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Targeting the A3 Adenosine Receptor for Cancer Therapy: Inhibition of Prostate Carcinoma Cell Growth by A3AR Agonist

PNINA FISHMAN[1,2], SARA BAR-YEHUDA[2], ETI ARDON1, LEA RATH-WOLFSON[3], FAINA BARRER, AVIVIT OHAION[1] and LEA MADI[1]

[1]Can-Fite Biopharma Ltd, Kiryat-Matalon, Petach-Tikva; [2]Laboratory of Clinical and Tumor Immunology, The Felsenstein Medical Research Center, Tel-Aviv University Sackler Faculty of Medicine, Rabin Medical Center, Petach-Tikva; [3]Pathology Department, Rabin Medical Center Campus Golda, Tel-Aviv University Sackler Faculty of Medicine

Abstract. *Background: Agonists to A3 adenosine receptor (A3AR) were shown to inhibit the growth of various tumor cell types. The present study demonstrates that a synthetic A3AR agonist, 1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purine-9-yl]-N-methyl-β-D-ribofura-nuronamide (IB-MECA), inhibits the growth of androgen-independent PC-3 prostate human carcinoma cells and illustrates the molecular mechanism involved. Materials and Methods: PC-3 prostate carcinoma cells were used. Cell growth was examined in vitro by the thymidine incorporation assay and in vivo by inoculating the tumor cells subcutaneously into nude mice and monitoring tumor size. The protein expression level in cells and tumor extracts was tested by Western blot analysis. Results: A decrease in the protein expression level of A3AR and the downstream effector PKAc was observed. Consequently, the GSK-3β protein level increased, resulting in the destabilization of β-catenin and the subsequent suppression of cyclin D1 and c-myc expression. IB-MECA treatment also induced down-modulation of the expression of NF-kB/p65, known to regulate the transcription of cyclin D1 and c-Myc. This chain of events occurred both in vitro and in vivo and suggests the use of the above-mentioned signaling proteins as markers to predict tumor cell response to A3AR activation. Conclusion: Taken together, we demonstrated that A3AR activation de-regulates the Wnt and the NF-kB signaling pathways resulting in the inhibition of prostate carcinoma cell growth.*

Activation of the Gi-protein-coupled A3AR has been involved in the inhibition of tumor cell growth (1-3). A3AR is highly expressed in tumor cells whereas low expression has been noted in a variety of normal cells (4-6). We recently examined the relationship between receptor fate upon activation and receptor functionality in melanoma cells. A3AR activation, with the synthetic agonist IB-MECA, induced rapid receptor internalization to the cytosol. The receptor was then degraded, subsequently re-synthesized and recycled to the cell surface to serve again as a functional receptor. These events generated the modulation of key proteins involved in the Wnt and the NF-kB signal transduction pathways. A decrease in cAMP production and expression of the downstream effector protein kinase A (PKA) and protein kinase B (PKB/Akt) was observed (7-9). We found that when PKA and PKB/Akt were inhibited, GSK-3β level was up-regulated. This led to the phosphorylation and ubiquitination of β-catenin and a decrease in the expression level of cyclin D1 and c-myc, resulting in melanoma cell growth inhibition (3). Moreover, a decline in the expression level of NF-kB was also noted consequent to PKB/Akt down regulation (9). These results were confirmed in an experimental murine model in which IB-MECA inhibited the growth of B16-F10 melanoma metastatic foci in the lung and the development of subcutaneous primary tumor (9). Interestingly, in tumor lesions derived from IB-MECA treated mice, A3AR expression and the level of key signaling proteins (GSK-3β, β-catenin, NF-kB, cyclin-D1 and c-Myc) were modulated in a pattern corresponding to that observed *in vitro*. These studies demonstrated that there is a direct correlation between A3AR activation, modulation of the signaling proteins and the inhibition of tumor cell growth. We therefore defined 5 of these proteins (PKA, GSK-3β, NF-kB cyclin-D1 and c-Myc) as protein markers to predict the response of tumor cells to A3AR activation both *in vitro* and *in vivo* (9).

Prostate cancer is a common disease in Western countries (10,11) and it is highly resistant to chemotherapy. There is still no effective cure for patients with advanced prostate cancer especially in cases of hormone-independent tumors (12). The molecular mechanisms involved in the initiation, progression and development of prostate cancer are largely unknown. Recently, the Wnt and the NF-kB signaling pathways have also

Correspondence to: Pnina Fishman, Ph.D., Laboratory of Tumor Immunology, Felsenstein Medical Research Center, Rabin Medical Center, Petach-Tikva, 49100, Israel. Tel: 972-3-9241114, Fax: 972-3-9249378, e-mail: pfishman@post.tau.ac.il

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been implicated in the development of prostate carcinoma (13,14). It thus led us to study the effect of IB-MECA on the growth of the human androgen-independent PC-3 prostatic carcinoma cell line and to follow-up the modulation of the 5 protein markers defined above, both *in vitro* and *in vivo*

Materials and Methods

Reagents. IB-MECA and MRS 1523 were purchased from RBI/Sigma (Natick, MA, USA). For both reagents, a stock solution of 10mM was prepared in DMSO and further dilutions in RPMI medium were performed. RPMI, fetal bovine serum (FBS) and antibiotics for cell cultures were obtained from Beit Haemek, Haifa, Israel. Rabbit polyclonal antibodies against murine and human PKAc, c-myc and GSK-3β were purchased from Santa Cruz Biotechnology Inc., Ca, USA. The human and murine rabbit polyclonal antibodies against murine and human cyclin D1 and Rel-65 NF-κB were purchased from Chemicon, Ca, USA. Rabbit polyclonal antibodies against murine and human A3AR were purchased from Alph Diagnostics, San Antonio, USA.

Tumor cells and proliferation assay. PC-3 cells derived from a human androgen-independent prostate cancer cell line (American Type Culture Collection, Manassas, Virginia, USA) were grown in RPMI 1640 penicillin, streptomycin, 2 mM. L-glutamine and 10% fetal bovine serum (FBS). The cells were maintained in T-75 flasks at 37˚C in a 5% CO_2 incubator and transferred to a freshly prepared medium twice weekly. For all studies serum-starved cells were used. FBS was omitted from the cultures for 18 hours and the experiment was carried out on monolayers of cells in RPMI medium supplemented with 1% FBS in a 37˚C, 5% CO_2 incubator.

[^{3}H]-thymidine incorporation assay was used to evaluate cell growth. PC-3 cells ($1.5x10^4$/ml) were incubated with IB-MECA (0.01μM-10μM) in 96-well microtiter plates for 24 hours. To test whether IB-MECA exerted its effect on tumor cells through binding to A3AR, an antagonist to A3AR, MRS-1523 (0.1μM), was added to the cell cultures in the presence of IB-MECA. Cultures of PC-3 cells that were incubated in the presence of MRS-1523 only served as controls. For the last 18 hours of incubation, each well was pulsed with 1μCi [^{3}H]-thymidine. The cells were harvested and the [^{3}H]-thymidine uptake was determined in an LKB liquid scintillation counter (LKB, Piscataway, NJ, USA). These experiments were repeated at least 10 times.

Western blot analysis. To detect the level of expression of A3AR, PKA, GSK-3β, β-catenin, c-myc and cyclin D1, protein extract from IB-MECA treated or untreated serum-starved PC-3 cells were utilized. The cells were incubated in the presence and absence of IB-MECA for 15 minutes at 37˚C. At the end of the incubation period, the cells were then rinsed with ice-cold PBS and transferred to ice-cold lysis buffer (TNN buffer, 50mM Tris buffer pH=7.5, 150mM NaCl, NP 40 0.5% for 20 minutes). Cell debris were removed by centrifugation for 10 minutes, at 7500xg. The supernatant was utilized for Western blot analysis. Protein concentrations were determined using the Bio-Rad protein assay dye reagent. Equal amounts of the sample (50μg) were separated by SDS-PAGE, using 12% polyacrylamide gels. The resolved proteins were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked with 1% bovine serum albumin and incubated with the desired primary antibody (dilution 1:1000) for 24 hours at 4˚C. The blots were then washed and incubated with a secondary antibody for 1hour at room temperature. Bands were recorded using BCIP/NBT color development kit (Promega, Madison,W1, USA). The densitometry of protein expression was normalized against β-actin and expressed as % of control (0-time).

In vivo studies. The mice were maintained on a standardized pelleted diet and supplied with tap water. Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Can-Fite BioPharma, Petah Tikva, Israel.

Nude male Balb/C mice, aged 2 months, weighing an average of 25g were obtained from Harlan Laboratories, Jerusalem, Israel. PC-3 prostate carcinoma cells ($2.5x10^6$) were subcutaneously injected into the flank of the mice. When the tumor reached 150-200mm^3 in size, the animals were randomly assigned into different experimental groups. Two types of experiments were set up:

A. a study in which the effect of IB-MECA on tumor growth was evaluated in mice in which the tumor reached a size of 150-200mm^3. Treatment was given orally once daily for 26 days. This experiment included two groups:

1. Vehicle
2. IB-MECA (10μg/kg body weight).

Tumor size (width (W) and length (L)) was measured twice weekly with a caliber and calculated according to the following formula: Tumor Size = $(W)^2xL/2$. Each group contained 10 mice.

B. a study in which the effect of IB-MECA on the expression of tumor markers was evaluated shortly after one treatment in tumor-bearing mice. This experiment included three groups:

1. Vehicle-control.
2. IB-MECA (10μg/kg body weight) given once. Mice were sacrificed after 2hours.
3. IB-MECA (10μg/kg body weight) given once. Mice were sacrificed after 24hours.

At the end of each experiment the mice were sacrificed and tumors were excised, protein extracts were prepared as described above and analyzed for the expression profile of A3AR and the marker proteins (PKA, NF-kB,GSK-3β, β-catenin and cyclin D1).

Statistical analysis. The results were evaluated using the Student's *t*-test, with statistical significance at $p<0.05$. Comparison between the mean value of different experiments was carried out.

Results

IB-MECA inhibits PC-3 growth in vitro and in vivo. To evaluate the direct anti-proliferative effect of IB-MECA on the human androgen-independent PC-3 prostatic carcinoma cell line *in vitro*, we used the thymidine incorporation assay. IB-MECA exerted a dose-dependent inhibitory effect on the prostate carcinoma cells. The inhibition of cell growth was statistically significant at all concentrations tested ($p<0.001$). The A3AR antagonist MRS1523 reversed the inhibitory effect of IB-MECA, demonstrating that tumor growth suppression was specifically mediated through A3AR (Figure 1a).

In vivo, the treatment with IB-MECA started when the subcutaneously transplanted PC-3 tumors had grown to a volume of 150-200 mm^3. As shown in Figure 2a and b, IB-MECA sup-

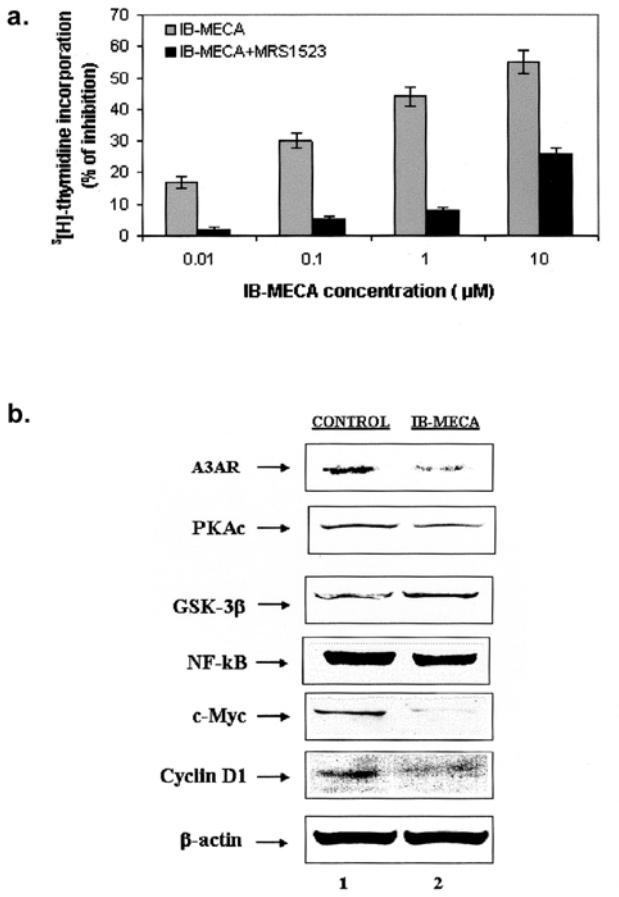


Figure 1. *(a) IB-MECA induces a dose-dependent inhibitory effect on the proliferation of PC-3 prostate carcinoma cells. PC-3 prostate carcinoma cells were depleted from serum for 18hours and treated with vehicle (control) or with various IB-MECA concentrations (0.01μM-10μM) in the presence of 1% FBS for 24hours. Cell proliferation was measured by [^{3}H]-thymidine incorporation assay. A3AR antagonist MRS-1523 (0.1μM) neutralized the inhibitory effect of IB-MECA. The data points are mean±SEM values from four independent experiments. (b) Expression level of A3AR and protein markers in PC-3 cells upon exposure to IB-MECA. Immunoblots showing the effect of 0.01μM IB-MECA on the expression level of A3AR, PKAc, GSK-3μ NF-kB, c-Myc and cyclin D1 in PC-3 prostate carcinoma cells. Serum starved cells (for 18 hours) were treated for 15 minutes with IB-MECA in the presence of 1% FBS.*

pressed growth of PC-3 tumors during the 26 days of treatment. At the end of the experiment, the mean volume of PC-3 tumors treated with IB-MECA was 69±37 mm^3, being significantly smaller than that in control group which measured 340±59 mm^3, the inhibition of tumor growth corresponding to 79.7% ($p<0.0001$, Figure 2a).

IB-MECA modulates tumor marker proteins upon A3AR activation. Shortly after A3AR activation with IB-MECA *in vitro*, the expression level of the receptor protein was down-regulated. Additional marker proteins, downstream to A3AR activation, were modulated, *i.e.*, PKA, NF-kB, c-Myc and cyclin D1 expression levels were decreased whereas GSK-3β level was up-regulated (Figure 1b).

In tumor lesions excised from mice treated daily for 26 days with IB-MECA, Western blot analysis revealed down-regulation of A3AR, PKAc, cyclinD1 and c-myc and up-regulation of GSK-3β expression level (Figure 2c). The level of the housekeeping protein β-actin did not change.

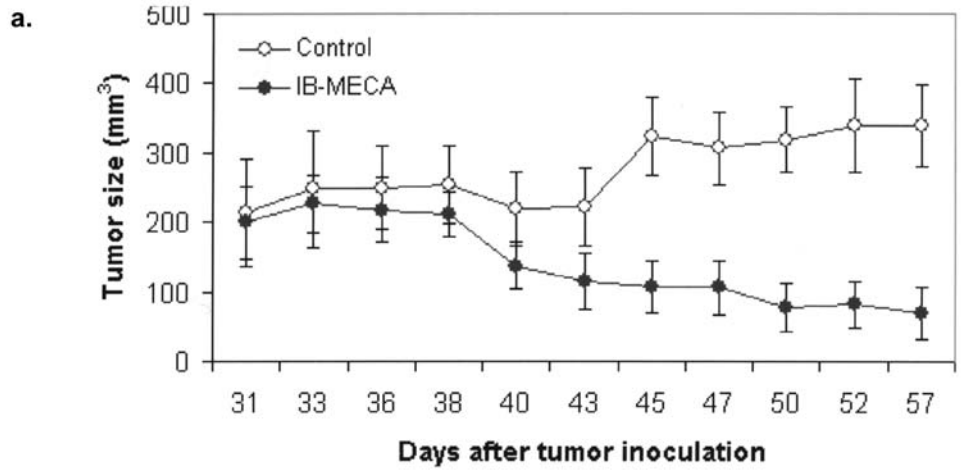
To explore the response of the above mentioned tumor proteins to one treatment of IB-MECA, mice with an already established tumor were treated only once with IB-MECA. Two hours after treatment, a marked down-regulation of A3AR, PKA, β-catenin, NF-kB, c-Myc and cyclin D1 was noted. Interestingly, 24 hours after IB-MECA administration, A3AR protein expression level was fully recovered to the control level, whereas the expression level of the other proteins was only partially recovered, and was lower than the control group.

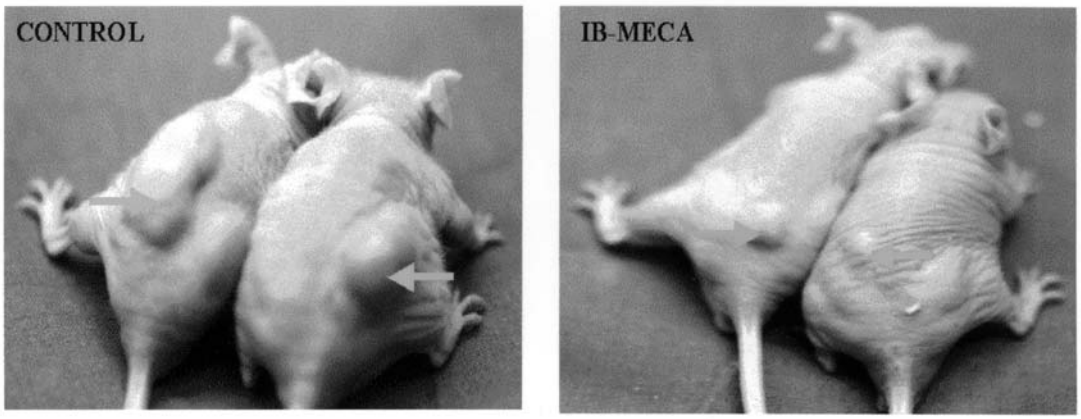
Discussion

The present study describes the ability of IB-MECA, a synthetic A3AR agonist, to inhibit the growth of prostate carcinoma cells in vitro and *in vivo*. A3AR belongs to the family of the Gi-protein-associated cell surface receptors. Receptor activation leads to internalization and the subsequent inhibition of adenylyl cyclase activity, cAMP formation and protein kinase Ac (PKAc) expression (15, 16). IB-MECA is a potent, stable and specific A3AR agonist due to a substitution at the N6 and 5' positions of adenosine. This structure protects the molecule against rapid metabolization by adenosine deaminase and further enhances its affinity to A3AR (17). A3AR expression level was found to be low in most body tissue, whereas tumor cells such as melanoma, T cell lymphoma and pineal tumor cells, significantly express A3AR (4-6). Receptor exhibition and spread is not the only factor determining cell response to a specific ligand. An additional parameter is the exhibition of A2A and A2B adenosine cell surface receptors, known to elicit opposite effects to that of A3AR. At high concentrations, A3AR agonists may also activate A2A and A2B adenosine receptors, affecting the balance of the response (18, 19).

In the present study, the dose-dependent growth inhibition observed in the PC-3 cells *in vitro* was obtained at low concentrations and was counteracted by the antagonist MRS1523. *In vivo*, IB-MECA generated the suppressive effect on tumor growth also at a low-dose (10μg/kg body weight). It is assumed that since IB-MECA possesses high affinity to A3AR (0.4nM), it activates this receptor exclusively at low concentrations.

Shortly upon IB-MECA activation, down-regulation of A3AR protein expression level was noted *in vitro*. This observation was confirmed in the *in vivo* studies in which we treat-





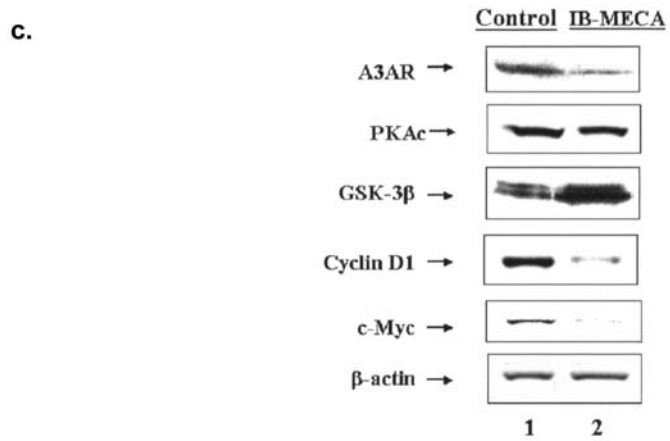


Figure 2. *(a) Inhibition of prostate carcinoma cell growth in mice and modulation of tumor protein markers in tumor lesions. (a) PC-3 prostate carcinoma cells (2.5x10^6) were subcutaneously injected into the flank of nude mice. One group was treated with IB-MECA (10μg/kg body weight) daily orally, starting when the tumor reached a size of 150-200 mm^3 and the other, treated with vehicle only, served as control. (b) Representative mice from the control (left) and the IB-MECA (right) treated mice, showing the difference in tumor size in the two groups. (c) Immunoblots showing the effect of IB-MECA on the level of A3AR, PKAc, GSK-3β, cyclin D1 and c-Myc in protein extracts derived from tumor lesions of prostate carcinoma bearing mice (description of the experiment is detailed in a).*

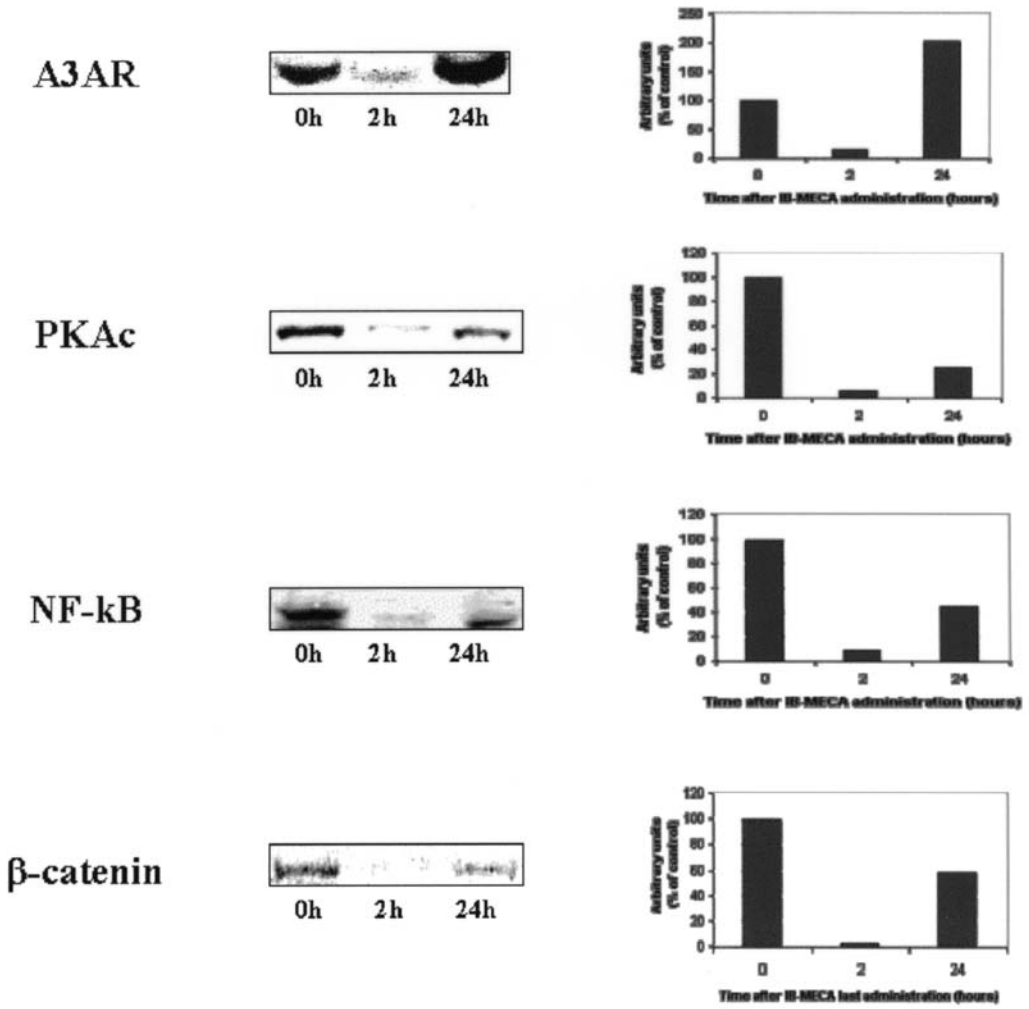


Figure 3. *Modulation of tumor protein markers in tumor lesions derived from IB-MECA-treated mice. The effect of IB-MECA (one treatment only, for 2 hours and 24 hours) on the expression of tumor protein markers was evaluated in tumor lesions excised from prostate carcinoma-bearing mice. Immunoblots showing the effect of IB-MECA on A3AR, PKAc, NF-kB, β-catenin, cyclin D1 are presented.*

ed tumor-bearing mice with IB-MECA. Receptor down-regulation is a general mechanism typical of Gi protein receptors. This family of receptors responds to ligand activation by receptor internalization (to the cytosol), degradation, re-synthesis and recycling to the cell surface (20). During these events, receptor desensitization/re-sensitization takes place and different signaling pathways are initiated (21, 22). We may suggest that the down-regulation of receptor expression in this study represents the rapid response of the prostate cells to agonist stimulation and the initiation of downstream responses.

Indeed, the prostate cells responded to A3AR activation by a decrease in PKAc level both *in vitro* and *in vivo*. PKAc is an effector protein involved in the initiation/regulation and cross talk between various signaling pathways. It phosphorylates and inactivates the enzyme GSK-3β (23), a key element in the Wnt signaling pathway (24). GSK-3β suppresses mammalian cell proliferation and survival by phosphorylating the cytoplasmic protein β-catenin, leading to its ubiquitination. GSK-3β in its inactive form does not phosphorylate β-catenin. The latter accumulates in the cytoplasm and subsequently translocates to the nucleus where it associates with Lef/Tcf to induce cyclin-D1 and c-myc transcription (25). In the present study we found that up-regulation of GSK-3β correlated with down-regulation of β-catenin, cyclin D1 and c-Myc. Davies *et al*. reported that there were no mutations within the binding regions between β-catenin and GSK-3β in PC-3 prostate carcinoma cells (26). Therefore, we concluded that there is an involvement of the Wnt pathway in the response of these cells to A3AR activation.

The expression level of NF-kB was down-regulated in both *in vitro* and *in vivo* studies. NF-kB is also linked to the effector protein PKAc. The most abundant form of NF-kB is a het-

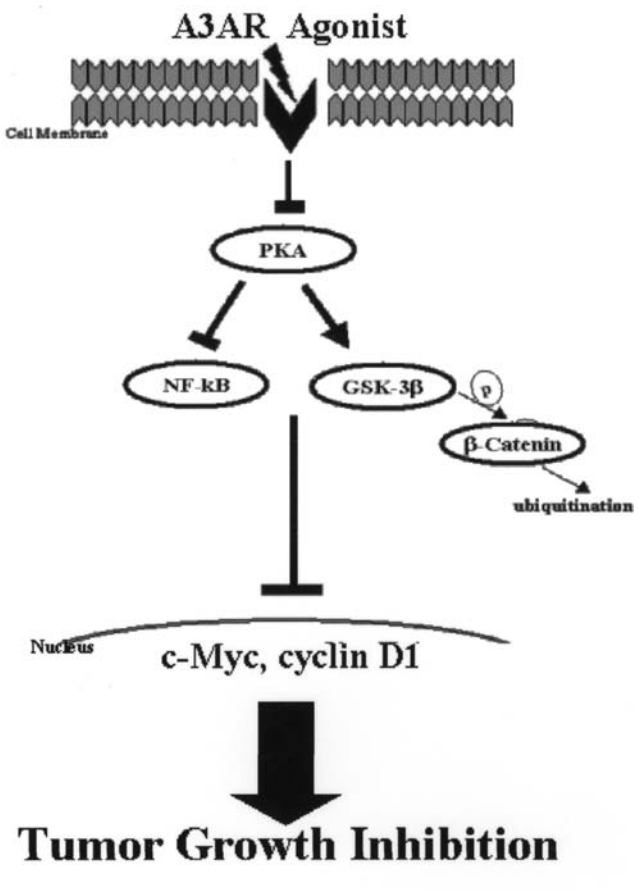


Figure 4. *Schematic representation of signaling pathways that mediate A3AR inhibition of melanoma cell growth.*

erodimer of p50 and p65 (Rel A) subunits in which the p65 contains the transcription activation domain. PKAc regulates the transcriptional activity of NF-kB by phosphorylating the p65 subunit of NF-kB, enabling its association with the co-activator CBP/p300 and the efficient transcriptional activity (27).

Previous reports have suggested that PC-3 prostate carcinoma cells and the androgen receptor-negative cell line (DU-145) have constitutive NF-kB activity (27, 28). Thus, the IB-MECA's capability to suppress NF-kB expression may serve as part of the mechanism through which it exerts an inhibitory effect on androgen-independent cells.

In vivo, the protein markers were significantly modulated upon a single or chronic exposure of the tumors to IB-MECA. One conclusion that can be drawn from this phenomenon is that these protein markers may serve as biomarkers for predicting the response of the tumor to IB-MECA in the host. These results provide a rationale to examine the protein markers in patients on IB-MECA treatment.

Collectively, these results suggest that IB-MECA inhibits the growth of prostate cancer cells *via* modulation of key proteins involved in the Wnt and NF-kB signaling pathway. These results corroborate our findings in other types of neoplasias (melanoma and colon carcinoma) and propose the use of A3AR agonists for the management of human prostate cancer.

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