The A_3 adenosine receptor agonist CF102 induces apoptosis of hepatocellular carcinoma via de-regulation of the Wnt and NF-κB signal transduction pathways

S. BAR-YEHUDA[1*], S.M. STEMMER[2*], L. MADI[1], D. CASTEL[1], A. OCHAION[1], S. COHEN[1], F. BARER[1], A. ZABUTTI[1], G. PEREZ-LIZ[3], L. DEL VALLE[3] and P. FISHMAN[1]

[1]Can-Fite BioPharma Ltd., Kiryat-Matalon; [2]Institute of Oncology, Davidoff Center, Rabin Medical Center, Sackler School of Medicine, Tel Aviv University, Petach Tikva, Israel; [3]Department of Neuroscience, Neuropathology Core & Center for NeuroVirology, Temple University, School of Medicine, Philadelphia, PA, USA

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Abstract. The A_3 adenosine receptor (A_3AR) is highly expressed in tumors and was suggested as a target for cancer treatment. In this study, we show that A_3AR 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-κB, known as a transcription factor of A_3AR. CF102, a synthetic highly selective agonist to A_3AR induced a marked dose response inhibition of tumor growth in N1S1 HCC tumor rats, via de-regulation of the NF-κB and the Wnt signal transduction pathways, resulting in apoptosis of tumor cells. Taken together, A_3AR 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_3AR 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 microsphere 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.

The small molecule sorefanib, blocking the RAF/MEK/ERK pathway, showed activity in unresectable HCC, with a statistical significant increased survival, but for a short term (2.6 months) when compared to placebo (13). This may pave the way for additional therapies based on modulation of signal transduction pathways to be developed.

Accumulative data have indicated that the Gi associated cell surface receptor, A_3 adenosine receptor (A_3AR) plays an important role in controlling tumor growth. The A_3AR was found to be highly expressed in different tumor cells in comparison with low expression in normal adjacent tissues (14). Targeting the A_3AR 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_3AR is a validated target in HCC by looking at the correlation between receptor expression and functionality. A_3AR 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 orthotropic model of HCC, demonstrating the functionality of the receptor. The role of NF-κB in mediating receptor expression and functionality was explored.

Materials and methods

Drugs. The A_3AR agonist 2-chloro-N^6-(3-iodobenzyl)-adenosine-5'-N-methyl-uronamide (CF102) was synthesized

Correspondence to: Professor Pnina Fishman, Can-Fite BioPharma, 10 Bareket St., P.O. Box 7537, Petach-Tikva 49170, Israel
E-mail: pnina@canfite.co.il

*Contributed equally

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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 A_3AR and the cell growth regulatory proteins PKB/Akt, IKK, NF-κB, TNF-α, GSK-3ß, LEF-1, ß-catenin, c-Myc, caspase 3 and ß-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% CO_2 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 laparatomy was performed and N1S1 cells ($5x10^6$/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, 100, 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 naive animals. Tumors were fixed in 10% formaldehyde for pathological staining. Protein extracts were subjected to Western blot analysis to evaluate the A_3AR 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 A_3AR 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 $MgCl_2$, 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 A_3AR 5'-ACGGTGAG GTACCACAGCTTGTG and 3'-ATACCGCGGGATGGCA GACC] were added.

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, stained with ethidium bromide and visualized with UV illumination. The specificity of the RT-PCR reaction was confirmed by size determination on 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 A_3AR 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

Table I. Characterization of HCC patients.

Patient no.	Age (years)	Disease duration (years)	Viral status	Cirrhosis	Previous treatment
1	70	5	HCV	Yes	Chemo-embolization x2 Radio-frequency ablation
2	38	4.16	HBV	Yes	Chemo-embolization Liver transplantation
3	71	3.8	HCV	Yes	Radio-frequency ablation
4	36	3	None	Yes	Supportive care
5	76	4.5	HBV	Yes	Chemo-embolization
6	80	5.75	HCV	Yes	Radio-frequency ablation
7	63	3.16	HCV	Yes	Chemo-embolization Resection
8	74	5.25	HCV	Yes	Chemo-embolization
9	63	4.5	None	No	Supportive care
10	79	3.8	HCV	No	Chemo-embolization
11	63	5.6	HBV	Yes	Chemo embolization
12	75	6.1	HCV	No	Radio-frequency ablation
13	62	4.1	HCV	Yes	Chemo-embolization
14	57	3	HBV	Yes	Liver transplantation
15	68	4.2	HCV	Yes	Raf kinase inh
16	67	3	HBV	No	Chemo-embolization
17	51	2.1	HBV	Yes	Raf kinase inh
18	71	2.25	HCV	No	No treatment
19	56	3	None	No	Liver transplantation
20	57	4.25	None	No	Chemo-embolization
21	80	4	HCV	Yes	Chemo-embolization

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 ß-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 de-paraffination 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 retreival. 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 A_3 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 22˚C. After washing with PBS, the slides were incubated with avidin-biotin complexes 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-

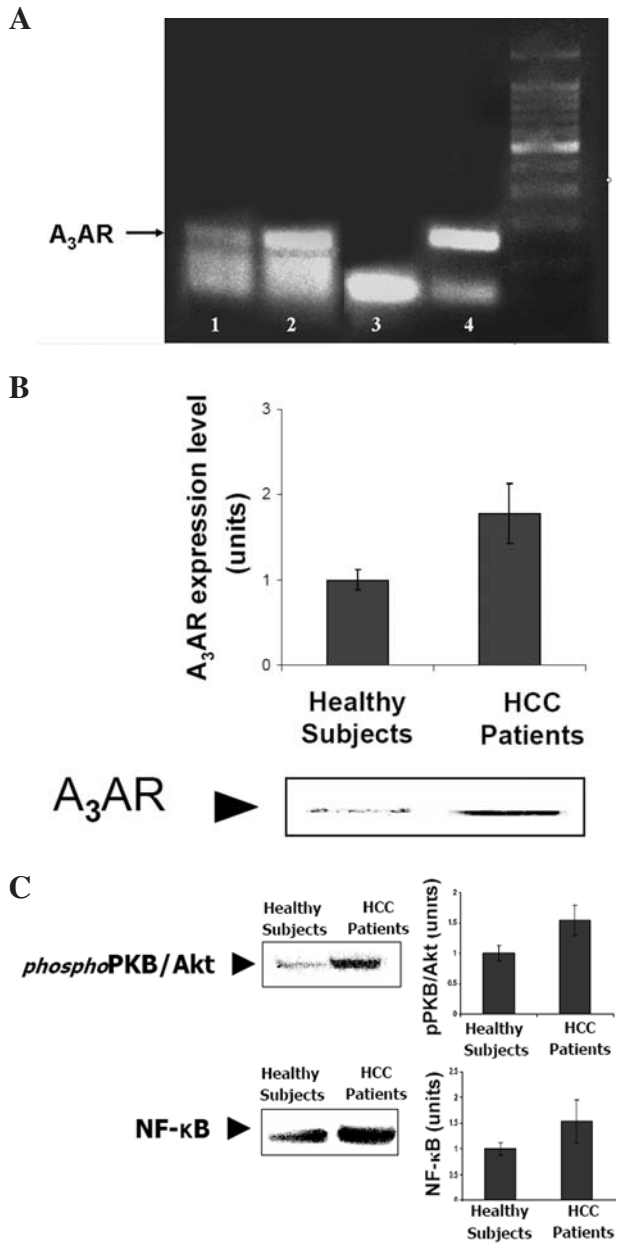

Figure 1. A_3AR expression level in tumor lesions and in PBMCs derived from HCC patients. (A) RT-PCR analysis was performed from paraffin-embedded slides of tumor tissues derived from HCC patients. (1, normal adjacent tissue; 2, tumor tissue; 3, negative control; 4, positive control). High A_3AR mRNA 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. A_3AR 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.

dase *In Situ* Detection Kit, Chemicon International). Briefly, sections were de-paraffinized an 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.

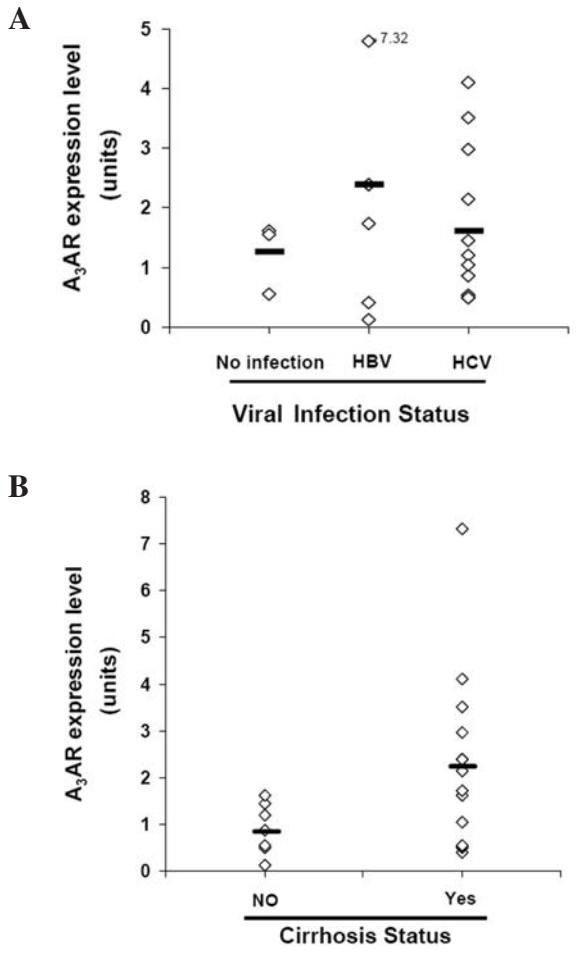

Figure 2. Analysis of A_3AR expression level in PBMCs derived from HCC patients with hepatitis or cirrhosis. (A) Comparison between the expression level of A_3AR in PBMCs derived from HCC patients that do not suffer from hepatitis to that of HCC patients with HBV or HCV infection. (B) Comparison between the expression level of A_3AR in PBMCs derived from HCC patients that do not suffer from cirrhosis to that of HCC patients with cirrhosis.

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

Results

A_3AR is highly expressed in tumor tissues and PBMCs derived from HCC patients. A_3AR mRNA expression levels in tumor tissues derived from HCC patients were tested in paraffin-embedded slides utilizing RT-PCR analysis. A_3AR 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 A_3AR expression level was noted in the tumor lesions vs. the adjacent non-neoplastic tissues (a representative blot is presented in Fig. 1A). The high expression level of the receptor was reflected in the PBMCs derived

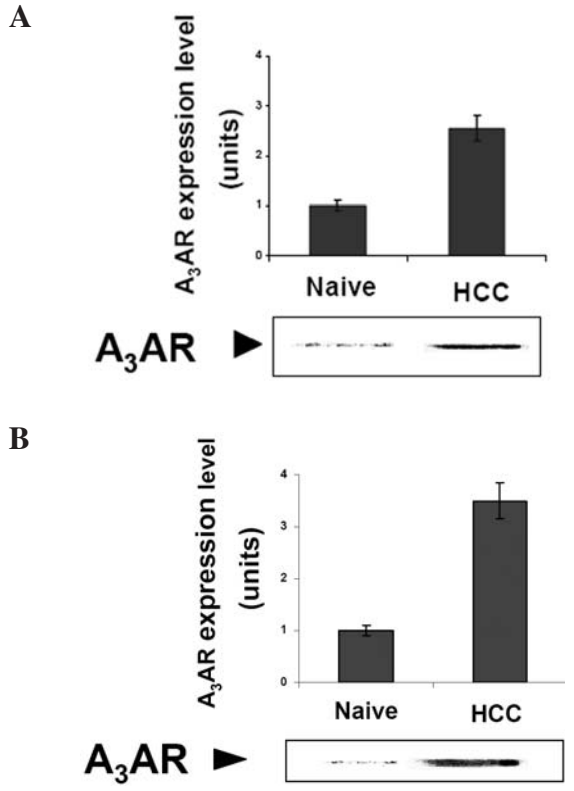

Figure 3. A_3AR expression levels in tumor tissues and PBMCs derived from HCC tumor-bearing rats. A_3AR expression level was examined by WB analysis in protein extracts of N1S1 tumor tissue samples and in PBMC's derived from tumor-bearing rats and compared to liver tissue and PBMCs derived from naïve rats. (A) A_3AR is over-expressed in tumor tissues derived from HCC tumor-bearing rats in comparison to liver tissue derived from naïve rat. (B) A_3AR was found to be over-expressed in PBMCs derived from the tumor-bearing rats vs. low expression in PBMCs derived from naïve animals.

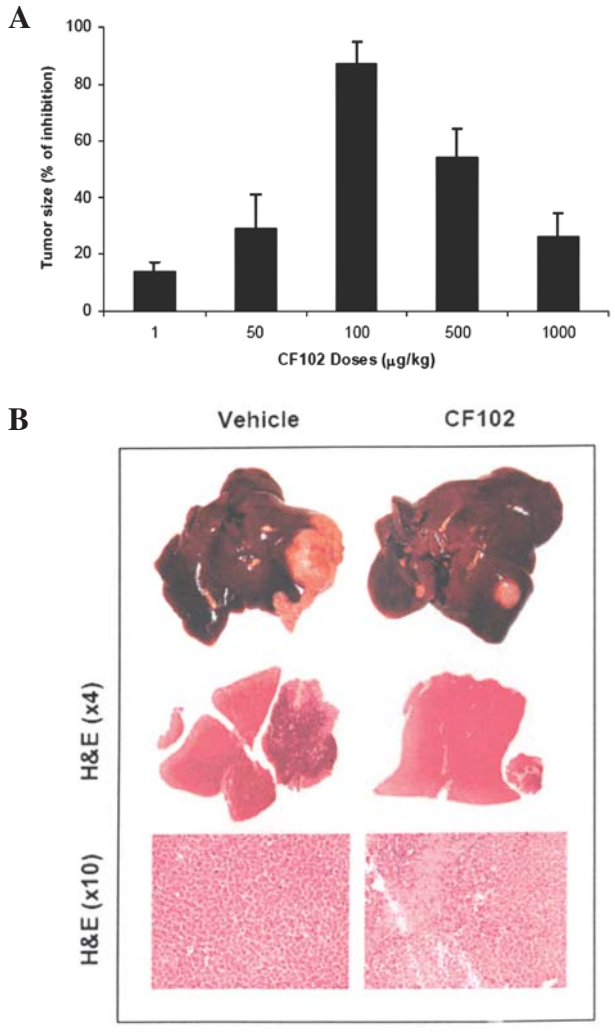

Figure 4. Effect of CF102 on the growth of HCC tumors in rat experimental model. N1S1 HCC cells ($5x10^6$/50 μl saline) were injected to the right hepatic lobe. Oral treatment with CF102 (at various doses, TID) was initiated 3 days after tumor inoculation and lasted till the end of the study. At the end of the study, the livers were excised and the tumors size was measured. (A) CF102 treatment exerted a bell shaped 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.

from HCC patients, i.e. 78% increase in the A_3AR 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 up-stream 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 A_3AR expression level vs. that of the PBMCs derived from HCC patients that were not viral infected was observed. A 30% increase in the A_3AR 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 A_3AR expression levels in comparison to HCC patients with no cirrhosis was found (Fig. 2B).

A_3AR is highly expressed in tumor tissues and PBMCs derived from HCC tumor-bearing rats. A_3AR 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 (Fig. 3B).

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 doses of 1, 50, 100, 500 and 1000 μg/kg) thrice daily 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

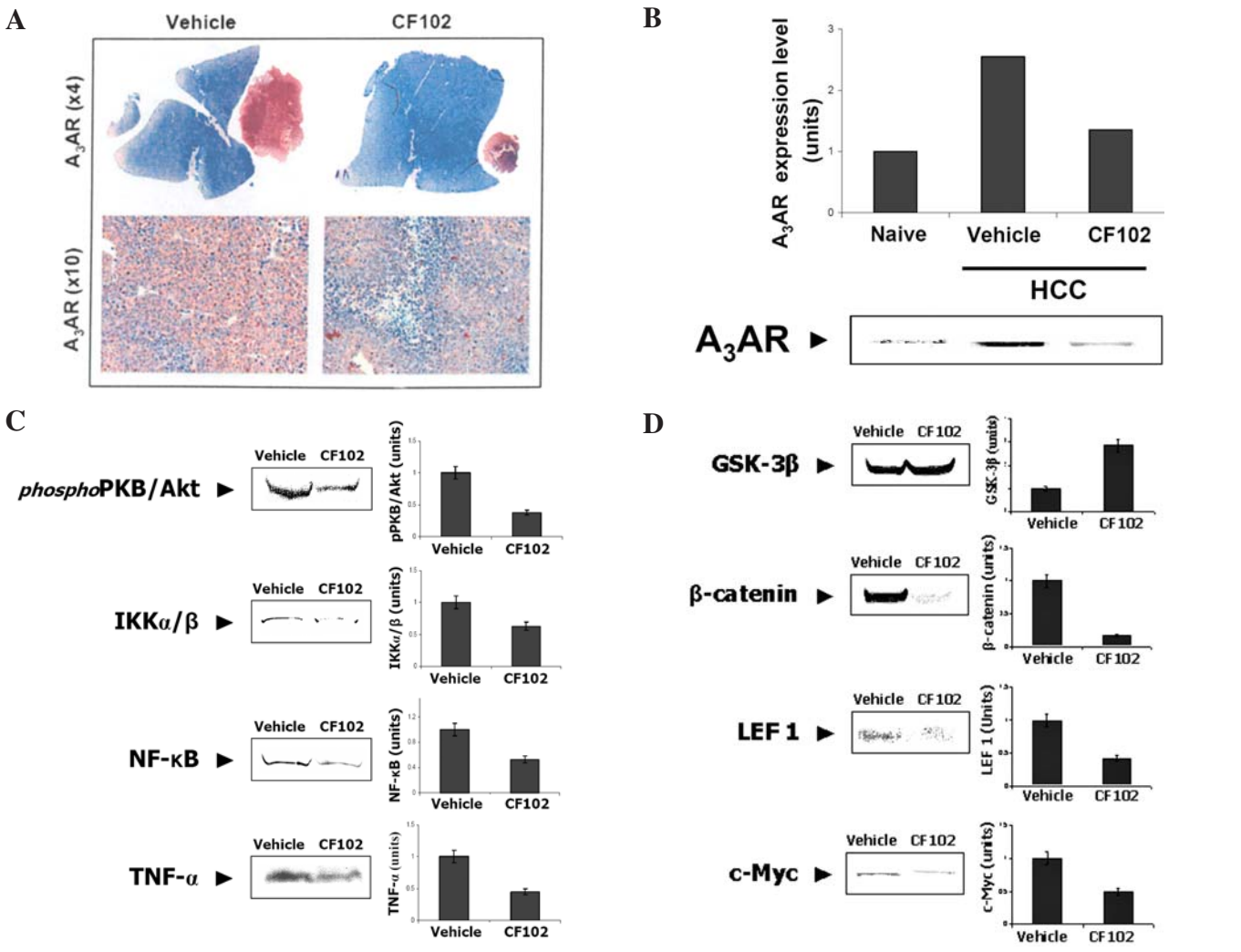

Figure 5. Effect of CF102 on the expression level of A_3AR and cell growth regulatory proteins extracted from HCC tumors. (A) Immunohistochemistry analysis preformed on N1S1 tumor tissues derived from vehicle and CF102 (100 μg/kg) treated rats revealed a reduction in A_3AR exhibition upon CF102 treatment. (B) Utilizing WB analysis on N1S1 tumor tissues derived from vehicle and CF102 (100 μg/kg) treated rats it was found that the A_3AR protein expression level was down-regulated upon treatment with CF102. (C) The expression levels of PKB/Akt, IKKα/ß, NF-κB, and TNF-α were reduced upon treatment with CF102. (D) The protein expression level of GSK-3ß was increased and a decreased level of ß-catenin, LEF/TCF and c-Myc was noted upon CF102 treatment.

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 eosinophyllic 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 preformed.

Effect of CF102 on expression level of A_3AR in N1S1 HCC tumors. Immunohistochemistry analysis for A_3AR 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 A_3AR exhibition was noted (Fig. 5A). The A_3AR protein expression level was down-regulated upon treatment with CF102 (Fig. 5B).

Effect of CF102 on the 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 IKKα/ß NF-κB, and TNF-α were decreased upon treatment with CF102 (Fig. 5C). In addition, the expression level of GSK-3ß was up-regulated, resulting in a decreased level of the down-stream proteins ß-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

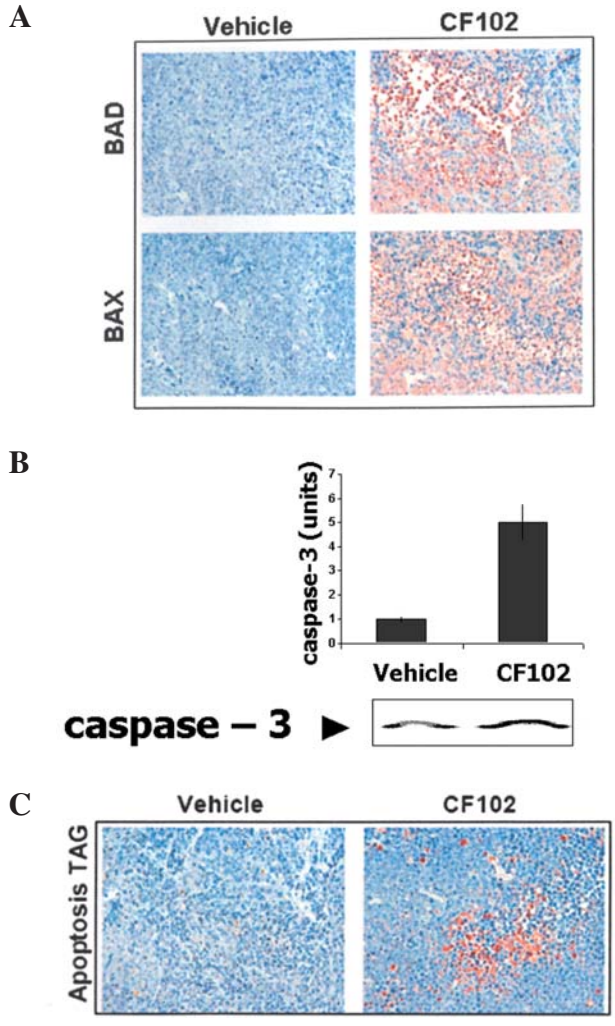

Figure 6. Effect of CF102 treatment on the apoptosis process. Upon CF102 (100 μg/kg) or vehicle treatment to N1S1 HCC tumor bearing rats protein extracts of the tumor tissues were subjected to immunohistological, TUNEL assay and WB analysis to evaluate the effect of CF102 on various proteins participating in the apoptosis process. (A) Increased expression levels of the pro-apoptotic proteins Bad and Bax was observed in tumor tissues derived from CF102-treated animals. (B) Increased level of caspase 3 was found in the tumors upon treatment with CF102. (C) Few apoptotic cells were detected by TUNEL assay in the vehicle-treated control group while abundant positive cells were observed in the tumors derived CF102-treated animals.

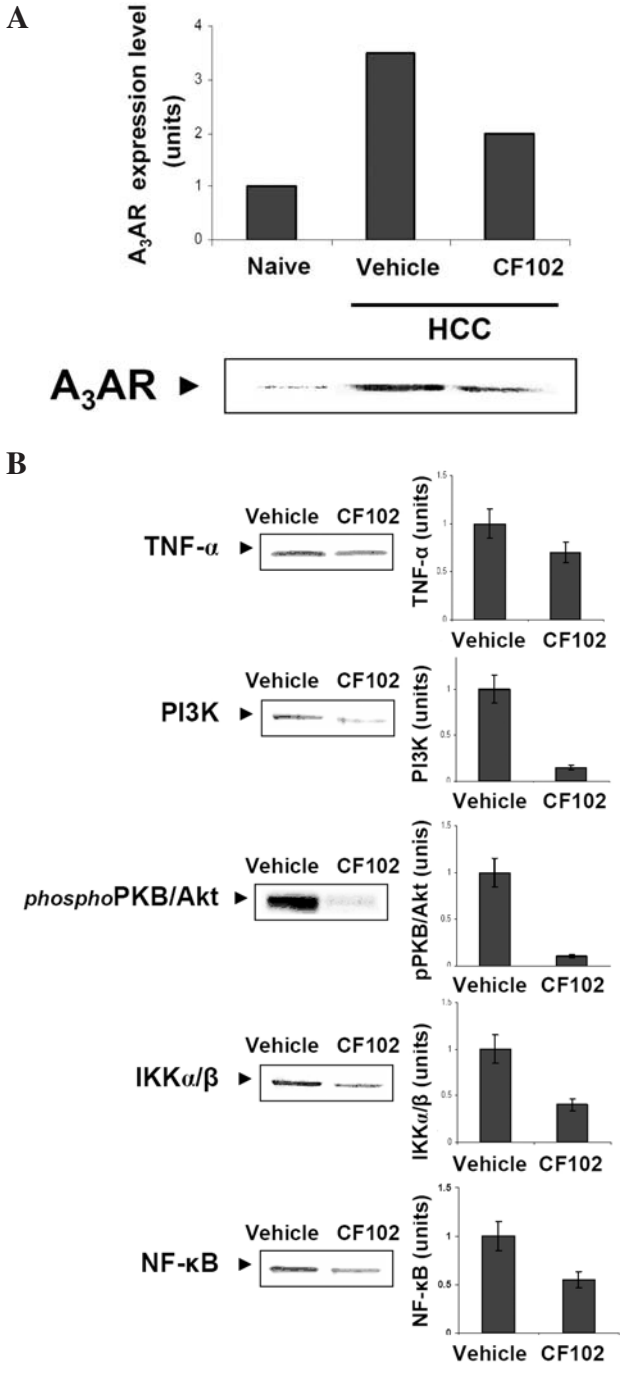

Figure 7. Effect of CF102 on the expression level of A_3AR in PBMCs derived from HCC tumor bearing rats. To evaluate the expression level of A_3AR and key signaling proteins up-stream to NF-κB, known to act as a transcription factor of A_3AR, WB analysis was performed on PBMCs derived from CF102 and vehicle-treated N1S1 HCC tumor bearing rats. (A) Upon CF102 treatment the expression level of A_3AR was decreased in PBMCs derived from tumor bearing rats in comparison to the level of the receptor in vehicle treated animals. (B) Expression level of TNF-α and the down-stream signaling key proteins, PI3K, PKB/Akt, IKK was down-regulated, resulting in decreased level of NF-κB.

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 A_3AR in PBMCs derived from HCC tumor bearing rats. Treatment with CF102 resulted in down-regulation of A_3AR 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 of NF-κB, known to act as a transcription factor of A_3AR. 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 TNF-α downstream signaling key proteins: PI3K, PKB/Akt, IKK, resulting in decreased level of NF-κB (Fig. 7B).

Discussion

This study presents data showing that A_3AR 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, A_3AR 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 A_3AR 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 A_3AR 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 A_3AR. Upon treatment with CF102 the expression levels of TNF-α and NF-κB were decreased, resulting in down-regulation of A_3AR expression both in PBMCs and the tumor tissue. Similar data were reported by Gessi *et al* showing that A_3AR 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 A_3AR 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 may be attributed to receptor de-sensitization or tachyphlaxis occurring upon treatment with higher agonist dose.

We further looked at cell growth regulatory proteins in the tumor lesions excised from CF102-treated and vehicle-treated control animals. The level of the cell survival protein PKB/Akt was found to be down-regulated in the treated animals. It has already been demonstrated in melanoma, colon and prostate carcinoma that activation of A_3AR decreased the PKB/Akt expression level (15-22). This kinase phosphorylates signaling proteins, which play a role in the NF-κB and the Wnt signal transduction pathways. Indeed, the levels of IKKα/ß and NF-κB were down-regulated in the CF102 treated N1S1 tumor-bearing animals. This event most probably prevented the nuclear translocation of NF-κB and the induction of TNF-α transcription, resulting in a low level of TNF-α in the CF102 treated animals. In addition CF102 treatment induced GSK-3ß up-regulation, a key component of the Wnt signaling pathway. The latter is known to phosphorylate ß-catenin and to induce its ubiquitination, thereby preventing its association with Lef/Tcf and the translocation of the complex to the nucleus (29,30). Indeed, in N1S1 tumor tissue lesions derived from CF102 treated animals the level of ß-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 ß-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 ß-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, A_3AR was found to be highly expressed in tumors and PBMCs of HCC patients and tumor-bearing animals. It was demonstrated that A_3AR 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 A_3AR 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, A_3AR could be suggested as a possible biological marker and a target to treat HCC.

References

1. Thomas MB and Zhu AX: Hepatocellular carcinoma: the need for progress. J Clin Oncol 23: 2892-2899, 2005.
2. Wilson JF: Liver cancer on the rise. Ann Intern Med 142: 1029-1032, 2005.
3. Ishii H, Okada S, Nose H, *et al*: Local recurrence of hepatocellular carcinoma after percutaneous ethanol injection. Cancer 77: 1792-1796, 1996.
4. Simonetti RG, Liberati A, Angiolini C and Pagliaro L: Treatment of hepatocellular carcinoma: a systematic review of randomized controlled trials. Ann Oncol 8: 117-136, 1997.
5. Goldberg SN, Gazelle GS, Solbiati L, Livraghi T, Tanabe KK, Hahn PF and Mueller PR: Ablation of liver tumors using percutaneous RF therapy. Am J Roentgenol 170: S1023-S1028, 1998.
6. Kouroumalis E, Skordilis P, Thermos K, Vasilaki A, Moschandrea J and Manousos ON: Treatment of hepatocellular carcinoma with octreotide: a randomised controlled study. Gut 42: 442-447, 1998.
7. Lau WY, Leung TW, Ho SK, *et al*: Adjuvant intra-arterial iodine-131-labelled lipiodol for resectable hepatocellular carcinoma: a prospective randomised trial. Lancet 353: 797-801, 1999.
8. Hemming AW, Cattral MS, Reed AI, van der Werf WJ, Greig PD and Howard RJ: Liver transplantation for hepatocellular carcinoma. Ann Surg 233: 652-659, 2001.

9. Poon RT, Fan ST, Lo CM, Ng IO, Liu CL, Lam CM and Wong J: Improving survival results after resection of hepatocellular carcinoma: a prospective study of 377 patients over 10 years. Ann Surg 234: 63-70, 2001.
10. Perrone F, Gallo C, Daniele B, *et al*: Cancer of Liver Italian Program (CLIP) Investigators. Cancer of Liver Italian Program (CLIP) Investigators. Tamoxifen in the treatment of hepatocellular carcinoma: 5-year results of the CLIP-1 multicentre randomised controlled trial. Curr Pharm Des 8: 1013-1019, 2002.
11. Llovet JM and Bruix J: Systematic review of randomized trials for unresectable hepatocellular carcinoma: chemoembolization improves survival. Hepatology 37: 429-442, 2003.
12. Hung H: Treatment modalities for hepatocellular carcinoma. Curr Cancer Drug Targets 5: 131-138, 2005.
13. Liu L, Cao Y, Chen C, *et al*: Tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer Res 66: 11851-11858, 2006.
14. Madi L, Ochaion A, Rath-Wolfson L, *et al*: The A3 adenosine receptor is highly expressed in tumor vs. normal cells: potential target for tumor growth inhibition. Clin Cancer Res 10: 4472-4479, 2004.
15. Fishman P, Madi L, Bar-Yehuda S, Barer F, Del Valle L and Khalili K: Evidence for involvement of Wnt signaling pathway in IB-MECA mediated suppression of melanoma cells. Oncogene 21: 4060-4064, 2002.
16. Fishman P, Bar-Yehuda S, Madi L and Cohn I: A3 adenosine receptor as a target for cancer therapy. Anticancer Drugs 13: 437-443, 2002.
17. Fishman P and Bar-Yehuda S: Pharmacology and therapeutic applications of A3 receptor subtype. Curr Top Med Chem 3: 463-469, 2003.
18. Fishman P, Bar-Yehuda S, Ardon E, Rath-Wolfson L, Barrer F, Ochaion A and Madi L: Targeting the A3 adenosine receptor for cancer therapy: inhibition of prostate carcinoma cell growth by A3AR agonist. Anticancer Res 23: 2077-2083, 2003.
19. Merimsky O, Madi L, Bar-Yehuda S and Fishman P: Modulation of the A3 adenosine receptor by low agonist concentration induced anti-tumor and myelostimulation effects. Drug Dev Res 58: 386-389, 2003.
20. Madi L, Bar-Yehuda S, Barer F, Ardon E, Ochaion A and Fishman P: A3 adenosine receptor activation in melanoma cells: association between receptor fate and tumor growth inhibition. JBC 278: 42121-42130, 2003.
21. Ohana G, Bar-Yehuda S, Arich A, *et al*: Inhibition of primary colon carcinoma growth and liver metastasis by the A3 adenosine receptor agonist CF101. Br J Cancer 89: 1552-1558, 2003.
22. Fishman P, Bar-Yehuda S, Ohana G, Barer F, Ochaion A, Erlanger A and Madi L: An agonist to the A3 adenosine receptor inhibits colon carcinoma growth in mice via modulation of GSK-3ß and NF-κB. Oncogene 23: 2465-2471, 2004.
23. Bar-Yehuda S, Madi L, Silberman D, Slosman G, Shkapenuk M and Fishman P: CF101, an agonist to the A3 adenosine receptor enhances the chemotherapeutic effect of 5-flurouracil in a colon carcinoma murine model. Neoplasia 7: 85-90, 2005.
24. Merighi S, Benini A, Mirandola P, *et al*: A3 adenosine receptor activation inhibits cell proliferation via phosphatidylinositol 3-kinase/Akt-dependent inhibition of the extracellular signal-regulated kinase 1/2 phosphorylation in A375 human melanoma cells. J Biol Chem 280: 19516-19526, 2005.
25. Nakamura K, Yoshikawa N, Yamaguchi Y, Kagota S, Shinozuka K and Kunitomo M: Antitumor effect of cordycepin (3'-deoxyadenosine) on mouse melanoma and lung carcinoma cells involves adenosine A3 receptor stimulation. Anticancer Res 26: 43-47, 2006.
26. Bunemann M, Lee KB, Pals-Rylaarsdam R, Roseberry AG and Hosey MM: Desensitization of G-protein-coupled receptors in the cardiovascular system. Annu Rev Physiol 61: 169-192, 1999.
27. Claing A, Laporte SA, Caron MG and Lefkowitz RJ: Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. Prog Neurobiol 66: 61-79, 2002.
28. Gessi S, Cattabriga E, Avitabile A, *et al*: Elevated expression of A3 adenosine receptors in human colorectal cancer is reflected in peripheral blood cells. Clin Cancer Res 10: 5895-5901, 2004.
29. Novak A and Dedhar S: Signaling through beta-catenin and Lef/Tcf. Cell Mol Life Sci 56: 523-537, 1999.
30. Ferkey DM and Kimelman D: GSK-3: new thoughts on an old enzyme. Dev Biol 225: 471-479, 2000.
31. Koizumi N, Hatano E, Nitta T, *et al*: Blocking of PI3K/Akt pathway enhances apoptosis induced by SN-38, an active form of CPT-11, in human hepatoma cells. Int J Oncol 26: 1301-1306, 2005.
32. Chiao PJ, Na R, Niu J, Sclabas GM, Dong Q and Curley SA: Role of Rel/NF-kappaB transcription factors in apoptosis of human hepatocellular carcinoma cells. Cancer 95: 1696-1705, 2002.
33. Arsura M and Cavin LG: Nuclear factor-kappaB and liver carcinogenesis. Cancer Lett 229: 157-169, 2005.
34. Fujie H, Moriya K, Shintani Y, *et al*: Frequent beta-catenin aberration in human hepatocellular carcinoma. Hepatol Res 20: 39-51, 2001.
35. Buendia MA: Genetic alterations in hepatoblastoma and hepatocellular carcinoma: common and distinctive aspects. Med Pediatr Oncol 39: 530-535, 2002.
36. Park JY, Park WS, Nam SW, *et al*: Mutations of beta-catenin and AXIN I genes are a late event in human hepatocellular carcinogenesis. Liver Int 25: 70-76, 2005.
37. Fiorentino M, Altimari A, Ravaioli M, *et al*: Predictive value of biological markers for hepatocellular carcinoma patients treated with orthotopic liver transplantation. Clin Cancer Res 10: 1789-1795, 2004.
38. Darnell JE Jr: Transcription factors as targets for cancer therapy. Nat Rev Cancer 2: 740-749, 2002.
39. Forde JE and Dale TC: Glycogen synthase kinase 3: a key regulator of cellular fate. Cell Mol Life Sci 64: 1930-1944, 2007.