect of VEGF on new capillary formations is facilitated by the concomitant stimulation of A_{2B} and A_3 receptors that induce the expression of angiopoietin-2 (Feoktistov et al. 2003). Furthermore, activation of the A_3 adenosine receptor subtype in glioblastoma and colon cancer cells stimulates VEGF expression (Merighi et al. 2006, 2007), whereas this receptor subtype promotes VEGF downregulation in PC12 pheochromocytoma cells (Olah and Roudabush 2000). Furthermore, the activation of A_3 receptors results in increased expression of angiopoietin-2 in mast and melanoma cells (Feoktistov et al. 2003; Merighi et al. 2005).

Although adenosine may contribute rather little to the increase in VEGF induced by hypoxia, it may contribute as much as 50% to angiogenesis (Adair 2005; Auchampach 2007). This could mean that adenosine acts also independently of VEGF, something that is not unlikely given the involvement of multiple cell types and multiple angiogenetic factors.

Recent studies indicate that pharmacologic inhibition of HIF-1α and particularly of HIF-regulated genes, that are important for cancer cell survival, may be useful to improve cancer treatment outcomes. However, the use of HIF-1 inhibitors as anticancer agents must occur within the conceptual framework of combination therapy, as administration of multiple agents simultaneously is essential for the successful treatment of human cancer (Semenza 2007). In this regard, it should be underlined that the A_3 adenosine receptor antagonists are able to block HIF-1α, Angiopoietin-2 and VEGF protein expression accumulation in hypoxia, indicating a new approach for the treatment of cancer, based on the cooperation between hypoxic and adenosine signals.

16.9 A_3 Receptor and the Immunosuppression

The resistance of many human cancers to immunotherapies has been attributed to the presence of immunosuppressive molecules located in tumor areas. Adenosine is present at elevated levels in hypoxic tissues due to an increased intracellular production and extracellular accumulation, as described above. This nucleoside activates cell surface receptors on T and NK cells that mediate cellular immune responses to tumor cells. It is well established that T cells recognize and destroy

cancer cells *in vitro* whilst fails to do so *in vivo*. This mechanism of tumor protection has been attributed to the immunosuppressive role played by adenosine that inhibits T lymphocytes activation, including adhesion to tumor cells and cytotoxic activity. Blay and coworkers have been the first to hypothesized that elevated levels of adenosine in solid tumors might be responsible for impaired destruction of tumor cells by immune effector cells. They identify the A_3 receptor subtype as the one responsible for these effects suggesting a role for A_3 antagonists in the immunotherapies of tumors (for review see Hoskin et al. 2008). Conversely, recently it has been reported that genetic deletion of immunosuppressive A_{2A} and A_{2B} receptors can prevent the inhibition of anti-tumor T cells thus suggesting a role for A_2 antagonists to improve full tumor rejection (Sitkovsky and Ohta 2005, Ohta et al. 2006). As described in detail in the chapter on immune cells (lymphocytes paragraph) of the present book the identification of adenosine receptor subtypes through which adenosine exerts its inhibitory effects on cell-mediated anti-tumor immune responses need further investigations at least for what concerns A_3 receptors and will allow us the development of specific pharmacologic approach to improve tumor rejection by antitumor cells.

16.10 Conclusions

To conclude, the efficacy of Cl-IB-MECA in several tumor animal models, especially HCC, prompted the introduction of this molecule into a program of pre-clinical and clinical studies. The excellent safety profile in pre-clinical animal studies and human Phase I in healthy subjects led to the initiation of Phase I/II studies in patients with HCC, currently ongoing.

On the other hand, from the results summarized in this chapter, it appears evident that also the future for generation of A_3 receptor antagonists in the treatment of human cancer can be considered promising, even though the clinical efficacy still remains to be demonstrated.

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