Abstract. The A\textsubscript{3} adenosine receptor (A\textsubscript{3}AR) is highly expressed in tumors and was suggested as a target for cancer treatment. In this study, we show that A\textsubscript{3}AR is highly expressed in tumor tissues and in peripheral blood mononuclear cells (PBMCs) derived from patients with HCC, as well as from HCC tumor-bearing rats. The high expression level of the receptor was directly correlated to overexpression of NF-\kappaB, known as a transcription factor of A\textsubscript{3}AR. CF102, a synthetic highly selective agonist to A\textsubscript{3}AR induced a marked dose response inhibition of tumor growth in N1S1 HCC tumor rats, via de-regulation of the NF-\kappaB and the Wnt signal transduction pathways, resulting in apoptosis of tumor cells. Taken together, A\textsubscript{3}AR is highly expressed in tumors and PBMCs of HCC patients and tumor-bearing rats. CF102 induced apoptosis and tumor growth inhibition. These data suggest A\textsubscript{3}AR as a novel targeted therapy to treat HCC.

Introduction

Primary liver cancer is one of the most deadly malignancies in the world. Africa and Southeast Asia have highest incidence of HCC and recently it has become the most common cause of cancer death in Japan. In the USA it is the most rapidly increasing type of cancer. Hepatitis C virus (HCV) infection, alcohol use, non-alcoholic fatty liver diseases, and androgenic steroid use are the most common factors of cirrhosis, which is the leading predisposing factor for HCC (1,2).

The current available treatment for primary liver cancer include liver transplantation, surgical resection, trans-arterial chemo-embolization (TACE), administration of intra-arterial iodine-131-lipiodol, per-cutaneous treatment by ethanol injection or radio-frequency ablation, intra-hepatic Y90 micro-sphere and intra-hepatic chemotherapy (3-11). Unfortunately, when the tumor metastasizes, there is no adequate therapy (12).

The primary resistance features of HCC, and the dysfunction of the liver due to the cirrhosis limits the safe administration of chemotherapy. For that reason, there is a major need to develop a new non-toxic effective systemic therapy for this lethal disease.

Accumulative data have indicated that the Gi associated cell surface receptor, A\textsubscript{3} adenosine receptor (A\textsubscript{3}AR) plays an important role in controlling tumor growth. The A\textsubscript{3}AR was found to be highly expressed in different tumor cells in comparison with low expression in normal adjacent tissues (14). Targeting the A\textsubscript{3}AR with synthetic agonists such as CF101 or CF102, small orally bio-available molecules, induced inhibition in the growth of melanoma, colon and prostate carcinoma both in vitro and in vivo (15-25).

The aim of this study was to explore whether A\textsubscript{3}AR is a validated target in HCC by looking at the correlation between receptor expression and functionality. A\textsubscript{3}AR expression level was examined in tumor tissues and PBMCs of HCC patients and tumor-bearing rats and was found to be highly expressed. CF102 markedly inhibited N1S1 HCC tumor growth in a rat orthotopic model of HCC, demonstrating the functionality of the receptor. The role of NF-\kappaB in mediating receptor expression and functionality was explored.

Materials and methods

Drugs. The A\textsubscript{3}AR agonist 2-chloro-N\textsuperscript{6}-(3-iodobenzyl)-adenosine-S\textsuperscript{N}-methyl-uronamide (CF102) was synthesized...
for Can-Fite BioPharma by Albany Molecular Research Inc., Albany, NY, USA. A stock solution was prepared in DMSO and further dilutions in PBS were performed to reach the desired concentration.

Rabbit polyclonal antibodies against A3AR and the cell growth regulatory proteins PKB/Akt, IKK, NF-xB, TNF-α, GSK-3β, LEF-1, β-catenin, c-Myc, caspase 3 and β-actin were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA.

**Blood sample collection from HCC patients and separation.** Blood samples were collected from healthy subjects and from HCC patients. The blood sample collection was approved by the hospital's ethics committee and an informed consent was signed prior to blood withdrawal. Patients' particulars are summarized in Table I. To separate PBMCs, heparinized blood (20 ml) was subjected to Ficoll hypaque gradient. The PBMCs were then washed with PBS and protein extracts were prepared as is detailed below.

**Tumor cells.** N1S1 rat HCC cell line (American Type Culture Collection, Manassas, Virginia) was grown in RPMI-1640 containing 2 mM L-glutamine and 10% fetal bovine serum (FBS). The cells were maintained in T-75 flasks at 37°C in a 5% CO2 incubator and transferred to a freshly prepared medium twice weekly.

**In vivo studies.** Male Sprague-Dawley rats, weighing an average of 200 g were obtained from Harlan Laboratories, Jerusalem, Israel. The animals 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. A subxyphoid laparotomy was performed and N1S1 cells (5x10⁶/50 μl saline) were injected to the right hepatic lobe. The rats were divided randomly into a control group which was treated with vehicle only and to 5 additional group in which the animals were treated with CF102 at doses of 1, 50, 150, 500 and 1000 μg/kg thrice daily. In each group 8-10 rats were included and the study was repeated three times.

Treatments were initiated on day 3 after tumor inoculation and lasted till day 15th. At the end of the study, the liver was excised and tumor size, i.e., width [W], length [L] and height [H] were measured and calculated according to the following formula: Tumor size = (Width x Height x Length)/2.

At the end of the study, the tumors and PBMCs were collected from the vehicle and the CF102 treated groups, as well as liver tissues and PBMCs from naïve animals. Tumors were fixed in 10% formaldehyde for pathological staining. Protein extracts were subjected to Western blot analysis to evaluate the A3AR and additional key signaling protein expression levels.

**RT-PCR analysis of formalin-fixed paraffin-embedded HCC tissue slides.** A retrospective study aimed to evaluate the expression levels of A3AR in tumor tissues was conducted as described earlier (14). Briefly, paraffin-embedded slides were prepared and upon histological examination of H&E stained sections, neoplastic and normal areas from each sample were marked. Non-stained sequential slides (20 μm thick) were marked for neoplastic and normal tissue based on the stained slides. Tissue sections on slides were de-paraffinized in xylene and re-hydrated by washing in serial dilutions of ethanol. Slides were used immediately or stored at -80°C until used. After re-hydration, 20 μl of solution A [1.25X PCR buffer (200 Mm Tris-HCl, 500 mM KCl), 6.25 mM MgCl₂, 5 U RNasin (Promega, Madison, WI), 2 mM DTT, 1 U RQ1 RNase-free DNase (Promega)] was directly applied to the marked areas. The equal neoplastic and non-neoplastic marked areas were completely scraped off the slide using a pipette tip and the neoplastic tissue or normal tissue were collected into different microcentrifuge tubes. The samples were treated with proteinase K at a final concentration of 0.1 mg/ml. The samples were incubated at 37°C for 1 h to allow for DNA digestion. Cell lysates were heated to 95°C for 15 min in order to inactivate DNase and proteinase K. Following centrifugation at 14,000 RPM for 5 min, 25 μl of the supernatant was transferred to separate tube and 1 μl of RT mixture [5 mM dNTPs, 2.5 μM random hexamer, 5 U RNasin, 100 U SuperScript One Step RT-PCR with Platinum Taq (Invitrogen) and the primers for A3AR 5'-ACGGTGAG L77729, L77730).

To separate PBMCs from naive and tumor-bearing rats, heparinized peripheral blood was subjected to a negative control lacking RNA, using standard techniques and by sequencing the sequences to that of the known sequences (ADORA3-L77729, L77730).

The RT reaction was performed at 45°C for 45 min, followed by heating to 99°C for 5 min. Fifty cycles of 94°C for 30 sec, 59°C for 45 sec and 73°C for 45 sec were performed. Products were electrophoresed on 2% agarose gels in comparison to a positive control from RNA extracted and to a negative control lacking RNA, using standard techniques and by sequencing the RT-PCR product and comparing the sequences to that of the known sequences (ADORA3-L77729, L77730).

Western blot analysis. To separate PBMCs from naive and tumor-bearing rats, heparinized peripheral blood was subjected to a density gradient centrifugation (Ficoll/Histopaque 1077 g/ ml). Tumor lesions were removed upon study termination in order to evaluate the expression level of A3AR and additional cell growth regulatory proteins A pool of 5 animals from each CF102-treated and un-treated group was used for any WB analysis, which was repeated 3 times from 3 different experiments. Cells (N1S1 and PBMCs) and tissue samples were rinsed with ice-cold PBS and transferred to ice-cold lysis buffer (TNN buffer, 50 mM Tris buffer pH 7.5, 150 mM NaCl, NP 40 0.5% for 20 min). Cell debris were removed by centrifugation for 10 min at 14,000 rpm. The supernatants were utilized for Western blot analysis. Protein concentrations were determined using the Bio-Rad protein assay dye reagent. Equal amounts of the sample (50 μg) were separated by SDS-PAGE, using 12% polyacrylamide gels. The resolved proteins were then electro-blotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked with 1% bovine serum albumin and incubated with the desired primary antibody (dilution 1:1000) for 24 h at 4°C. Blots were then washed.
and incubated with a secondary antibody for 1 h at room temperature. Bands were recorded using BCIP/NBT color development kit (Promega). The optical density of the bands was quantified using an image analysis system and were calculated against the optical density of the corresponding housekeeping protein β-actin which was used to assure the equal loading of the protein. Based on this a graph comparing the different samples is presented. Data presented in the different figures are representative of at least three different experiments.

**Immunohistochemistry.** After tumor induction and treatment as described above, the liver and tumor were fixed in 10% buffered formalin and embedded in paraffin. Serial sections of 4-6 μm in thickness were prepared in a microtome, and mounted onto electromagnetically charged slides. The first section was stained with hematoxylin and eosin for routine histological evaluation. Immunohistochemistry was performed using the Avidin-Biotin-Peroxidase methodology according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Our modified protocol includes deparaffination in xylene and re-hydration through graded alcohols in the following order: 100, 90 and 70%. The sections were then heated at 95°C in pre-warmed citrate buffer pH 6.0 (3.84 g of citric acid anhydrous in 2 l of water) for non-enzymatic antigen retrieval. Endogenous peroxidase was quenched with 20% hydrogen peroxidase in methanol for 20 min. The tissue sections were blocked with 5% normal horse serum in 0.1% PBS-BSA for 2 h. Next, the sections were incubated with primary antibodies in 0.1% PBS-BSA overnight at room temperature. Primary antibodies utilized for the present study were all mouse monoclonal antibodies and included anti-adenosine A3 receptor (Clone SP4056P, Acris Antibodies, 1:100 dilution), anti-Bad (Clone C7, Santa Cruz Biotechnology, 1:250 dilution), and anti-Bax (Clone B9, Santa Cruz, 1:100 dilution). After thoroughly washing with PBS, sections were incubated with a biotinylated secondary antibody (1:250 dilution Vector Elite) in 0.1% PBS-BSA for 1 h at room temperature. The peroxidase was developed with a diaminobenzidine substrate for 3 min (Sigma-Aldrich). Finally, the sections were counterstained with hematoxylin, dehydrated through graded alcohols, cleared in xylene and mounted with Permount (Fisher Scientific, Waltham, MA). For negative control, the primary antibody was omitted.

**TUNEL assay.** Apoptosis was examined via DNA fragmentation via TUNEL assay by using the ApopTag methodology according to the manufacturer's instructions (ApopTag Peroxi-
In Situ Detection Kit, Chemicon International). Briefly, sections were de-paraffinized and re-hydrated and endogenous peroxidase quenched as described for immunohistochemistry. Then the slides were pre-treated with Proteinase K for 15 min at room temperature and treated with an equilibration buffer and Tdt enzyme for 10 sec and 1 h respectively. Finally sections were incubated with an anti-digoxigenin conjugate, washed with PBS and developed with DAB, counterstained with hematoxylin and mounted.

Statistical analysis. Results were analyzed by the Student’s t-test, with statistical significance at p<0.05.

Results

A3AR is highly expressed in tumor tissues and PBMCs derived from HCC patients. A3AR mRNA expression levels in tumor tissues derived from HCC patients were tested in paraffin-embedded slides utilizing RT-PCR analysis. A3AR protein expression levels were tested in PBMCs utilizing WB analysis. Patients’ particulars are summarized in Table I. The mean age of the patients was 64.62±2.7 and disease duration was 4.58±3.7. Out of the patient population, 83% suffered from viral infection, 67% were HCV infected and the rest (33%) were HBV infected. In addition, 61% of the HCC patients suffered from cirrhosis, and most of them had a concomitant viral infection and cirrhosis.

High A3AR expression level was noted in the tumor lesions vs. the adjacent non-neoplastic tissue. (A) RT-PCR analysis was performed from paraffin-embedded slides of tumor tissues derived from HCC patients. (1, normal adjacent tissue; 2, tumor tissue; 3, negative control; 4, positive control). High A3AR mRNA expression level was noted in the tumor lesion vs. adjacent non-neoplastic tissue. (B) Blood samples were collected from healthy subjects and from HCC patients. PBMCs were separated by Ficoll Hypack gradient and subjected for WB analysis. A3AR was highly expressed in the PBMCs in comparison to low expression in healthy subjects. (C) Expression levels of NF-κB and PKB/Akt were also up-regulated in PBMCs derived from HCC patients.
from HCC patients, i.e., 78% increase in the A3AR expression level of the receptor was noted in the HCC patients when compared to healthy subjects (Fig. 1B). NF-κB, known to act as a transcription factor for the receptor and its upstream modulator PKB/Akt were found to be up-regulated in PBMCs derived from HCC patients (Fig. 1C).

An interesting point to note is that in PBMCs derived from HCC patients that were infected with HBV an increase of 92.7% in the A3AR expression level vs. that of the PBMCs derived from HCC patients that were not viral infected was observed. A 30% increase in the A3AR expression level was noted in PBMCs derived from HCC patients that were infected with HCV vs. the HCC patients that were not viral infected (Fig. 2A). In addition, in PBMCs derived from patients with cirrhosis an increase of 48% in A3AR expression levels in comparison to HCC patients with no cirrhosis was found (Fig. 2B).

**A3AR is highly expressed in tumor tissues and PBMCs derived from HCC tumor-bearing rats.** A3AR was found to be over-expressed in tumor tissues derived from N1S1 HCC tumor-bearing rats in comparison to liver tissue derived from naive rats (Fig. 3A). Similar to the human data, the receptor was also found to be over-expressed in PBMCs derived from tumor-bearing rats when compared with low expression in PBMCs derived from naive animals.

**Effect of CF102 treatment on the growth of HCC tumors in a rat orthotopic experimental model.** N1S1 HCC cells (5x10⁶/50 μl saline) were injected to the right hepatic lobe. Oral treatment with CF102 (at various doses, TID) was initiated 3 days after tumor inoculation and lasted till the end of the study (15 days after tumor inoculation). CF102 treatment exerted a bell shape dose-dependent inhibitory effect on the development of tumors in the liver with maximal effect at a dose of 100 μg/kg. (B) Tumors derived from vehicle and CF102 (100 μg/kg) treated rats were subjected to histopathological analysis upon hematoxylin and eosin staining. The tumors in the control group were loaded with neoplastic cells with a central hyperchromatic nuclei and moderate eosinophyllic cytoplasm. In the CF102 (100 μg/kg)-treated remarkably reduced, several irregular areas of necro-apoptosis with abundant picnotic and fragmented nuclei were observed.

**Effect of CF102 treatment on the growth of HCC tumors in a rat orthotopic experimental model.** N1S1 cells were inoculated into the liver and oral treatment with CF102 (at various doses, TID) was initiated 3 days after tumor inoculation and lasted till the end of the study (15 days after tumor inoculation). CF102 treatment exerted a bell shape dose-dependent inhibitory effect on the development of tumors in the liver with maximal effect at a dose of 100 μg/kg (92.8±6.9% of inhibition) (Fig. 4A). Macroscopic pictures and histological montages depicted in Fig. 4B demonstrate...
the dramatic reduction of tumor size in a representative liver derived from CF102 (100 μg/kg)-treated animals compared to tumor in a liver derived from vehicle-treated control group, in which the size of the tumor is considerably larger. Histologically, the tumors in the control group were characterized by solid sheaths of neoplastic cells with a central hyperchromatic nuclei and moderate eosinophilic cytoplasm. In addition to the size reduction, in the CF102 treated group, the tumors showed several irregular areas of necroapoptosis in which picnotic and fragmented nuclei are abundant (Fig. 4B). The data displayed in Fig. 4 represent one of the 3 studies that were performed.

**Effect of CF102 on expression level of A3AR in N1S1 HCC tumors.** Immunohistochemistry analysis for A3AR showed elevated levels of the receptor in the tumor, in contrast to the liver in which expression is conspicuous. Upon CF102 (100 μg/kg) treatment a reduction in A3AR exhibition was noted (Fig. 5A). The A3AR protein expression level was down-regulated upon treatment with CF102 (Fig. 5B).

**Figure 5.** Effect of CF102 on the expression level of A3AR and cell growth regulatory proteins extracted from HCC tumors. (A) Immunohistochemistry analysis performed on N1S1 tumor tissues derived from vehicle and CF102 (100 μg/kg) treated rats revealed a reduction in A3AR exhibition upon CF102 treatment. (B) Utilizing WB analysis on N1S1 tumor tissues derived from vehicle and CF102 (100 μg/kg) treated rats it was found that the A3AR protein expression level was down-regulated upon treatment with CF102. (C) The expression levels of PKB/Akt, IKKa/β, NF-κB, and TNF-α were decreased upon treatment with CF102. (D) The protein expression level of GSK-3β was increased and a decreased level of β-catenin, LEF/TCF and c-Myc was noted upon CF102 treatment.

**Effect of CF102 on expression level of cell growth regulatory proteins extracted from HCC tumors.** WB analysis of N1S1 tumor protein extracts from CF102 (100 μg/kg) treated animals revealed down-regulation of the of PKB/Akt compared to vehicle treated animals. The expression levels of the down-stream signaling proteins IKKa/β NF-κB, and TNF-α were decreased upon treatment with CF102 (Fig. 5C). In addition, the expression level of GSK-3β was up-regulated, resulting in a decreased level of the down-stream proteins β-catenin, LEF/TCF and c-Myc (Fig. 5D). As a result apoptosis was induced as was evidenced in the different assays. Up-regulation of the pro-apoptotic proteins Bad and Bax was found by immunohistochemistry staining of the tumors derived from CF102-treated animals, whereas in tumors derived from vehicle-treated animals, no expression of the mentioned proteins was found (Fig. 6A). Increased level of caspase-3 was found in the tumors upon treatment with CF102 (Fig. 6B). In TUNEL assays very few apoptotic cells could be identified in the tumors derived from vehicle-treated control group while there were abundant positive cells in the
CF102-treated tumors, particularly in and around the areas of cell death (Fig. 6C). The modulation of the above mentioned proteins indicated that both the NF-κB and the Wnt signaling pathways are involved in mediating CF102’s inhibitory effect, resulting in apoptosis of the tumor cells.

**Effect of CF102 on the expression level of A3AR in PBMCs derived from HCC tumor bearing rats**. Treatment with CF102 resulted in down-regulation of A3AR expression level in PBMCs derived from HCC tumor-bearing rats (Fig. 7A). To investigate the molecular mechanisms involved with receptor down-regulation in the PBMCs we looked at the expression level of key signaling proteins up-stream to NF-κB, known to act as a transcription factor of A3AR. The expression of TNF-α, a cytokine which induces NF-κB and its serum levels is significantly elevated in HCC patients was decreased upon CF102 treatment. This was followed by down-regulation in the expression level of NF-κB downstream signaling key proteins, PI3K, PKB/Akt, IKK, resulting in decreased level of NF-κB (Fig. 7B).
Discussion

This study presents data showing that A3AR is highly expressed in the tumor tissue and in PBMCs of HCC patients. The high expression level of the receptor was directly correlated to up-regulated expression level of the transcription factor, NF-κB. This observation was also found in tumor tissue and in PBMCs derived from N1S1 HCC tumor-bearing rats.

Upon treatment with CF102, A3AR expression level was down-regulated in the tumor lesions excised from N1S1 tumor-bearing rats and in the PBMCs. It is well established that Gi protein receptors are internalized to early endosomes upon 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. Former studies have shown that exposure of the A3AR to the specific agonist, IB-MECA resulted in receptor internalization/externalization in B16-F10 melanoma cells (20,26,27).

The data showing direct correlation in the A3AR expression between tumor tissue and PBMCs suggest that receptor expression in the PBMCs mirrors receptor status in the tumor tissue. It may be that TNF-α up-regulation give rise to an increase in the expression level and activity of NF-κB, known to act as a transcription factor of A3AR. Upon treatment with CF102 the expression levels of TNF-α and NF-κB were decreased, resulting in down-regulation of A3AR expression both in PBMCs and the tumor tissue. Similar data were reported by Gessi et al showing that A3AR is up-regulated in both colon carcinoma tissue and PBMCs of patients with colon carcinoma. This group has further demonstrated that upon tumor removal receptor level was down-regulated in the PBMCs (28).

Additional important finding of this study is that CF102, a synthetic A3AR agonist, inhibited in a bell shape dose-dependent manner the growth of N1S1 HCC cells in the orthotopic rat model. The maximal inhibitory effect was observed at a dose of 100 μg/kg. The bell shape response was demonstrated in correlation to the nuclear localization of β-catenin and the translocation of the Lef/Tcf complex to the nucleus (29,30). Indeed, in N1S1 tumor tissue lesions derived from CF102 treated animals the level of β-catenin and Lef/Tcf was down-regulated. Both, Lef/Tcf and NF-κB act as the transcription factors of gene products such as c-Myc, which contribute to cell cycle progression. c-Myc was found to be down regulated in the N1S1 tumor lesion extracts derived from CF102 treated animals.

The involvement of the Wnt and the NF-κB signal transduction pathways in the etiology of liver cancer has been already described. Blocking of the PI3K-PKB/Akt pathway was shown to be efficacious in overcoming chemoresistance of HCC (31); NF-κB may play an important role in the pathogenesis of human HCC (32,33). Mutations in β-catenin are very common in HCC, especially when there is an association with hepatitis C infection (34-36); poor prognosis was demonstrated in correlation to the nuclear localization of β-catenin (37).

The NF-κB and the Wnt signal transduction pathways are involved in modulating a wide range of cellular processes including differentiation, growth, motility and apoptosis (38,39).

In this study, we have shown that de-regulation of NF-κB and the Wnt signal transduction pathways results in the induction of apoptosis in tumor tissues derived from CF102-treated N1S1 HCC tumor-bearing rats. This was demonstrated by an increase in the expression levels of pro-apoptotic proteins Bad, BAX as well as capase-3 and an increased apoptotic signal as was demonstrated in the TUNEL assay.

In conclusion, A3AR was found to be highly expressed in tumors and PBMCs of HCC patients and tumor-bearing animals. It was demonstrated that A3AR activation by CF102, an orally bioavailable small molecule, resulted in HCC tumor growth inhibition. Tumor response to the treatment was accompanied by down-regulation of A3AR expression level both in the tumor tissue and in the PBMCs. The mechanism of action included de-regulation of the Wnt and the NF-κB signaling pathways accompanied by induction of apoptosis. Thus, A3AR could be suggested as a possible biological marker and a target to treat HCC.

References


