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Research Paper

Imidazole inhibits B16 melanoma cell migration via degradation of β-catenin

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Abstract

Objectives In the present study, we determined whether or not imidazole affects B16 murine melanoma cell migration to prevent melanoma metastasis.
Methods To determine the effects of imidazole on melanoma cell migration, B16 cells were treated with imidazole at various concentrations, and the migration was measured using a scratch migration assay.
Key findings Imidazole did not exhibit cytotoxic effects on B16 cells at a concentration below 100 μM. The anti-migratory activity of imidazole was determined by the scratch migration assay. Our results showed that imidazole significantly inhibits B16 cell migration. It is known that the Wnt/β-catenin signalling pathway regulates the progression of melanocytic tumours and determines the prognosis in cutaneous melanomas. Western blot analysis demonstrated that imidazole increases phosphorylation of β-catenin and subsequent degradation of β-catenin. Moreover, inhibition of melanoma cell migration by imidazole was restored by MG132, a proteasome inhibitor, via inhibition of β-catenin degradation.
Conclusions Imidazole inhibits B16 cell migration through β-catenin degradation, suggesting that imidazole is a potential candidate for the treatment of metastatic melanoma.
Keywords imidazole; melanoma; metastasis; MG132; Wnt

Introduction

Malignant melanoma is known to develop from melanocytes.[1] Because malignant melanoma frequently metastasises to other organs, such as lymph nodes, lung, liver, brain, and bone, this aggressive cancer is responsible for 80% of skin cancer deaths.[2] If melanoma is detected at an early stage, it can be removed and/or treated easily with surgery, biological therapy, chemotherapy or radiotherapy. In contrast, when melanoma is diagnosed at an advanced stage, it is very hard to treat with current therapies because of spread to other organs.[2]

Dacarbazine (5-(3,3-dimethyl-1-triazenyl)imidazole-4-carboxamide), a derivative of imidazole, is a chemotherapeutic agent for the treatment of melanoma and has been a US Food and Drug Administration approved drug for 40 years.[3] However, dacarbazine provides complete remission in only 5% of melanoma patients.[3] Several studies have tested new anticancer candidates for superior efficacy in melanoma therapy. Imidazole (Figure 1) is a heterocyclic aromatic organic compound with the formula $C_3H_4N_2$. This ring system is present in important biological building blocks, such as histidine and histamine. It has been reported that imidazole and its analogues inhibit the proliferation of melanoma cells.[4,5] Recently, several studies have revealed that cimetidine (a histamine-2 (H_2) receptor antagonist), imidazo[1,2-*a*]quinoxaline and imidazo[1,5-*a*]quinoxaline (analogues of imiquimod) exhibit potent anticancer activity *in vitro* and *in vivo*.[6–8] Based on these studies, we reasoned that the structure of imidazole plays a key role in the anticancer effects of the compounds. Hence, we determined whether or not imidazole has an influence on the migration of melanoma cells because cancer cell proliferation and migration are important for cancer metastasis.

Recent studies have shown that the Wnt/β-catenin pathway is implicated in the progression of melanoma.[9,10] In principle, the Wnt/β-catenin pathway is crucial for normal development

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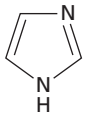

Figure 1 The structure of imidazole

of multicellular organisms via control of cell fate, proliferation and cell motility.[11] Wnt/β-catenin signalling is necessary and sufficient to drive neural crest cells to differentiate into melanocytes via direct control of transcriptional targets, such as microphthalmia-associated transcription factor.[11,12] Interestingly, although enhancing mutations in the β-catenin pathway are rare in melanomas, the Wnt5a-mediated non-canonical pathway has been implicated in melanoma metastasis.[9] Other studies have reported that alterations in membranous cadherin and catenin expression occur late in the biological progression of melanomas, such as regional lymph node metastasis.[13] Moreover, these same genes activated by Wnt/β-catenin signalling are antagonized by Wnt5a, and melanoma progression is associated with Wnt/β-catenin signalling.[9] Consequently, the Wnt/β-catenin signalling pathway can provide a possible target in the development of new drugs for melanomas.

In this study, we determined the effects of imidazole on B16 murine melanoma cells. To determine the antimigratory effect of imidazole on B16 melanoma cells, the mobility of B16 cells was analysed using a scratch migration assay. We also evaluated the effect of imidazole on migration-related signalling pathways, such as β-catenin, Akt and glycogen synthase kinase 3β (GSK-3β). Our study demonstrates that imidazole induces the antimigratory effects on B16 melanoma cells via β-catenin degradation.

Materials and Methods

Materials

Imidazole (Figure 1) was obtained from Sigma (St Louis, MO, USA). Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132) was purchased from Calbiochem (San Diego, CA, USA). Antibodies that recognise Akt, phospho-specific Akt (Ser473), GSK-3β, phospho-specific GSK-3β (Ser9), β-catenin and phospho-specific β-catenin (Ser33/37/Thr41) were obtained from Cell Signaling (Danvers, MA, USA). Anti-actin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell cultures

B16 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 50 μg/ml of streptomycin and 50 μg/ml of penicillin at 37°C in 5% CO_2.

Cell viability

Cell viability was assessed using the crystal violet staining assay.[14] After treatment with imidazole (0–100 μM) for 24 h, the culture medium was removed. Cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature, then rinsed four times. The crystal violet retained by the adherent cells was extracted with 95% ethanol and absorbance was determined at 590 nm using an ELISA reader (VERSAMax; Molecular Devices, Sunnyvale, CA, USA).

Cell adhesion analysis

Cell adhesion analysis was performed in six-well plates that had been precoated with poly-L-lysine (0.01%). B16 cells were suspended in serum-free DMEM. The cell suspension (1×10^5) was treated with or without different concentrations of imidazole in a volume of 1 ml/well, and then seeded into pre-coated wells. After a 30-min incubation at 37°C, the wells were washed three times with Dulbecco's phosphate buffered saline (DPBS). Following incubation overnight, adhesion was quantified by the crystal violet staining assay.

Scratch migration assay

For the scratch migration assay, cells were grown in six-well plates to 100% confluence and serum-starved for 24 h. Perpendicular wounds were made by dragging a sterile plastic tip across the cell plates and washed with DPBS. These plates were replenished with fresh medium alone or medium containing imidazole at various concentrations (0–100 μM) for 6 h. Cell migration was monitored under a phase contrast microscope (Olympus Optical Co., Tokyo, Japan) using an ocular grid at 0 and 6 h, and photographed using a DCM300 digital camera (Scopetek, Inc., Hangzhou, China), which was supported by ScopePhoto software (Scopetek, Inc.). To further confirm the inhibitory effect of imidazole on cell migration, B16 cells were pretreated with MG132 (25 μM) for 30 min, then treated with imidazole (50 μM) for 6 h. Cell migration was calculated using the following formula:

$$\text{cell migration} = (0\text{ time wound width} - \text{final wound width})/10$$

The migration rate (%) is represented as the percentage migration, with migration in the untreated control as 100%. All experiments were performed in triplicate and repeated at least three times.

Western blotting

The protein samples were prepared in mammalian protein extraction reagent (Pierce, Rockford, IL, USA) containing a complete protease inhibitor mixture (Roche, Mannheim, Germany). Samples were separated on 12% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene fluoride membranes, which were blocked with 5% dried milk in phosphate buffered saline containing 0.4% Tween 20. The blots were incubated with the appropriate primary antibodies at a dilution of 1:1000. Membrane-bound primary antibodies were detected using secondary antibodies conjugated with horseradish peroxidase and chemiluminescent substrate (Pierce). The images of the blotted membranes were obtained using a LAS-1000 lumino-image analyser (Fuji Film, Tokyo, Japan).

Statistics

The statistical significance of the differences between groups was assessed by analysis of variance (ANOVA), followed by the Student's *t*-test. P values less than 0.01 were considered significant.

Results

Imidazole has no influence on B16 cell viability

To examine the cytotoxicity of imidazole, B16 cells were treated with imidazole at 0–100 μM for 24 h. Cell viability was assessed by the crystal violet staining assay. As shown in Figure 2, imidazole did not exhibit cytotoxic effects in B16 cells. To further investigate the effect of imidazole on the viability of normal skin cells, HaCaT keratinocytes were treated with imidazole at 0–100 μM. Consistent with the findings in B16 cells, imidazole exhibited no cytotoxicity compared with the control (data not shown).

Imidazole inhibits B16 cell migration

To evaluate whether imidazole prevents B16 cell migration, the scratch migration assay was used. Migratory potentials were monitored after imidazole treatment for 6 h. Representative photographs of the scratch migration assay are shown in Figure 3a. Imidazole significantly inhibited the cell migration at 1–100 μM (Figure 3b). These data indicate that imidazole has potential inhibitory effects on B16 cell migration. Proliferation is a prerequisite for the migration and metastasis of cancer cells. Thus, we next investigated whether imidazole prevents cell proliferation or cell attachment in B16 cells. However, imidazole treatment did not have an effect on cell proliferation or attachment (data not shown).

Imidazole induces β-catenin degradation by activation of GSK-3β

To determine how imidazole prevents cell migration without affecting cell proliferation and attachment, Western blot analyses were performed for phosphorylation of β-catenin and Akt/GSK-3β, which are known migration-related signalling pathways. The phosphorylation of β-catenin was specifically activated 1 h after imidazole treatment (Figure 4a). Moreover, imidazole decreased the level of β-catenin protein in a dose-dependent fashion (Figure 4a). The activated (dephosphorylated) form of GSK-3β results in the degradation of β-catenin via phosphorylation of β-catenin.[15] Imidazole led to Akt activation, and also induced phosphorylation of GSK-3β at 3 h (Figure 4a and b). Thus, phosphorylated β-catenin was abolished by GSK-3β phosphorylation (inactivation).

We next used MG132, a selective proteasome inhibitor, to further verify the degradation of β-catenin by imidazole. MG132 prevented the degradation of β-catenin, as well as inhibition of cell migration induced by imidazole (Figure 5a and b). These results further confirm that increased proteasomal degradation of β-catenin occurs in imidazole-treated cells and decreased β-catenin is responsible for inhibition of migration by imidazole.

Discussion

In the present study, we demonstrated that the antimigratory effects of imidazole may include the degradation of β-catenin by regulating the phosphorylation of GSK-3β. In principle, cell migration is a basic process involved in organogenesis during development, normal homeostasis of adult tissues, and tissue regeneration and repair after injury.[16] In contrast to normal cells, some tumour cells, like melanomas, acquire the ability to migrate out of the initial tumour.[16] Melanoma is the most important malignancy induced by radiation in Caucasians.[1] The frequency of melanoma increases with age and chronic sun exposure.[1] Many studies have

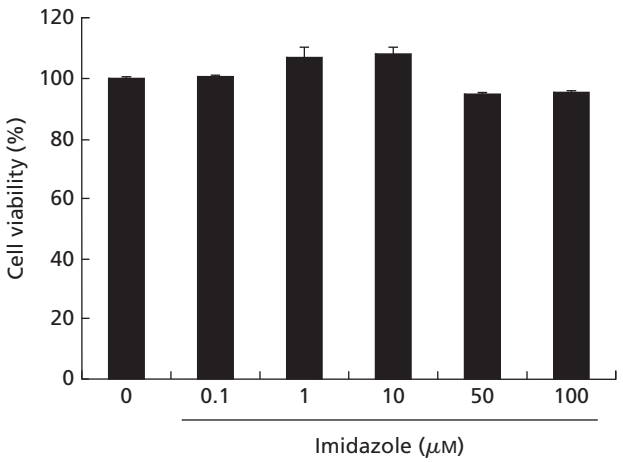

Figure 2 Effects of imidazole on B16 cell viability. After serum starvation, B16 cells were treated with imidazole. Cell viability was measured by the crystal violet assay. Imidazole was not cytotoxic in B16 cells. The data represent the means ± SD of triplicate assays expressed as percentages of the control

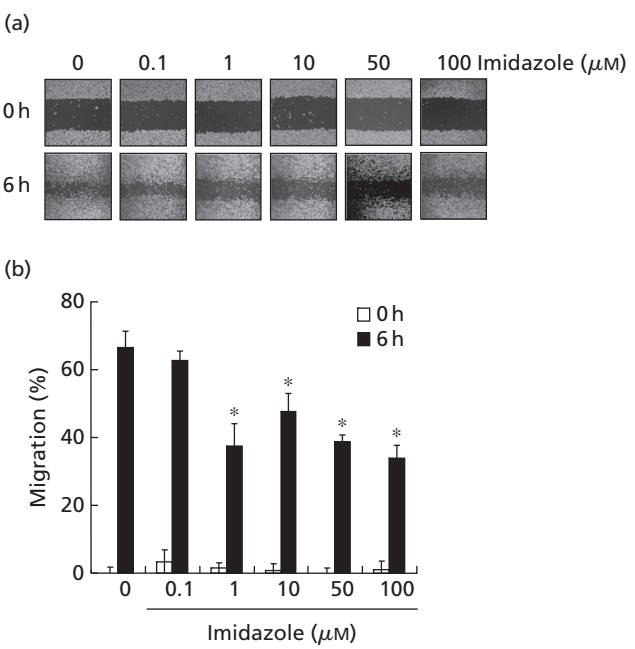

Figure 3 Effects of imidazole on the migration of B16 cells. Wounds were made, as described in the Materials and Methods section. B16 cells were treated with imidazole. (a) Phase contrast photographs were taken using a digital video camera. (b) The quantitative rate of cell migration is given in the graph. Data represent the means ± SD of 10 randomly chosen fields expressed as percentages of the control. *$P < 0.01$ compared to the untreated control

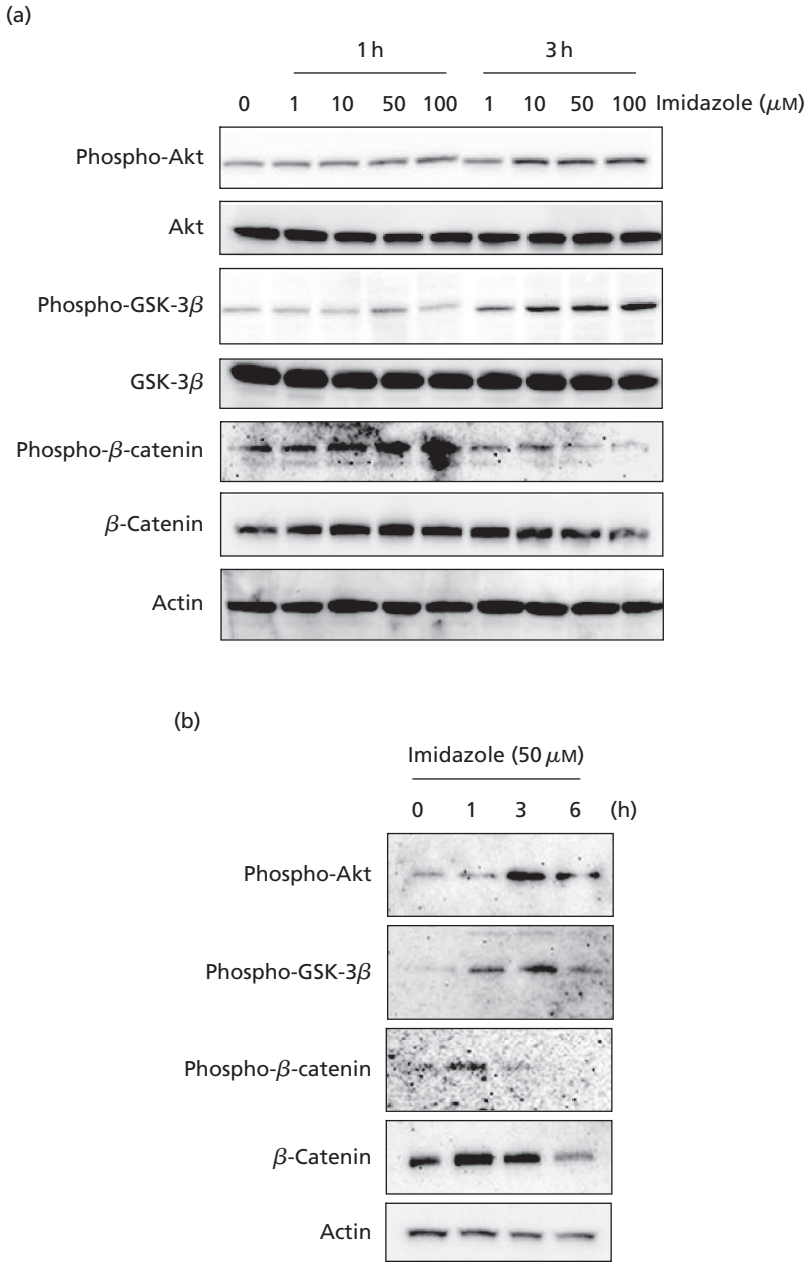

Figure 4 Effects of imidazole on cell migration-related signalling pathways. After serum starvation, B16 cells were treated with imidazole. Cells were harvested at the time points indicated. (a) Protein samples were then subjected to Western blot analysis with antibodies against phospho-specific Akt, phospho-specific GSK-3β, phospho-specific β-catenin or β-catenin. Equal protein loadings were confirmed by reaction with actin, Akt or GSK-3β antibodies. (b) Protein samples were then subjected to Western blot analysis with indicated antibodies. Equal protein loadings were conformed by reaction with anti-actin antibody

investigated the effectiveness of a large variety of anticancer drugs in the treatment of melanoma, such as various chemotherapies, immunotherapies and photodynamic therapy.[2,17] Nevertheless, melanoma remains a lethal entity. Moreover, billions of dollars have been spent on clinical treatments with unsatisfactory results.

The specific effect of imidazole on melanoma migration is not understood, although a previous report has suggested that imidazole and imidazoline analogues can inhibit proliferation in melanoma cells.[5,7,8] These studies suggested that imidazole rings may serve as an important source for melanoma treatment. Furthermore, these observations indicated that imidazole-containing substances could affect the cell migration and interrupt the metastatic cascade. Based on this concept, we also demonstrated that imidazole exhibited an inhibitory effect on the migration of B16 cells, although it did not affect proliferation and attachment (data not shown). Interestingly, these results were unexpected because a

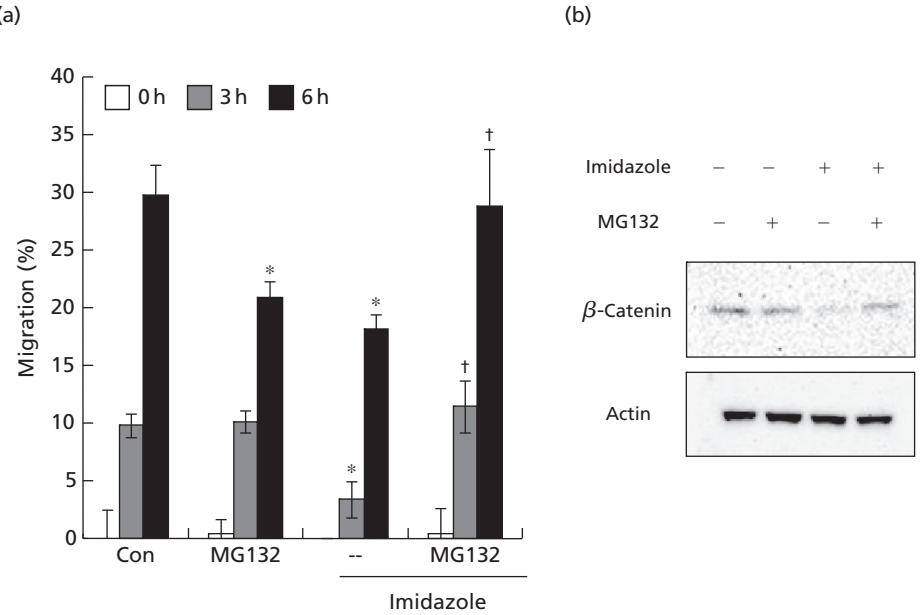

Figure 5 Prevention of the anti-migratory effect of imidazole by MG132. (a) Quantitative rate of cell migration is given in the graph. Cells were treated with imidazole (50 μM) in the presence or absence of MG132 (25 μM). Wounds were made as described in the Materials and Methods section. Data represent the means ± SD of 10 randomly chosen fields, expressed as percentages of the control. $^{*}P < 0.01$ compared to the untreated control (Con) and $^{\dagger}P < 0.01$ compared to the imidazole-treated group. (b) Cells were pretreated with MG132 (25 μM). Cells were harvested after 6 h of imidazole (50 μM) treatment, and protein samples were then subjected to Western blot analysis with an antibody against β-catenin. Equal protein loadings were confirmed by blotting with anti-actin antibody

previous study showed that imidazole and its analogues inhibit proliferation of melanoma cells.[5] Based on these contradictory results, we postulated the possible involvement of EphB4 signalling in cell migration of melanoma. Activation of the EphB4 receptor with its ligand ephrinB2 regulates the migration of melanoma cells.[18] Another study has reported that ephrinB2 induced inhibition of VEGF-induced cell migration without affecting VEGF-stimulated cell proliferation.[19]

Activation of the Wnt/β-catenin pathway is important for progression of melanocytic tumours and prognosis in cutaneous melanoma.[12] The dual role of β-catenin in melanoma tumorigenesis is not understood, but nuclear accumulation of β-catenin is associated with transcription of target genes, including tumorigenesis-related proteins, such as c-Myc, cyclin D_1 and matrix metalloproteinase-7.[12] Recent studies have demonstrated that some drugs (vitamins, lithium, curcumin and flavonoids) inhibit the Wnt/β-catenin signalling pathway.[20] Indeed, cytotoxic T-lymphocyte antigen-4 (CTLA-4) is expressed in human melanoma tumours, and anti-CTLA-4 antibody can be used as a treatment for melanoma via Wnt/β-catenin signalling.[21] Cordycepin, derived from *Cordyceps sinensis*, a parasitic fungus on the larvae of *Lepidoptera*, also inhibits the proliferation of B16-BL6 mouse melanoma cells by activation of the Wnt pathway.[22] In agreement with these findings, we also showed that imidazole strongly induces GSK-3β-mediated β-catenin phosphorylation and degradation (Figure 4). In addition, MG132 treatment further confirmed that the antimigratory effect of imidazole occurs via degradation of β-catenin (Figure 5). A previous study has demonstrated that MG132 inhibits invasion of human malignant pleural mesothelioma cells.[23] Thus, MG132 alone showed an influence on cell migration at 6 h (Figure 5a).

These data are consistent with previous findings that treatment with ciglitazone, a ligand of peroxisome proliferator-activated receptor (PPAR) γ, inhibits the Wnt/β-catenin pathway by proteasomal degradation of β-catenin.[24] In addition, it has also been reported that PPAR α activation decreases metastatic activity of melanoma cells in hamsters.[25,26] The antimigratory effects of imidazole for melanoma treatment need further in-vivo studies.

Conclusions

The present study has demonstrated that imidazole prevents cell migration through degradation of β-catenin. Overall, our findings suggest that imidazole may offer a potential target of the Wnt/β-catenin signalling pathway. From these results, we suggest that chemical substances whose structures include imidazole should be pursued in the research and development strategy for a new antimigratory drug against melanoma.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts on interest to disclose.

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References

1. MacKie RM. Incidence, risk factors and prevention of melanoma. *Eur J Cancer* 1998; 34(Suppl 3): S3–S6.
2. Chin L *et al*. Malignant melanoma: genetics and therapeutics in the genomic era. *Genes Dev* 2006; 20: 2149–2182.
3. Serrone L *et al*. Dacarbazine-based chemotherapy for metastatic melanoma: thirty-year experience overview. *J Exp Clin Cancer Res* 2000; 19: 21–34.
4. Sheets MR *et al*. Superelectrophilic chemistry of imidazoles. *J Org Chem* 2009; 74: 2502–2507.
5. Chen J *et al*. Synthesis and antiproliferative activity of imidazole and imidazoline analogs for melanoma. *Bioorg Med Chem Lett* 2008; 18: 3183–3187.
6. Fujikawa T *et al*. Cimetidine inhibits epidermal growth factor-induced cell signaling. *J Gastroenterol Hepatol* 2007; 22: 436–443.
7. Moarbess G *et al*. In vitro and in vivo anti-tumoral activities of imidazo[1,2-a]quinoxaline, imidazo[1,5-a]quinoxaline, and pyrazolo[1,5-a]quinoxaline derivatives. *Bioorg Med Chem* 2008; 16: 6601–6610.
8. Szincsak N *et al*. Cimetidine and a tamoxifen derivate reduce tumour formation in SCID mice xenotransplanted with a human melanoma cell line. *Melanoma Res* 2002; 12: 231–240.
9. Bachmann IM *et al*. Importance of P-cadherin, beta-catenin, and Wnt5a/frizzled for progression of melanocytic tumors and prognosis in cutaneous melanoma. *Clin Cancer Res* 2005; 11: 8606–8614.
10. Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 2004; 303: 1483–1487.
11. Larue L, Delmas V. The WNT/Beta-catenin pathway in melanoma. *Front Biosci* 2006; 11: 733–742.
12. Chien AJ *et al*. Activated Wnt/beta-catenin signaling in melanoma is associated with decreased proliferation in patient tumors and a murine melanoma model. *Proc Natl Acad Sci USA* 2009; 106: 1193–1198.
13. Sanders DS *et al*. Alterations in cadherin and catenin expression during the biological progression of melanocytic tumours. *Mol Pathol* 1999; 52: 151–157.
14. Kim DS *et al*. Indole-3-carbinol enhances ultraviolet B-induced apoptosis by sensitizing human melanoma cells. *Cell Mol Life Sci* 2006; 63: 2661–2668.
15. Ikeda S *et al*. Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin. *Embo J* 1998; 17: 1371–1384.
16. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; 100: 57–70.
17. Choudhary S *et al*. Photodynamic therapy in dermatology: a review. *Lasers Med Sci* 2009; 24: 971–980.
18. Yang NY *et al*. The EphB4 receptor-tyrosine kinase promotes the migration of melanoma cells through Rho-mediated actin cytoskeleton reorganization. *J Biol Chem* 2006; 281: 32574–32586.
19. Sturz A *et al*. EphB4 signaling is capable of mediating ephrinB2-induced inhibition of cell migration. *Biochem Biophys Res Commun* 2004; 313: 80–88.
20. Takahashi-Yanaga F, Sasaguri T. The Wnt/beta-catenin signaling pathway as a target in drug discovery. *J Pharmacol Sci* 2007; 104: 293–302.
21. Shah KV *et al*. CTLA-4 is a direct target of Wnt/beta-catenin signaling and is expressed in human melanoma tumors. *J Invest Dermatol* 2008; 128: 2870–2879.
22. Yoshikawa N *et al*. Cordycepin (3′-deoxyadenosine) inhibits the growth of B16-BL6 mouse melanoma cells through the stimulation of adenosine A3 receptor followed by glycogen synthase kinase-3beta activation and cyclin D1 suppression. *Naunyn Schmiedebergs Arch Pharmacol* 2008; 377: 591–595.
23. Yuan BZ *et al*. Proteasome inhibitor MG132 induces apoptosis and inhibits invasion of human malignant pleural mesothelioma cells. *Transl Oncol* 2008; 1: 129–140.
24. Grabacka M *et al*. PPAR gamma regulates MITF and beta-catenin expression and promotes a differentiated phenotype in mouse melanoma S91. *Pigment Cell Melanoma Res* 2008; 21: 388–396.
25. Grabacka M *et al*. Inhibition of melanoma metastases by fenofibrate. *Arch Dermatol Res* 2004; 296: 54–58.
26. Grabacka M *et al*. Peroxisome proliferator-activated receptor alpha activation decreases metastatic potential of melanoma cells in vitro via down-regulation of Akt. *Clin Cancer Res* 2006; 12: 3028–3036.