Oncogene (2002) 21, 4060–4064

www.nature.com/onc

SHORT REPORTS

Evidence for involvement of Wnt signaling pathway in IB-MECA mediated suppression of melanoma cells

Pnina Fishman*[,1,2], Lea Madi[2], Sara Bar-Yehuda[1], Faina Barer[2], Luis Del Valle[3] and Kamel Khalili[3]

[1]*Laboratory of Clinical and Tumor Immunology, The Felsenstein Medical Research Center, Tel Aviv University, Sackler Faculty of Medicine, Rabin Medical Center, Petach-Tikva 49100, Israel;* [2]*Can-Fite Biopharma, Ltd, Kiryat-Matalon, Petach-Tikva, Israel;* [3]*Center for Neurovirology and Cancer Biology, College of Science and Technology, Temple University, 1900 North 12th Street, 015-96, Room 203, Philadelphia, Pennsylvania, PA 19122, USA*

The A3 adenosine receptor, A3AR, belongs to the family of Gi proteins, which upon induction, suppresses the formation of cAMP and its downstream effectors. Recent studies have indicated that activation of A3AR by its agonist, IB-MECA, results in growth inhibition of malignant cells. Here we demonstrate the ability of IB-MECA to decrease the levels of protein kinase A, a downstream effector of cAMP, and protein kinase B/Akt in melanoma cells. Examination of glycogen synthase kinase 3β, GSK-3β, whose phosphorylation is controlled by protein kinase A and B, showed a substantial decrease in the levels of its phosphorylated form and an increase in total GSK-3β levels in IB-MECA treated melanoma cells. This observation suggests that the treatment of cells with IB-MECA augments the activity of GSK-3β in the cells. Evaluation of β-catenin, a key component of Wnt signaling pathway which, upon phosphorylation by GSK-3β rapidly ubiquitinates, showed a substantial decrease in its level after IB-MECA treatment. Accordingly, the level of β-catenin responsive cell growth regulatory genes including c-*myc* and cyclin D1 was severely declined upon treatment of the cells with IB-MECA. These observations which link cAMP to the Wnt signaling pathway provide mechanistic evidence for the involvement of Wnt pathway via its key elements GSK-3β and β-catenin in the anti-tumor activity of A3AR agonists.
Oncogene (2002) **21,** 4060–4064. doi:10.1038/sj.onc.1205531

Keywords: A3 adenosine receptor; IB-MECA; cAMP; Wnt pathway; GSK-3β

Introduction

Adenosine binds to A1, $A2_a$, $A2_b$, and A3 G protein-associated receptors and induces a series of signaling pathways that control several biological events (Linden, 1991; Stiles, 1990). Results from recent studies illustrated that adenosine has the ability to inhibit the proliferation of various tumor cells, including melanoma (Ohana *et al*., 2000; Fishman *et al*., 1998). Pharmacological studies utilizing antagonists to the various adenosine receptors revealed that A3AR plays a key role in the adenosine-induced inhibition of tumor cell proliferation (Fishman *et al*., 2000). These observations provided a rationale for examining the anti-cancer activity of synthetic agonists to A3AR, a Gi protein coupled receptor. In this respect, our early efforts showed that the A3AR agonists, 1-Deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purine-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA) and its 2-chloro derivative, Cl-IB-MECA, have the ability to inhibit the growth of B16-F10 melanoma cells through cell cycle arrest in the G0/G1 phase. Moreover, these compounds suppressed tumor cell growth *in vivo* when administered to mice inoculated with the B16-F10 melanoma cells (Bar-Yehuda *et al*., 2001; Fishman *et al*., 2001).

Activation of A3AR is known to inhibit adenylyl cyclase activity and cAMP formation (Zhao *et al*., 2000). cAMP modulates the level and activity of protein kinase A, PKA, and protein kinase B, PKB/Akt, both of which play a central role in modulation of a variety of extracellular signals (Filippa *et al*., 1999; Sable *et al*., 1997). PKA contains a catalytic subunit, PKAc, which dissociates from the parent molecule upon activation with cAMP. Recent studies have demonstrated that PKAc phosphorylates and inactivates glycogen synthase kinase 3β (GSK-3β), a serine/threonine kinase that regulates glycogen synthesis in response to various stimuli (Fang *et al*., 2000). GSK-3β also serves as a direct substrate of PKB/Akt that induces its phosphorylation and inactivation (Cross *et al*., 1995). GSK-3β acts as a key element in the Wnt signaling pathway by dictating cell fate during embryogenesis and tumorigenesis. In its active form, GSK-3β suppresses mammalian cell proliferation and survival by phosphorylating the cytoplasmic protein, β-catenin. The phosphorylated form of β-catenin became the target for degradation by the ubiquitin pathway. Upon phosphorylation, GSK-3β loses its ability to

*Correspondence: P Fishman; E-mail: pfishman@post.tau.ac.il
Received 10 November 2001; revised 22 January 2002; accepted 26 March 2002

modify β-catenin, hence, this protein accumulates in the cytoplasm and subsequently translocates to the nucleus where it associates with LEF/TCF to induce transcription of cyclin D1 and c-*myc* (Ferkey and Kimelman, 2000; Novak and Dedhar, 1999; Morin, 1999). Earlier studies suggested the involvement of the Wnt signaling pathway in the pathogenesis of malignant melanoma (Bonvini *et al*., 2000; Robbins *et al*., 1996).

In this study, we demonstrate that activation of A3AR in melanoma cells decreases cAMP, thereby preventing the activation of both PKA and PKB/Akt. This event diminishes phosphorylation of GSK-3β and by maintaining this protein in its active form, leads to inhibition of cell proliferation via the Wnt pathway.

In the first series of experiments we examined the effect of the A3AR agonist, IB-MECA, on the proliferation of the melanoma cell line, B16-F10 by [^{3}H]thymidine incorporation assay. As shown in Figure 1, increasing concentrations of IB-MECA in the media results in a decrease in the growth of B16-F10 cells. Of note, while no evidence for apoptosis was obtained at these concentrations, treatment of cells with IB-MECA at the higher concentrations, i.e. 100 μM, caused apoptosis in these cells (data not shown). The antagonist to A3AR, MRS 1523, was able to block anti-proliferative activity of IB-MECA, suggesting that the observed tumor growth inhibition is mediated through A3AR. To investigate the mechanism whereby IB-MECA causes cell growth inhibition, we focused our attention on the signaling pathway downstream to A3AR. As activation of A3AR decreases the production of cAMP, we determined the level of cAMP in the control and IB-MECA treated cells by ELISA. Our results showed a marked decrease in the level of cAMP in IB-MECA treated cells (0.5 ± 0.041 pg/μl) compared to the control cells (4.2 ± 0.31 pg/μl) verifying the ability of IB-MECA to affect adenylyl cyclase activity. Examination of PKA, whose expression and activity is regulated by cAMP, revealed that at 10 and 20 min after serum stimulation, treatment of the cells with IB-MECA significantly decreased the level of PKA in B16-F10 cells (Figure 2a, top). The level of the control housekeeping protein, β-actin, remained constant in treated and untreated cells (Figure 2a, bottom). Evaluation of PKB/Akt showed no significant decrease in the level of the phosphorylated form of PKB/Akt after 15 and 30 min serum stimulation in the cells treated with IB-MECA. However, at 60 min following serum induction, a drastic decline in the level of phosphorylated form of PKB/Akt is observed in the treated cells (Figure 2b). Examination of the total level of PKB/Akt indicated no major differences in the level of expression of this protein during IB-MECA treatment (Figure 2c).

Since GSK-3β, a component of the Wnt signaling pathway presents one potential target for PKA and PKB/Akt (Li *et al*., 2000; Moule, 1997), in the next series of experiments we examined the level of GSK-3β by Western blot technique. As shown in Figure 3

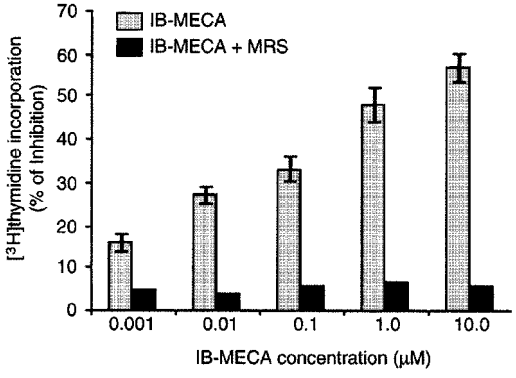

Figure 1 IB-MECA inhibits proliferation of B16-F10 melanoma cells. B16-F10 melanoma cells were maintained in RPMI without serum for 18 h. After 18 h, 1.5×10^4 cells were transferred to 96-well microtiter plates containing 1 ml of fresh medium containing 1% fetal bovine serum (FBS) in the absence and presence of various concentrations of IB-MECA (0.001 to 10 μM) (RBI/Sigma, Natick MA, USA) for 24 h. During the last 6 h of incubation, cells were labeled with 1 μCi of [^{3}H]thymidine. Cells were harvested and the [^{3}H]thymidine incorporation was determined in an LKB liquid scintillation counter. In parallel experiments, MRS 1523 at a concentration of 0.01 μM was added during the 24 h incubation. Data points are mean $\pm$ s.e.m. values from four independent experiments

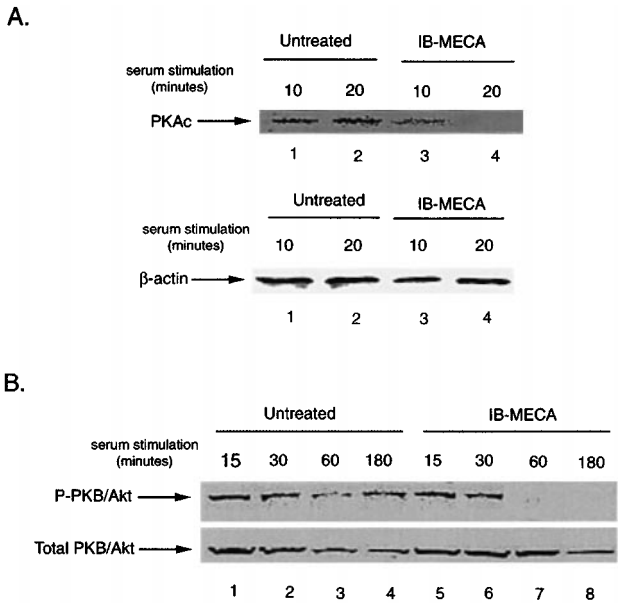

Figure 2 IB-MECA decreases the level of PKA and phosphorylated PKB/Akt protein levels in melanoma cells. (**a**) Protein extracts were prepared from untreated and IB-MECA treated (0.01 μM) cells after 10 and 20 min serum stimulation by the method described previously (Ausubel *et al*., 1989). Approximately 50 μg of protein were analysed by Western blot using rabbit polyclonal antibodies that recognize PKAc and β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The arrow points to the position of a band corresponding to PKAc. (**b**) Fifty micrograms of protein extract prepared from untreated and IB-MECA treated cells after 15, 30, 60, and 180 min serum stimulation, were analysed by Western blot using antibodies that recognize the phosphorylated form of PKB/Akt (top) and total PKB/Akt (bottom). The arrow points to the position of the specific bands

(Panel a, top), the level of GSK-3β was gradually decreased between 15 to 180 min serum stimulation (compare lanes 1 to 3). Under similar conditions, treatment of cells with IB-MECA led to a marked increase in the level of GSK-3β after 15 min serum stimulation (compare lane 1 to lane 4), and this level remained unchanged at 60 and 180 min after serum stimulation (compare lane 4 with lanes 5 and 6). The level of the reference housekeeping protein, β-actin, remained constant in the untreated and IB-MECA treated cells (Figure 3a, middle). As phosphorylation of GSK-3β by PKB/Akt results in inactivation of this protein, we examined the level of the phosphorylated form of GSK-3β in the control and IB-MECA treated cells. Our results showed a noticeable decrease in the level of phosphorylated GSK-3β in IB-MECA treated cells (Figure 3a, bottom). We also investigated the level of GSK-3β by immunocytochemistry using anti-GSK-3β antibody. As illustrated in Figure 3b, treatment of the cells with IB-MECA caused a noticeable decrease in the number of cells (top panels) and a significant enhancement in the amount of GSK-3β in the cells (bottom panels). As before, the antagonist to A3AR was able to reverse the negative effect of IB-MECA upon PKA expression and decreased the elevated level of GSK-3β, which was seen upon IB-MECA treatment (data not shown). These observations further verify that the modulation of PKA and GSK-3β is mediated by A3AR.

In light of these results, in the next series of experiments we examined the level of β-catenin, the well-known substrate of GSK-3β upon treatment of cells with IB-MECA. Results from Western blot analysis revealed a drastic decrease in the level of β-catenin upon treatment of the cells with IB-MECA (Figure 4a). Again, the level of β-actin remained unchanged. Evaluation of β-catenin by immunohistochemistry indicated strong staining of β-catenin in the control cells and very weak staining in the cells treated with IB-MECA. These observations demonstrate that treatment of cells with IB-MECA decreases the level of β-catenin in the cells. The observed reduced level of β-catenin in the IB-MECA treated cells may be due to the rapid ubiquitination of this protein by GSK-3β (Novak and Dedhar, 1999), whose level is increased in IB-MECA treated cells.

As stated earlier, the stability of β-catenin in cells results in its nuclear import through its partnership

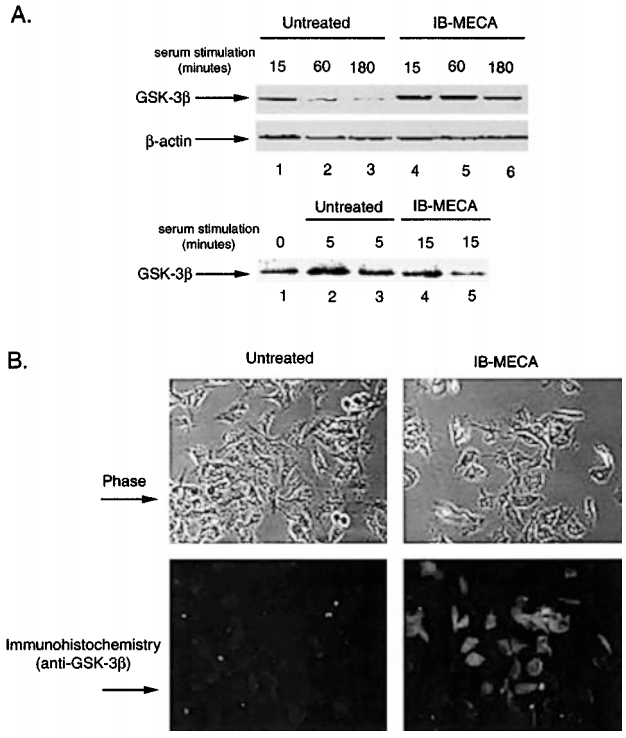

Figure 3 Expression of GSK-3β upon treatment of melanoma cells with IB-MECA. (**a**) Protein extracts (50 μg) from untreated and IB-MECA treated cells after 15, 60, and 180 min serum stimulation were analysed by Western blot using antibodies that recognize total GSK-3β (top) or the housekeeping protein, β-actin (middle). Examination of phosphorylated GSK-3β was carried out by Western blot analysis of protein extract after 5 and 15 min serum starvation in the untreated and IB-MECA treated cells using antibody that specifically recognize the phosphorylated form of GSK-3β. (**b**) Immunocytochemistry of untreated and IB-MECA treated melanoma cells using antibodies that recognize total GSK-3β cells. Briefly, cells were cultured on poly-L-lysine coated glass chamber slides in the absence or presence of IB-MECA. Immunocytochemistry was performed using a fluorescent system (immunofluorescence Kit, Vector Laboratories). Enhanced staining of the cells with anti-GSK-3β antibody upon treatment with IB-MECA is shown in bottom pictures, whereas the top pictures depict a phase-microscopic view of the cells

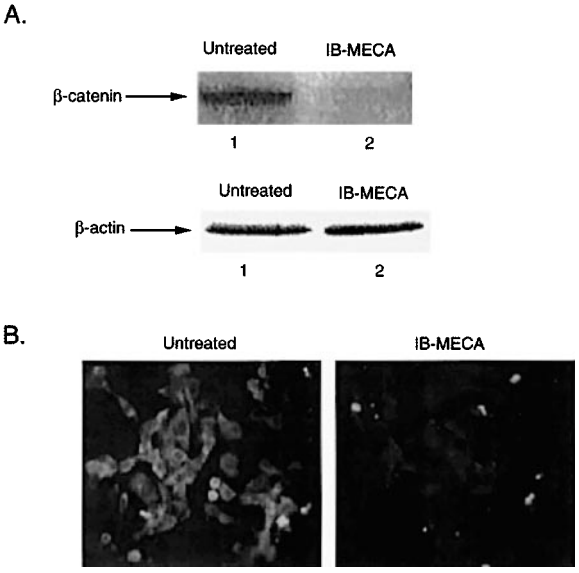

Figure 4 Decrease in the level of β-catenin upon treatment of cells with IB-MECA. (**a**) Western blot analysis of 50 μg of protein extracts from untreated and IB-MECA treated cells using anti-β-catenin antibody or anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to procedures described earlier (Gan *et al*., 2001). The position of β-catenin and β-actin are shown by the arrows. (**b**) Immunocytochemistry of untreated and IB-MECA treated cells depicting the level of β-catenin in the cells. The procedure for seeding and staining of cells has been described previously (Gan *et al*., 2001)

with LEF/TCF where it stimulates expression of genes such as cyclin D1 and c-*myc*. This event can eventually lead to uncontrolled cell growth and proliferation (Ferkey and Kimelman, 2000). The reduced level of β-catenin in IB-MECA treated cells prompted us to investigate the level of c-*myc* and cyclin D1 by Western blot analysis. As shown in Figure 5, the intensity of the band corresponding to c-*myc* and cyclin D1 was significantly less in cells treated with IB-MECA, suggesting down-regulation of c-*myc* and cyclin D1 by IB-MECA in melanoma cells. As before, this effect is specific for c-*myc* and cyclin D1, as the level of β-actin remains unchanged upon treatment. Furthermore, the observed inhibition by IB-MECA is at the transcription level as our results from RNase Protection Assay showed a similar decline in the level of transcript corresponding to cyclin D1 and cyclin D2, but not cyclin A2, cyclin B2, and cyclin D3 upon IB-MECA treatment (Figure 5c).

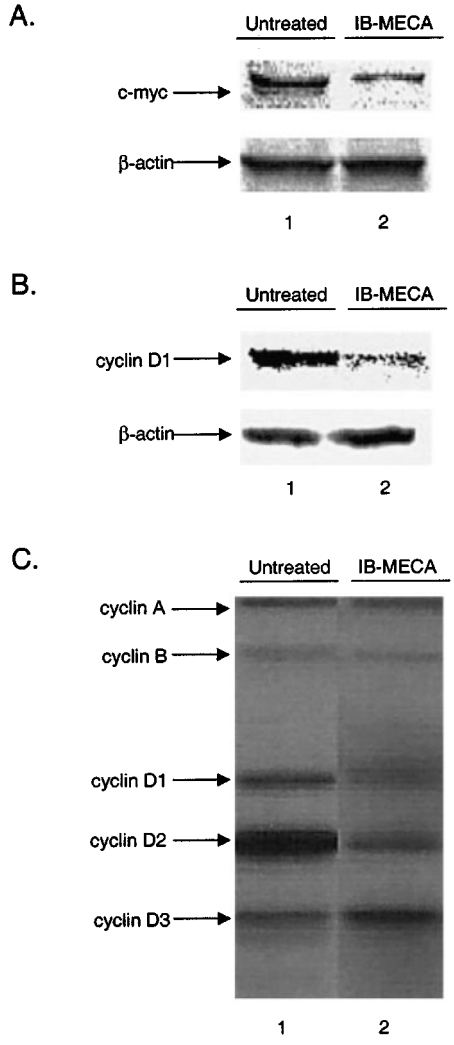

Figure 5 Expression of cyclin D1 and c-*myc* in cells treated with IB-MECA. Western blot analysis of protein extracts (50 μg) from untreated and IB-MECA treated cells using anti-c-*myc* (**a**) and anti-cyclin D1 (**b**) antibodies. RNase Protection Assay in untreated and IB-MECA treated melanoma cells (**c**). The assay was performed according to the instructions provided by the supplier (Pharmingen, San Diego, CA, USA) using 10 μg of RNA. The positions of various cyclins, A, B, D1, D2, and D3, are shown

In this study, we identified a potential mechanism which mediates suppression of melanoma cell growth by A3AR agonist, IB-MECA. The Wnt signaling pathway which is highly active during embryogenesis through modulation of several key cell cycle proteins, plays an important role in the development of various tumors (McEwen and Peifer, 2000). In a number of neoplastic cells including malignant melanoma, the central protein of this pathway, GSK-3β, loses its ability to phosphorylate β-catenin, an event that leads to ubiquitination of β-catenin in the cells (Morin, 1999; Bonvini *et al*., 2000; Robbins *et al*., 1996). At least two effector proteins, i.e. PKB/Akt and PKA, by controlling the level of expression and activity of GSK-3β, may affect the regulatory events associated with the Wnt pathway. For example, both PKB/Akt and PKA which phosphorylate GSK-3β at serine 9 and 21 can render this protein to its inactive form (Fang *et al*., 2000; Cross *et al*., 1995; Li *et al*., 2000). On the other hand, cAMP, by dissociating the PKAc unit from its parent molecule, potentiates PKA (Fang *et al*., 2000). Recent studies have provided evidence suggesting that cAMP can also activate PKB/Akt (Filippa *et al*., 1999), a key kinase which is commonly activated in response

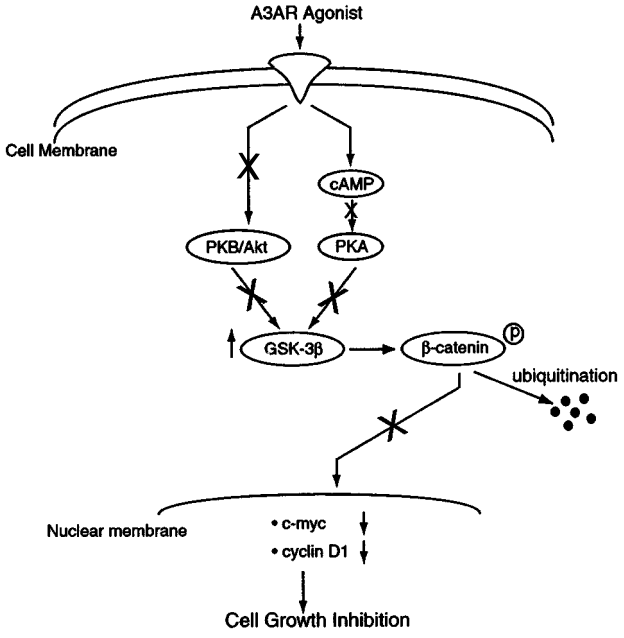

Figure 6 Proposed schematic representation of signaling pathways that mediate A3AR inhibition of melanoma cell growth. The interaction of IB-MECA with A3AR results in transmission of signals to PKA and PKB/Akt that results in an inhibition of GSK-3β phosphorylated form and the enhancement of its level in the cells. The elevated GSK-3β by phosphorylating β-catenin causes ubiquitination in the cytoplasm, thus, preventing its nuclear import. These events cause suppression of cyclin D1 and c-*myc* expression that eventually cause cell growth suppression

to stimulation by various growth factors through a phosphatidylinositol 3′ kinase, PI-3-kinase, dependent pathway (Moule *et al*., 1997). These observations along with our results presented here provide a model which describes the potential mechanism whereby IB-MECA suppresses growth of tumor cells. According to this model, activation of A3AR by IB-MECA decreases formation of cAMP which either directly or indirectly inhibits the levels of PKA and PKB/Akt (Figure 6). Inhibition of PKA and PKB/Akt, in turn, results in reduced levels of the inactive form of GSK-3β, thus permitting this kinase to phosphorylate β-catenin. As phosphorylation of β-catenin results in rapid degradation of this protein, cells which are treated with IB-MECA show a reduced level of β-catenin. A decrease in the β-catenin levels in IB-MECA treated B16-F10 cells eventually leads to suppression of c-*myc* and cyclin D1 expression, genes whose transcription can be augmented upon nuclear import of β-catenin and its partner TCF/LEF-1. In fact, earlier results demonstrated overexpression of both cyclin D1 and c-*myc* in melanoma cells (Caballero *et al*., 2001; D'Agnano *et al*., 2001; Miracco *et al*., 2000). Down-regulation of both c-*myc* and cyclin D1, the two major downstream targets in the Wnt pathway which controls cell proliferation, upon IB-MECA treatment is consistent with the involvement of the Wnt pathway in IB-MECA mediated suppression of tumor cell growth. It is also important to note that GSK-3β, c-*myc* PKA, and PKB/Akt may also act through alternative pathways to exert the cytostatic effect upon tumor cells. Earlier reports indicated that GSK-3β directly phosphorylates cyclin D1 on thr 286 which triggers its rapid turnover (Diehl *et al*., 1998). Thus it is likely that IB-MECA, by altering the state of cyclin D1 phosphorylation reduces the level of this protein in the cells. The decrease in the level of c-*myc* may also influence telomerase activity in the cells as earlier studies pointed to reactivation and stabilization of telomerase upon c-*myc* overexpression (Cerni, 2000). In support of this notion our previous results demonstrated that treatment of B16-F10 cells with IB-MECA reduces the telomerase activity in the cells (Fishman *et al*., 2000). Experiments are in progress to investigate the effect of IB-MECA on deregulation of other important biological pathways which are involved in the control of cell proliferation.

Acknowledgments
We thank Dr Jennifer Gordon for her insightful contributions to this work and Ms C Schriver for editorial assistance And preparation of the manuscript.

References

Ausubel F, Brent R, Kingston R, Moore D, Siedman JG, Smith JA and Struhl K. (1989). *Current Protocols in Molecular Biology*. New York: John Wiley and Sons. pp. 342–345.

Bar-Yehuda S, Barer F, Volfsson L and Fishman P. (2001). *Neoplasia,* **3,** 125–131.

Bonvini P, Hwang SG, El-Gamil M, Robbins P, Kim JS, Trepel J and Neckers L. (2000). *Biochim. Biophys. Acta.,* **1495,** 308–318.

Caballero OL, Sidransky D and Merbs SL. (2001). *Invest. Ophthalmol. Vis. Sci.,* **42,** 1679–1684.

Cerni C. (2000). *Mutat. Res.,* **462,** 31–47.

Cross DA, Alessi DR, Cohen P, Andjelkovich M and Hemmings BA. (1995). *Nature,* **378,** 785–789.

D'Agnano I, Valentini A, Fornari C, Bucci B, Starace G, Felsani A and Citro G. (2001). *Oncogene,* **20,** 2814–2825.

Diehl JA, Cheng M, Roussel MF and Sherr CJ. (1998). *Genes Dev.,* **12,** 3499–3511.

Fang X, Yu SX, Lu Y, Bast Jr RC, Woodgett JR and Mills GB. (2000). *Proc. Natl. Acad. Sci. USA,* **97,** 11960–11965.

Ferkey DM and Kimelman D. (2000). *Dev. Biol.,* **225,** 471–479.

Filippa N, Sable CL, Filloux C, Hemmings B and Van Obberghen E. (1999). *Mol. Cell. Biol.,* **19,** 4989–5000.

Fishman P, Bar-Yehuda S and Wagman L. (1998). *Cancer Res.,* **58,** 3181–3187.

Fishman P, Bar-Yehuda S, Barer F, Multani AS and Pathak S. (2001). *Exp. Cell Res.,* **269,** 230–236.

Fishman P, Bar-Yehuda S, Ohana G, Pathak S, Wasserman L and Multani AS. (2000). *Eur. J. Cancer,* **36,** 1452–1458.

Gan D-D, Reiss K, Carrill T, Del Valle L, Croul S, Giordano A, Fishman P and Khalili K. (2001). *Oncogene,* **20,** 4864–4870.

Li M, Wang X, Meintzer MK, Laessig T, Birnbaum MJ and Heidenreich KA. (2000). *Mol. Cell. Biol.,* **20,** 9356–9363.

Linden J. (1991). *FASEB J.,* **5,** 2668–2676.

McEwen DG and Peifer M. (2000). *Curr. Biol.,* **10,** 562–564.

Miracco C, Pacenti L, Santopietro R, Biagioli M, Fimiani M, Perotti R, Rubegni P, Pirtoli L and Luzi P. (2000). *Int. J. Cancer,* **88,** 411–416.

Morin PJ. (1999). *Bioessays,* **21,** 1021–1030.

Moule SK, Welsh GI, Edgell NJ, Foulstone EJ, Proud CG and Denton RM. (1997). *J. Biol. Chem.,* **272,** 7713–7729.

Novak A and Dedhar S. (1999). *Cell Mol. Life Sci.,* **56,** 523–537.

Ohana G, Bar-Yehuda S, Barer F and Fishman P. (2000). *J. Cellular Physiol.,* **186,** 19–23.

Robbins PF, El-Gamil M, Li YF, Kawakami Y, Loftus D, Appella E and Rosenberg SA. (1996). *Exp. Med.,* **183,** 1185–1192.

Sable CL, Filippa N, Hemmings B and Van Obberghen E. (1997). *FEBS Lett.,* **409,** 253–257.

Stiles GL. (1990). *Clin. Res.,* **38,** 10–18.

Zhao Z, Makaritsis K, Francis CE, Gavras H and Ravid K. (2000). *Biochim. Biophys. Acta.,* **1500,** 280–290.