The Anti-Cancer Effect of A_3 Adenosine Receptor Agonists: A Novel, Targeted Therapy

P. Fishman[1,*], K.A. Jacobson[2], A. Ochaion[1], S. Cohen[1] and S. Bar-Yehuda[1]

[1]*Can-Fite BioPharma Ltd., Kiryat-Matalon, Petah -Tikva, 49170, Israel and* [2]*Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes & Digestive & Kidney Diseases, National Institutes of Health, Bethesda, Maryland, USA*

Abstract: The A_3 adenosine receptor (A_3AR) is highly expressed in various human solid tumor cells whereas low expression is found in the adjacent normal tissues. Activation of the A_3AR with synthetic highly selective agonists, such as IB-MECA, Cl-IB-MECA or LJ529, induces tumor growth inhibition of melanoma, lymphoma, breast, hepatoma, prostate and colon carcinoma cells both *in vitro* and *in vivo*. Two molecular events take place upon receptor activation and include: a. receptor internalization and subsequent degradation, followed by decreased receptor mRNA and protein expression level. b. modulation of down-stream signal transduction pathways, including those related to Wnt and NF-κB. Subsequently, the levels of cyclin D1 and c-Myc are decreased leading to tumor growth inhibition. IB-MECA synergizes with chemotherapeutic agents to yield an additive anti-tumor effect and protects against myelotoxicity induced by chemotherapy. Taken together, A_3AR agonists may be suggested as a new family of orally bioavailable compounds to be developed as potent inhibitors of malignant diseases.

Key Words: A_3 Adenosine receptor, synthetic agonists, anti-cancer effect, PKB/Akt, Wnt, NF-κB.

INTRODUCTION

The limited response of malignant tumors to conventional chemotherapy led to the development of new approaches based on the understanding of special targets involved in the proliferation of tumor cell motility, invasion and metastasis. Utilizing agents that will bind to and modulate the activity of receptors that are over-expressed on the surface of tumor cells can reduce damage to normal tissues and increase the efficacy of the drugs. Therefore, targeted therapies that specifically involve tumor cell-surface receptors and downstream signal transduction pathways have been developed. The earliest receptor targeted by this approach was the estrogen receptor in breast cancer. Monoclonal antibody drugs against members of the growth receptor family were recently developed for breast and colon cancer malignancies [1-3].

Most of the agents targeting cell-surface proteins developed lately can be classified into two main categories: monoclonal antibodies and small molecules. Small molecules are preferred in the development of targeted therapy for cancer due to better tumor penetration, avoidance of host immune effects, and the ability to systematically modify the molecular structure providing stability and greater flexibility in drug design, thus increasing specificity [4,5].

Adenosine (9-β-D-ribofuranosyladenine), a purine nucleoside, mediates various physiological cellular activities, such as cell growth, differentiation and cell death [6,7]. This small molecule is released into the extracellular environment from activated or metabolically stimulated cells and binds to selective G-protein-coupled membrane receptors, designated A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors [8]. Adenosine induces a direct anti-prolifera-tive effect on various tumor cell types at micromolar (μM) concentrations. Indirectly, it affects tumor development *via* its capability to affect cytokine release, cell migration, angiogenesis and chemotaxis. Moreover, adenosine induces activation or suppr-ession of T killer or natural killer cells which affect tumor cell development [9-13].

It has been shown that the A_3AR is abundantly expressed in tumor vs. normal cells, a characteristic which prompted investi-gators to examine the effects of specific agonists to this receptor on the growth of normal and tumor cells. The rationale was that targeting the receptor with a low dose of highly selective and specific agonist, will exclusively affect A_3AR. Various types of solid tumor cells were inhibited upon receptor activation whereas normal proliferating cells such as bone marrow or fibroblasts were stimulated [14, 15]. This specific response served as the initiative to assess the pharmacological effects of synthetic A_3AR ligands on the growth of various solid tumor cells in experimental animal models.

This review will focus on the activity of synthetic A_3AR agonists which possess high affinity and selectivity to the A_3AR. Their inhibitory effect on tumor growth *in vitro* and *in vivo* and the mechanism involved are presented.

I. THE TARGET

A. A_3AR - Member of the Adenosine Receptor Family

Medicinal chemistry has provided thousands of adenosine analogues that are potent selective activators of specific receptors of this group. These include widely-used agonists such as : CCPA which is an A_1AR selective agonist possess-

*Address correspondence to this author at the Can-Fite BioPharma, 10 Bareket St., P.O.Box 7537, Petach-Tikva 49170, Israel; Tel: 972-3-9213501; Fax: 972-3-9213567; E-mail: pnina@canfite.co.il

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ing a Ki of 0.8 nM to the A_1AR, a Ki of 2,300 nM to the $A_{2A}AR$ and a Ki of 32 nM to the A_3AR; CGS21680 which is an $A_{2A}AR$ selective agonist possessing a Ki of 290 nM to the A_1AR, a Ki of 27 nM to the $A_{2A}AR$ and a Ki of 67 nM to the A_3AR; IB-MECA which is an A_3AR selective agonist possessing a Ki of 51 nM to the A_1AR, a Ki of 2,900 nM to the $A_{2A}AR$ and a Ki of 1.8 nM to the A_3AR; NECA which activates both $A_{2A}AR$ and $A_{2B}AR$ possessing a Ki of 14 nM to the A_1AR, a Ki of 20 nM to the $A_{2A}AR$ and a Ki of 25 nM to the A_3AR [16].

The four members of this G protein-coupled family of adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3,, were classified by both functional characterization and gene cloning experiments [16]. The A_1 adenosine receptors inhibit adenylyl cyclase (AC) and activate phospholipase C (PLC), while both A_{2A} and A_{2B} adenosine receptors are coupled positively to AC to mediate the stimulation of cAMP generation [17-21]. The A_3AR was the subtype most recently cloned and is distinct in that it exhibits dramatic species differences in both tissue distribution and pharmacological responses [22-25]. The A_3AR is negatively coupled to adenylyl cyclase as shown by its ability to mediate inhibition of forskolin-induced generation of cAMP in Chinese hamster ovary (CHO) cells that were stably transfected with the cloned receptors. This leads to a decreased level of the down-stream kinase PKA, an initiator of various signal transduction pathways, which acts as the effector of cAMP [26-28]. The basal adenosine level in interstitial fluid is between 30 and 300 nM and may increase by several orders of magnitude during hypoxia and ischemia, thus there may be varying degrees of stimulation of the A_3AR by endogenous adenosine. It was previously shown that the cell response to a given adenosine agonist (either the natural ligand adenosine or a synthetic analogue) is strongly affected by the number of cell surface receptors. Since most normal cells express low levels of the A_3AR, this AR subtype is most probably inactive under physiological conditions except when adenosine levels are increased upon stress to the tissue [29-34].

B. A_3AR is Highly Expressed in Tumor Cells

Low A_3AR expression was detected in most body normal tissues, while high levels were found in various tumor cells, including astrocytoma, HL-60 leukemia, B16-F10 and A378 melanoma, human Jurkat T cell lymphoma and murine pineal neoplastic cells [35-42]. In recent studies, we compared side-by-side A_3AR expression levels in tumors vs. normal tissue. High A_3AR mRNA expression levels were found in melanoma, colon, breast, renal, ovarian, small cell lung and prostate carcinoma, as well as in hepatoma, in comparison to the normal adjacent and normal relevant tissue derived from healthy subjects. Moreover, receptor mRNA was over-expressed in the regional lymph node metastases in comparison to the primary tumor tissue [12].

A support for the above notion that the A_3AR is highly expressed in the tumor vs. adjacent normal tissue was obtained from the analysis of fresh tumors of colon and breast carcinoma. Protein analysis, utilizing Western blot, revealed that in more than 60% of the samples, higher A_3AR expression was found in the tumor vs. adjacent normal tissue. This was further strengthened by information obtained from a database search showing a 2.3-fold increase in the expression of A_3AR in human colon adenoma vs. normal colon tissue utilizing microarray analysis (Princeton University database). A search in the CGAP (The Cancer Genome Anatomy project; SAGE Genie; Virtual Northern Legend) based on SAGE revealed that the A_3AR was abundant under normal conditions in the brain, kidney, lung, germ cells, placenta and retina, however, brain, lung and pancreatic tumors expressed more A_3AR in the malignant than the relevant normal tissues. Additional searching in Expression Viewer (HCNC; CleanEX) based on ESTs revealed that the relative expression for the A_3AR was 1.6-fold higher in all cancer tissues compared to normal tissues [12]. Moreover, it was found that the A_3AR expression level tends to increase according to the stage of the disease, i.e., lower expression in colorectal carcinoma at stage I or II and higher in stage III or IV. Also, the A_3AR expression level was found to be higher in large adenomas in comparison to small size adenomas. Interestingly, the elevated A_3AR expression level in colorectal carcinoma tumors was reflected in peripheral blood mononuclear cells (PBMNC) of the same individuals. Colorectal cancer patients exhibited a high expression level of the receptor in their PBMNC in comparison to PBMNC derived from healthy subjects. The high expression level of the receptor returned to the level noted in healthy subjects upon surgical resection of the tumor [14]. High receptor expression may be a result of up-regulation in the level of TNF-α which thereby induces increase in NF-κB level. The latter was recently found in bio-informatic studies to be present in the promoter of the A_3AR gene. It does induces the transcription of A_3AR.

C. Receptor Fate Upon Chronic Activation

It is well established that G_i protein-coupled receptors are internalized into lysosomes and early endosomes following agonist binding. Early endosomes serve as the major site of receptor recycling, whereas the late endosomes are involved in the delivery of the internalized receptor to the lysosomes, thus sorting it to degradation [43-45]. Thus, it seems that chronically targeting a G_i protein-coupled receptor may lead to loss of a functional receptor from the cell surface. Confocal microscopy and radioligand binding studies with B16-F10 melanoma cells showed that, shortly after IB-MECA treatment, the receptor was internalized and subsequently sorted to the endosome and lysosome. Receptor degradation then occurred followed by re-synthesis and recycling of the receptor to the cell surface [37]. Moreover, in tumor lesions excised from melanoma, colon or prostate carcinoma-bearing mice treated chronically with IB-MECA, the A_3AR was down-regulated temporarily shortly after treatment but was fully expressed 24 hours later [36, 37]. Taken together these results suggest that receptor is internalized and degraded upon agonist activation and re-synthesized and recycled to the cell surface to act as a functional receptor. This may lead to the conclusion that complete tachyphylaxis does not occur upon chronic activation of A_3AR, suggesting the latter as a target to combat tumor cell growth. This mechanism of receptor down-regulation can also explain how antagonists to the A_3AR can induce the same anti-tumor effect of the agonist. In fact the antagonist, *via* blocking the A_3AR will evoke the same effect generataed by the agonist, leading to tumor growth inhibition. The approach of utilizing antagonists to combat cancer was lately presented by Borea *et al.* [46].

II. THE LIGANDS

The natural ligand adenosine has a relatively low affinity at the A_3AR, under physiological conditions, in comparison to its synthetic analogues and in comparison to adenosine binding to A_1 and $A_{2A}AR$ subypes [47,48]. In addition adenosine has a short half-life *in vivo* of approximately 20 sec, thus it cannot be considered as an anticancer drug candidate. The group of Jacobson *et al.* was the first to synthesize high affinity and selectivity A_3AR agonists. Fig. (1) depicts the chemical formula of three synthetic A_3AR agonists, IB-MECA, Cl-IB-MECA and LJ-529, all of which showed anticancer effects [49-52]. The A_3AR affinity and selectivity of IB-MECA was enhanced by further structural modifications, i.e. the addition of a 2-chloro on the adenine ring or the subsequent substitution of the ribose oxygen with sulfur, leading to Cl-IB-MECA and LJ-529, respectively.

Cl-IB-MECA displayed a *Ki* value of 0.33 nM and showed 2500- and 1400-fold selectivity for the rat A_3 receptor versus rat A_1 and A_{2A} ARs, respectively. Later, this derivative was found to have a *Ki* value of 1.4 nM at the human and 150- and 3800-fold selecti-vity in comparison to the human A_1 and A_{2A} receptors, respectively [47, 53]. This nucleoside is now being used extensively as a pharmacological tool for studying A_3 receptors that is independent of the species being studies. LJ-529 (also designated LJ-568), which is the 4′-thio analogue of Cl-IB-MECA, was synthesized by Jeong *et al.* by an entirely different route from the 4′-oxonucleosides, starting with D-gulonic γ-lactone. It was slightly more potent than Cl-IB-MECA in binding affinity with a *Ki* value of 0.38 nM at the human A_3AR and was also selective in comparison to human A_1 and human A_{2A} receptors by 510-and 580-fold, respectively. All three nucleoside derivatives had potency >10 μM at the human $A_{2B}AR$. In functional assays in transfected CHO cells, they were full agonists in the human A_3 receptor-mediated inhibition of cyclic AMP: The relative maximal efficacies under similar conditions were reported as: 100% (IB-MECA); 100% (Cl-IB-MECA) and 114 ± 9% (LJ-529).

III. ANTI CANCER EFFECTS

A. *In Vitro*

Both tumor and normal cells are inhibited by micromolar concentrations of A_3AR agonists. The inhibitory effect of IB-MECA and Cl-IB-MECA was demonstrated in HL-60 myeloid leukemia, Jurkat lymphoma, ADF astrocytoma, A375 human melanoma cells and Li-7A human hepatoma cells. The first demonstration of apoptosis induced by an A_3AR agonist was in HL-60 cells. This growth inhibitory effect occurred only at micromolar concentrations of Cl-IB-MECA, and was later shown to involve, at least partially, a non-AR-mediated mechanism [54]. In A375 human melanoma cells and Li-7A hepatoma cells, the anti-proliferative activity was partially abolished by a selective A_3AR antagonist. At lower, nanomolar concentrations, the A_3AR agonists protected cells against apoptosis [36, 55-61]. The anti-apoptotic effects of A_3AR activation in basophilic leukemia 2H3 mast cells have been studied mechanistically by Linden and coworkers [62].

Interestingly, in breast cancer cells that do not express the A_3AR, micromolar concentrations of agonists (LJ529 and IB-MECA) induced apoptosis independent of the presence of the A_3AR. This phenomenon was explained by the ability of IB-MECA to down-regulate the estrogen receptor thereby inducing the anti-cancer effect. However, there was no difference in the response of estrogen receptor-positive (MCF-7; T47D) or negative (MDA-MB-231; SK-BP-3) cells to the agonist. It was also suggested that in those cells that do not express the A_3AR, the agonist may enter the cells *via* nucleoside transporters and induce a growth inhibitory effect intracellularly. Although one study negates this approach (by demonstrating that dipyridamole, a nucleoside transporter inhibitor, was not able to neutralize the agonist effect) it is still an intriguing theory, which warrants further investigation [52, 63, 64].

IB-MECA and Cl-IB-MECA also induced cell proliferation inhibition and apoptosis of normal cells, such as rat astrocytes, cardiac myocytes and CHO cells transfected with the A_3AR [64-66].

A differential effect on tumor and normal cell growth was observed at nanomolar concentrations of IB-MECA and Cl-IB-MECA. Melanoma (B16-F10), hepatoma (N1S1), Lymphoma (Nb2-11C, Yac-1), Leukemia (K562), colon (HCT-116), prostate (PC3 and LnCap) and pancreatic (Mia-PaCa) carcinoma cells responded with proliferation inhibition. However, normal cells, such as murine or human bone marrow cells, were stimulated upon receptor activation with A_3AR agonists. The specificity of this response was demon-

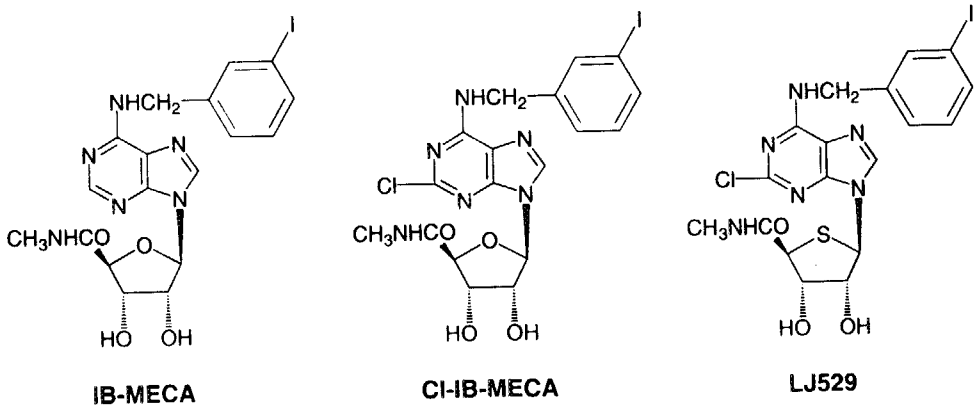

Fig. (1). The structure of 3 widely used selective A_3AR agonists.

strated in some of the cell lines by the introduction of an A_3AR antagonist to the culture system, which abrogated the inhibitory effect of the agonist. This differential effect may be explained by the high vs. low expression level in the tumor and normal cells, respectively. It seems that in cells over-expressing the A_3AR, a low agonist concentration will exclusively activate the A_3AR, leading to tumor growth inhibition whereas at high agonist concentrations both tumor and normal cell growth will be inhibited in a non-specific manner [5,14, 53, 68-74].

B. *In Vivo*

Oral administration of A_3AR agonists was shown to be efficacious in inhibiting the development of tumor growth utilizing various animal experimental models. IB-MECA inhibited tumor development in primary and metastatic murine and rat models, which included melanoma and colon and hepatocellular carcinoma. In xenograft models IB-MECA and Cl-IB-MECA inhibited the growth of human colon and prostate carcinoma (Table 1) [34,35,68,71,74,75]. Moreover, xenograft breast carcinoma tumors were inhibited upon treatment with the agonist LJ529. All A_3AR agonists examined exerted an inhibitory effect at μg/kg dose range. Interestingly, at a dose range of 50-5000 μg/kg, Chang *et al.* showed the same anti-tumor effect, suggesting that when a receptor-saturating concentration of agonist is administered *in vivo*, an increased agonist dose will not intensify the effect [52].

Table 1. A3AR Agonist Inhibit the Development of Tumor Growth in Various *In Vivo* Models

Tumor Type	Cell Line
Colon carcinoma	CT-26, HCT-116
Melanoma	B16-F10
Prostate carcinoma	PC3, Ln-CAP
Pancreatic Carcinoma	BxPC3
Hepatoma	N1S1

IV. MECHANISM OF ACTION

It is well established that A_3AR activation inhibits adenylyl cyclase activity and cAMP formation, which subsequently results in down-regulation of the effector protein kinase A (PKA). As a result, protein kinase B, the PKB/Akt, known to be phosphorylated by PKA is also down-regulated. In addition, a decreased expression level of PI3K was also reported in some tumor cells upon treatment with IB-MECA, thereby down-regulating the PKB/Akt levels. Two signaling pathways, related to Wnt and NF-κB, seem to play a major role in mediating the anti-cancer response of A_3AR agonists [35, 70, 75].

The Wnt signaling pathway controls cell fate during embryogenesis and tumorigenesis. Glycogen synthase kinase-3β (GSK-3β), a key element of the Wnt pathway, 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 [77-79].

PKB/Akt is also known to control 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 [77-79].

Overall, the Wnt and the NF-κB pathways are interconnected up-stream on the junction of PKB/Akt and down-stream at the level of cyclin D1 and c-Myc known to regulate cell cycle progression and apoptosis.

Interestingly, activation of the A_3AR by specific agonists results in a decreased expression level of PKA and PKB/Akt, which on one hand up-regulates both GSK-3β and ubiquitination of β-catenin, preventing its association with LEF/TCF and the transcription of cyclin D1 and c-Myc. On the other hand, the decreased level of PKB/Akt prevents the phosphorylation of IKK, thus maintaining the IκB-NF-κB complex in its sequestered form. This also results in a decreased cyclin D1 and c-Myc expression level (Fig. 2). De-regulation of these two pathways was observed in melanoma, hepatoma, colon, prostate and breast carcinoma leading to cell growth inhibition. *In vivo* studies carried out with melanoma and colon and prostate carcinoma confirmed that treatment of tumor-bearing animals with IB-MECA yielded tumor growth inhibition *via* de-regulating the Wnt and the NF-κB pathways. This was concluded based on the analysis of key signaling proteins extracted from tumor tissues upon IB-MECA treatment [34-36, 70].

V. COMBINATION OF CHEMOTHERAPY AND A_3AR AGONISTS

NF-κB and the upstream kinase PKB/Akt are highly expressed in chemo-resistance tumor cells and play a major role in hampering apoptosis of malignant cells. Since A_3AR agonists were shown to down regulate PKB/Akt and NF-κB protein expression level, their ability to sensitize tumor cells to chemotherapy was examined. IB-MECA enhanced the cytotoxic effect of 5-fluorouracil (5-FU) in colon carcinoma cells both *in vitro* and *in vivo*. Furthermore, myelotoxicity was suppressed in the 5-FU-treated animals, as was evidenced by normal white blood cell and neutrophil counts. This myeloprotective effect was described by Bar-Yehuda *et al.* and is attributed to the differential effect of A_3AR agonists and their ability to induce granulocyte-colony-stimulating factor (G-CSF) production [68,74, 80-82]. *In vivo* studies by Hofer *et al.* administering both 5-FU and IB-MECA in mice confirmed the potential of using this agonist for cancer therapeutics and demonstrated that the dosing and timing of IB-MECA treatment determined its effectiveness in stimulating granulopoiesis under conditions of myelosuppression [83, 84].

Interestingly, an A_3AR antagonist was also found to exert similar effect on tumor cells by enhancing the chemothera-

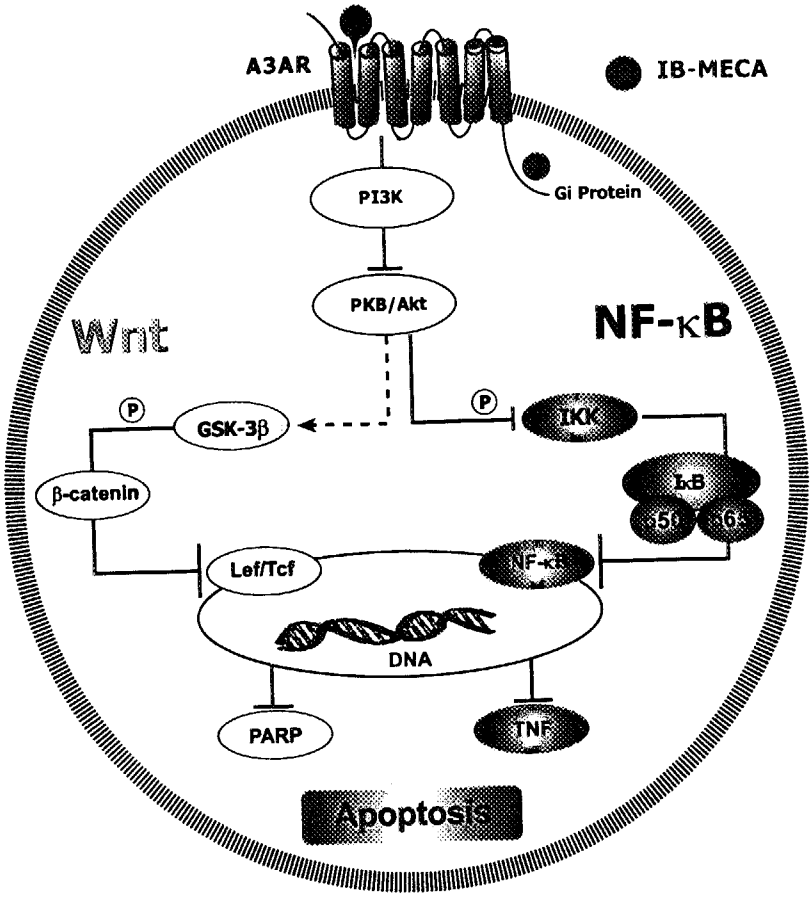

Fig. (2). The mechanism of the A_3AR-induced growth-inhibitory effect in tumor cells: Involvement of the NF-κB and Wnt signaling pathway.

peutic index of taxol and vindesine in a human melanoma cell line [85].

CONCLUSIONS

The A_3AR, which is expressed differentially on tumor and normal cells, may be suggested as a specific target to inhibit tumor cell growth. Highly selective A_3AR agonists exert anti-tumor as well as myeloprotective activity and synergize with chemotherapy to enhance the anti-tumor effect. The ability to utilize nucleoside derivatives as orally bioavailable synthetic agonists to target this receptor suggests the development of this distinctive family of small molecules as anti-cancer agents. Indeed, the A_3AR agonist CF102 (Cl-IB-MECA) is being now developed by the Biotechnology company Can-Fite BioPharma as a candidate to treat Hepatocellular carcinoma.

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ABBREVIATIONS

A_3AR	=	A_3 adenosine receptor
GSK-3β	=	Glycogen synthase kinase-3β
μM	=	Micromolar
nM	=	Nanomolar
NF-κB	=	Nuclear Factor – kappa B
PBMNC	=	Peripheral blood mononuclear cells
PKA	=	Protein Kinase A
PKB/Akt	=	Protein Kinase B/Akt

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