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Original Article

Reduction behavior induced by HL010183, a metformin derivative against the growth of cutaneous squamous cell carcinoma

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Abstract: Metformin is a biguanide widely prescribed as a first-line antidiabetic drug in type 2 diabetes mellitus patients. Animal and cellular studies support that metformin has a strong anti-proliferative effect on various cancers. Herein, we report that metformin derivative, HL010183 significantly inhibited human epidermoid A431 tumor xenograft growth in nu/nu mice, which in turn is associated with a significant reduction in proliferative biomarkers PCNA and cyclins D1/B1. Enhanced apoptotic cell death and an increase in Bax: Bcl2 ratio supported the tumor growth reduction. The mechanism of the drug effects appears to be dependent on the inhibition of nuclear factor kappa B (NFkB) and mTOR signaling pathways. Reduced enhancement of NFkB transcriptional target proteins, iNOS/COX-2 together with decreased phosphorylation of NFkB inhibitory protein IKBa were also observed. Further, AKT signaling activation was evaluated by the reduced phosphorylation at Ser473. In addition, a concomitant decrease in mTOR signaling pathway was also estimated from the reduced phosphorylation at mTOR regulatory proteins p70S6K and 4E-BP-1. Along with this, decreased phosphorylation of GSK3b, which is carried out by AKT kinases was also observed. Overall results suggested that HL010183 interrupt SCC growth via NFkB and mTOR signaling pathways.

Keywords: Metformin, HL010183, cutaneous squamous cell, apoptosis

Introduction

Metformin is a well-established antidiabetic drug with proven efficacy coupled with an overall favorable safety profile and low cost. Beyond glucose lowering, metformin has shown promising beneficial preliminary results regarding cancer development and progression. Metformin has been shown in some studies to decrease the risk of cancer as well as improve cancer mortality in diabetic population to levels below that of nondiabetic subjects [1-3]. A number of laboratory studies have demonstrated an overall favorable effect of metformin on cancer. Indeed, metformin (using high concentrations of up to 16 mM, while the therapeutic plasma levels in humans is 2.8-15 mM (0.465-2.5 mg/L) has been shown to inhibit proliferation of various histological types of human lung cancer cell lines [4]. An experiment examined the effect of metformin (with high concentrations of up to 10 mM) on proliferation of endometrial cancer cell lines [5]. Metformin significantly inhibited growth in a dose-dependent manner of the endometrial cancer cell lines. Treatment of hepatocellular carcinoma cell lines with metformin (using high concentrations of up to 10 mM) showed a decrease of cancer cells growth [6]. Indeed, metformin has been shown to have antiproliferative effects on both acute myeloid leukemia and acute promyelocytic leukemia cells [7, 8]. Moreover, in vitro invasion of human endometrial adenocarcinoma cells was attenuated by metformin while using concentrations which are normally achieved in human serum [9]. Another experiment indicated a growth inhibition of medullary thyroid cancer cells by metformin (using high concentrations of up to 5 mM) [10]. An anticancer effect was also observed by metformin (with high concentrations of 5-20 mM) on head and neck squamous carcinoma cell lines [11]. In addition, a favorable effect was also seen by metformin on human breast cancer cells and mouse fibrosarcoma cells, even at low concentrations of metformin comparable to those

achieved normally in human plasma [12]. Beyond reducing the risk of cancer, metformin may also improve the efficacy of chemotherapy. Indeed, metformin has been shown to enhance the sensitivity of endometrial cancer cells to cisplatin and paclitaxel [13, 14].

A number of possible mechanisms regarding the favorable effects of metformin on cancer have been proposed [15, 16]. Metformin acts by activating the AMPK pathway via an LKB1 dependent mechanism. LKB1 has been identified in studies as a tumor suppressor protein [17]. AMPK is activated by the increase of the intracellular AMP/ATP ratio in three distinct ways, which are antagonized by increased ATP levels [18]. First, AMP binds to regulatory sites on the AMPK γ-subunits which lead to conformational changes that allosterically activate AMPK. This typically leads to a fivefold or less activation of AMPK. Second, AMP facilitates the phosphorylation of the a-subunit at a specific threonine residue (Thr172) [19]. This phosphorylation of Thr172 leads to at least a 50 to 100-fold activation of AMPK. Third, the binding of AMP to AMPK prevents the dephosphorylation of Thr172 by phosphatases [20]. Furthermore, in studies of various cancer cell lines, the metformin-mediated activation of the LKB1AMPK pathway has been shown to inhibit the activation of the mammalian target of rapamycin (mTOR) and protein synthesis [21, 22]. Moreover, metformin has also shown an AMPK independent pathway of inhibiting mTOR via decreasing the levels of IGF-1 [23, 24]. In addition, metformin inhibited mTOR activity in the absence of tuberous sclerosis complex proteins 1 and 2 (TSC1/2) and AMPK by suppressing RAG GTPases, which are involved in mTOR activation [25]. mTOR plays a major role in carcinogenesis and its activation is linked with cancer progression and poor outcomes [26]. Furthermore, as described above, metformin lowers hepatic gluconeogenesis in the absence of both AMPK and LKB-1 by reducing hepatic energy [27]. Moreover, metformin has exhibited an apoptosis-inducing effect in lung cancer cells via activation of the JNK/p38 MAPK pathway and the upregulation of the growth arrest and deoxyribonucleic acid (DNA) damage inducible gene 153 (GADD153) [28]. As a result an antiproliferative effect with metformin treatment may be observed. Based on the aforementioned facts, we tested whether Metformin derivative, HL010183 administration retards the growth of cutaneous SCCs in a human tumor xenograft highly immunosuppressed nu/nu murine model. In this study, human A431 epidermoid carcinoma cells were utilized for developing xenograft tumors.

Materials and methods

Reagents and antibodies

HL010183 was synthesized according to the literature report [29] with slight modification. Dimethyl-N-cyanodithioiminocarbonate, N,N-dimethylguanidine sulfate, potassium carbonate (K_2CO_3), m-chloroperbenzoic acid (m-CPBA), 2-propylaniline, 1,4-dioxane were purchased Sigma Aldrich company (Brockville, Ontario, Canada). The primary antibodies Cyclin B1, cdc2, p38, p-Akt1/2/3(Ser473), Akt1/2/3, p-GSK3b, GSK3b were purchased from Santa Cruz Biotechnology Inc. (Dallas, Texas, USA). Bax, Bcl-2 (C-21), iNOS, P44/42 MAPK (ERK1/2), p-ERK, p-p38, p-PI3K 85Kda, PI3K 85Kda, PI3K 110Kda, mTOR, mTOR (Ser 2448), mTOR (Ser 2481), p-p70S6 kinase, p70S6 kinase, p-4E-BP-1, 4E-BP-1, p-AMPK and AMPK were purchased from Cell Signaling Technology (Beverly, Minneapolis, USA). GLUT1 and GLUT4 were purchased from Abcam, (Danvers, Massachusetts, USA) COX-2 from Cayman chemical (Ann Arbor, Michigan, USA) and Cyclin D1 from Thermo Fisher Scientific Company (Fremont, California, USA). All other chemicals and solvents used were of analytical grade and obtained from Xi'an Chemical Co., Ltd. (Boaji, Shaan'xi, China).

Cell lines

Human epidermoid carcinoma, A431 cells were obtained from Creative Bioarray (Shirley, New York, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U mL^{-1} of penicillin, and 100 μg mL^{-1} of streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Tumor xenograft study

3-5 weeks old Female athymic NCr-nu/nu mice of 25-30 g were purchased from NCI-Frederick Animal Production Program (Frederick, MD). Animals were housed under standard conditions of fluorescent lighting, 12 h per day at room temperature, and relative humidity of

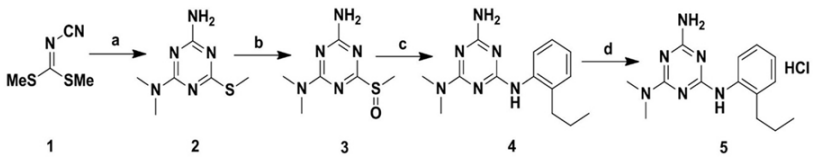

Figure 1. Reagents and conditions: A. N,N-dimethylguanidine sulfate, 40% K2CO3 solution, DMSO, 120°C, 2 h; B. m-CPBA, CH_2Cl_2, 0°C to room temp, 12 h; C. 2-propylaniline, 1,4-dioxane, reflux, 15 h; D. 4 M HCl in dioxane, room temp, 2 h.

45-55%. Animals were divided into two groups of six mice each. Each mouse from both groups received 5 × 10^6 cells in 200 μL of PBS subcutaneously in both flanks. Starting 24 h post-tumor cell inoculation, group I mice received an injection of vehicle (PBS) whereas each mouse in group II were given 5 mg of HL010183 in PBS (i.p.) 5 days per week for 3 weeks. Tumors of about > 1 mm in diameter were measured by digital calipers twice weekly and tumor volumes were calculated using the formula volume = length × width × height, plotted as a function of days on test. At the final stage of the experiment, mice were sacrificed and tumors were harvested for analysis and the study was approved by the ethics review board of affiliated hospital of Hebei University of Engineering.

Tumor xenograft histology and immunohistochemistry

All tumor xenograft tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 5 μm sections. Tissue slides were stained with hematoxylin and eosin (H&E) for histology. Proliferation cell nuclear antigen (PCNA) staining of formalin-fixed tumor tissue was performed by Vectastain ABC kit (Vector Laboratories Inc., Burlingame, California, USA) as per manufacturer's instructions. Sections were counterstained with Harris hematoxylin (Sigma-Aldrich, Shangai, China), dehydrated and mounted using Permount (Zhejiang HiSun Minsheng Pharmaceutical Co., Ltd, Zhejiang, China).

TUNEL assay

TUNEL assay in tumor xenograft tissue was performed using in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction. Sections were counterstained with DAPI and mounted.

Western blot analysis

The tumor tissue was homogenized in ice cold lysis buffer (50 mM Tris pH 7.5, 1% Triton X-100, 0.25% NaF, 10 mM, β-glycerol phosphate, 1 mM EDTA, 5 mM sodium pyrophosphate, 0.5 mM Na_3VO_4, 10 mM DTT, 1% PMSF and protease inhibitors). The homogenate was centrifuged at 13 000 g for 20 min at 4°C and then the supernatant was aliquotted and stored at 80°C. For western blotting, 40-80 μg proteins were resolved on 8-12% polyacrylamide gel (BioRad, Burlingame, California, USA). The proteins were transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) and then the membranes were incubated with primary antibody overnight at 4°C. After washing with TBST the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, Illinois, USA) for 1 h. The immune-complex was detected with chemiluminescent substrate (Pierce, Rockford, Illinois, USA) and was exposed to HyBlot CL autoradiography film (Denville Scientific Inc., New Jersey, USA). Membranes were then stripped and reprobed with β-actin antibody to verify equal protein loading. In instances where a blot is stripped multiple times and probed with different antibodies, but the data are presented as a part of more than one figure, the same β-actin image was placed at the bottom of these different figures. Relative density of western blot bands was analyzed by using IMAGE J software.

Statistical analysis

Statistical analysis was performed using Microsoft Excel software. The significance between two test groups was determined using Student's t-test. A *P*-value of < 0.05 was considered to be significant.

Results

Synthesis of HL010183

HL010183 was synthesized based on reaction **Figure 1**. HL010183 was obtained after several steps. In the first step, dimethyl-N-cyanodithi-

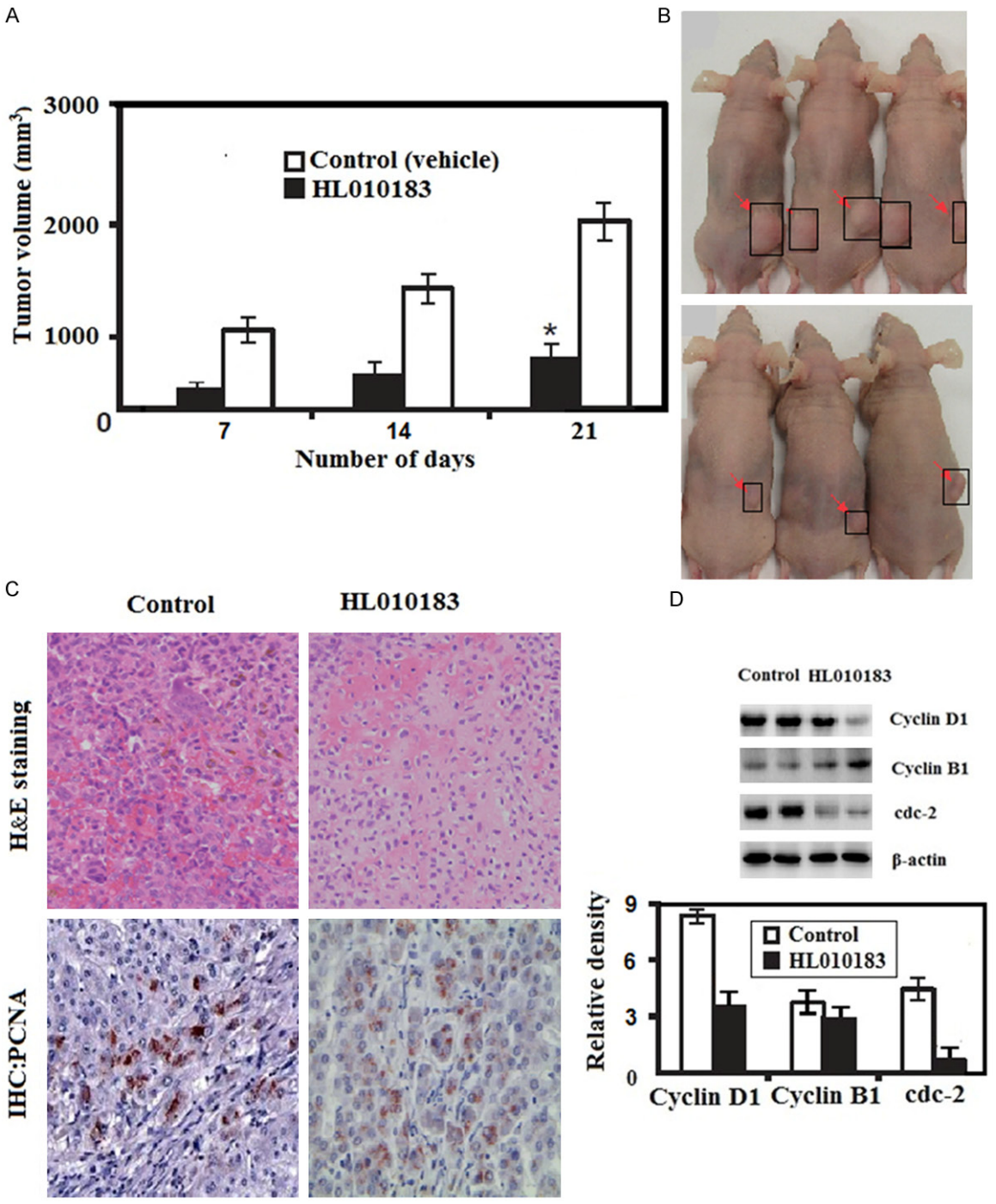

Figure 2. HL010183 reduces SCC growth by dampening cell cycle progression and blocking proliferation. Each mouse was subcutaneously injected with 5 × 10^6 cells in PBS on both flanks. Two days later, either vehicle (150 μL) or HL010183 (5 mg per mouse in 150 μL PBS; i.p.) was given daily for 5 days per week for 3 weeks. A. Average tumor volume (mm^3) ± SEM/mouse; B. Representative pictures of mice showing xenograft tumors. The A431 tumor xenograft tissues were harvested at the termination of the experiment. Tumor lysates were subjected to Western blot analysis; C. H&E staining and immunohistochemical analysis of proliferation marker PCNA in paraffin-fixed tumor tissue sections. Metformin treatment resulted in a reduction in the number of PCNA-positive cells (magnification 20 ×); D. The expression levels of cyclin D1, cyclin B1 and cdc2 from different xenograft tumor groups. Relative density of bands was analyzed using IMAGE J software, normalized to the respective β-actin band intensities to account for sample loading variation, and shown as a bar graph. Statistical significance of difference between control and metformin groups was analyzed by Student's t-test. β-actin was used to confirm equal loading of the samples.

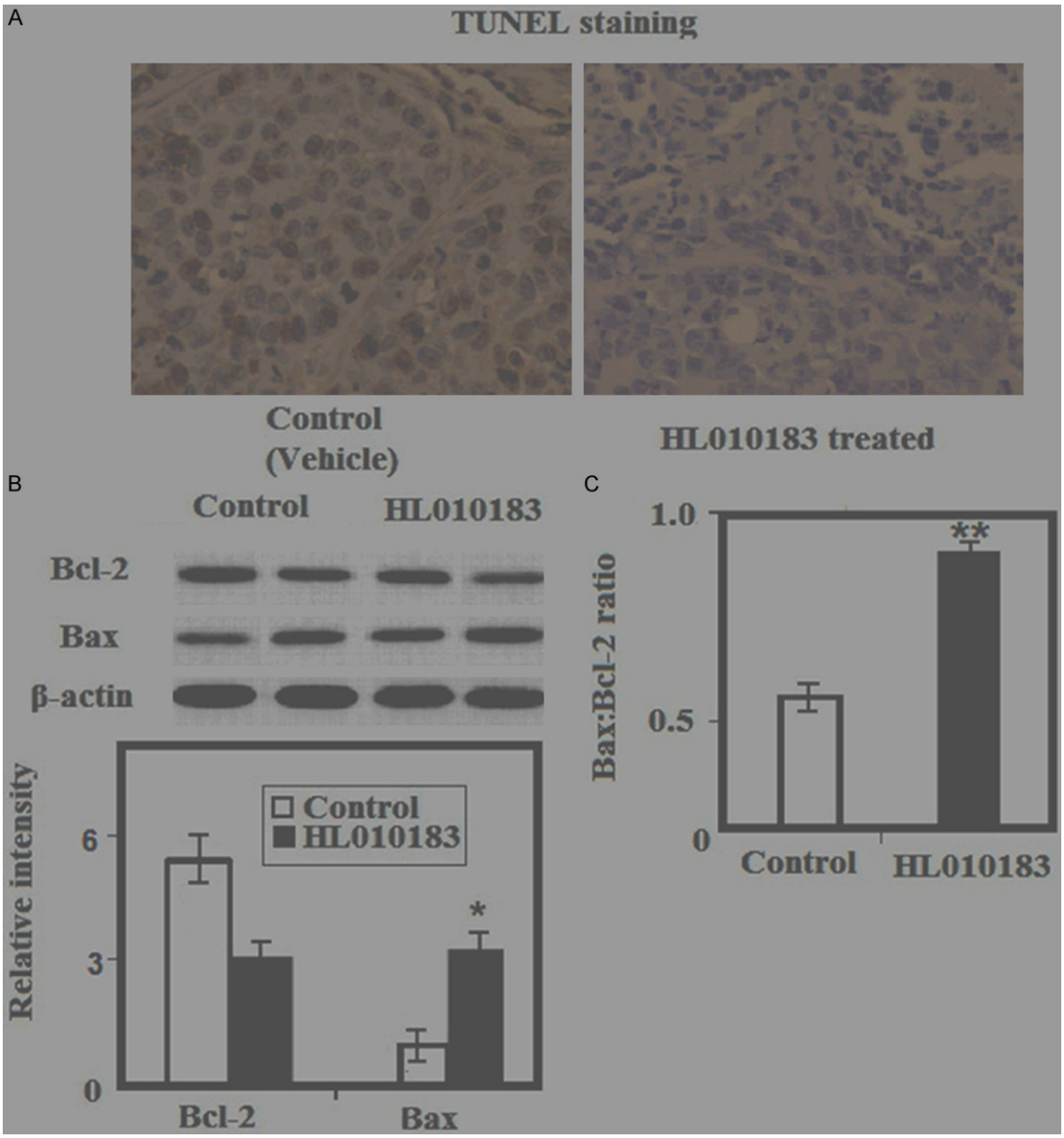

Figure 3. HL010183 enhances Bax: Bcl2 ratio and apoptosis as evidenced by the accumulation of TUNEL-positive cells in metformin-treated SCCs. A. Effect of HL010183 on the number of TUNEL-positive cells in tumor xenograft tissue harvested at the termination of experiment; B. Western blotting showing expression of pro-apoptotic Bax and antiapoptotic Bcl2; C. Bax: Bcl2 ratio was calculated by densitometric analysis data and expressed as mean ± SE of three individual values. β-actin was used to confirm equal loading of the samples. Relative density of bands was analyzed using IMAGE J software, normalized to the respective β-actin band intensities to account for sample loading variation, and shown as a bar graph $*P < 0.05$ and $**P < 0.001$.

oiminocarbonate (1) with N,N-dimethylguanidine sulfate in the presence of potassium carbonate (K_2CO_3) yielded the triazine compound 2, which upon subsequent oxidation with m-chloroperbenzoic acid (m-CPBA) at room temperature afforded the sulfonyl compound 3. This was then treated with 2-propylaniline in 1,4-dioxane under reflux condition, provided compound 4 which was followed by treatment with hydrochloric acid (HCl) yielded compound 5 (HL010183), in a quantitative yield.

Mp: 147°C; ^{1}H NMR (300 MHz, DMSO-d_6) d 9.82 (br s, 1H), 8.02 (br s, 2H), 7.12-7.29 (m,

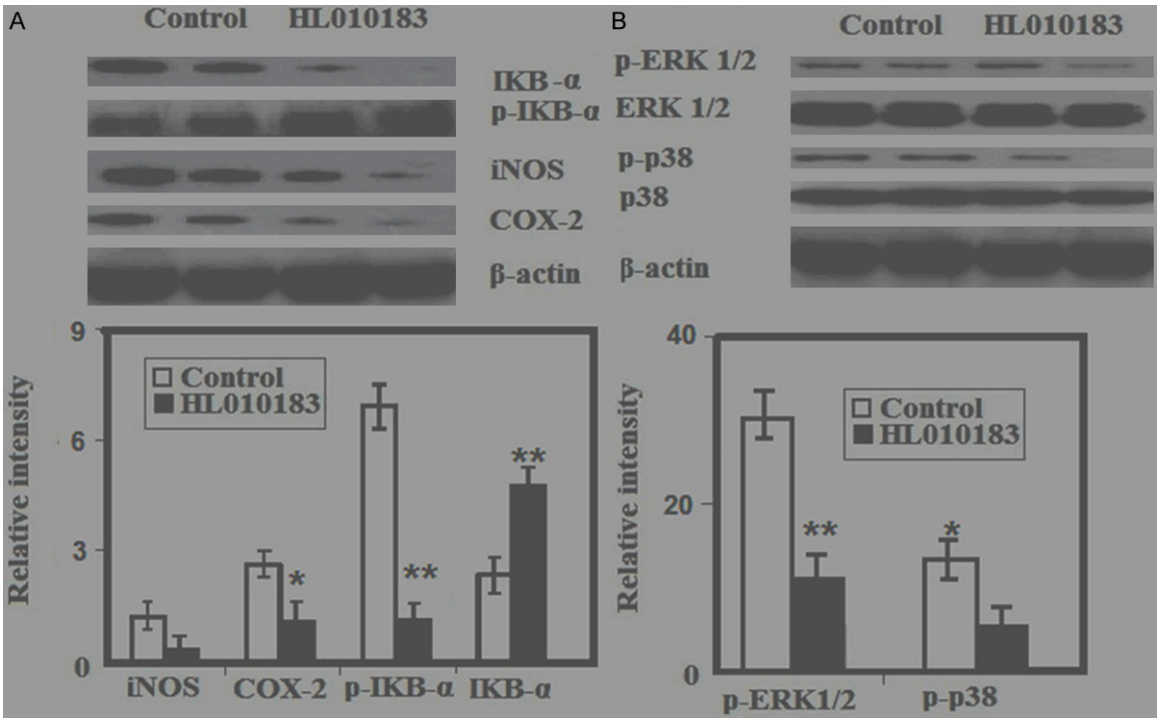

Figure 4. HL010183 targets NFjB and MAPK signaling pathways. The A431 tumor xenograft tissues were harvested at the termination of the experiment, and tumor lysates were subjected to analyses of phosphorylation of ERK1/2 and p38 protein using western blot analysis. A. The expression levels of p-Ikbα, Ikbα, iNOS and COX-2 proteins; B. The phosphorylated forms of ERK1/2 and p38. β-actin was used to confirm equal loading of the samples $*P < 0.05$ and $**P < 0.001$.

4H), 3.14 (s, 6H), 2.48 (s, 2H), 1.41 (s, 2H), 0.97 (s, 3H). MS (ESI) m/z: 273.4.

Inhibition of tumor growth and cell cycle regulatory proteins in A431 xenografts

HL010183 inhibition of tumor growth and cell cycle regulatory proteins was assessed in A431 human epidermoid tumor xenografts using nu/nu mice. The animals used here were first implanted with A431 cells followed by dividing into two sections receiving vehicle or HL-010183. Treatment with HL010183 significantly reduced the development of xenograft tumors in these highly immunosuppressed mice and the tumor volumes were also reduced over a period of 3 days-3 weeks (**Figure 2A**, **2B**). At the end of the experiment, tumor volume in HL010183-treated mice was reduced by 58.7%. The mean tumor volume in HL-010183-treated mice was 663.5 ± 179.0 mm^3 as compared with 1735.2 ± 636.1 mm^3 in vehicle-treated controls ($P < 0.05$). No significant alteration in the body weights of mice treated with HL010183 or vehicle was identified (data not shown). H&E staining showed similar histology between tumors developed in HL010183 treated animals and vehicle-treated controls (**Figure 2C**). However, these results showed that HL010183 treatment reduced the expression of proliferation related biomarkers. As shown in **Figure 1C** immunohistochemistry was also analyzed via PCNA expression. Also, the G1-associated cyclin D1, G2/M progression-associated cyclin B1 and its partner kinase cdc2 were found to decrease in the HL010183 treated group when compared to that of controls (**Figure 2D**).

Apoptosis and Bax: Bcl2 enhancement ratio in xenograft human SCCs

HL010183 induced apoptosis was assessed using TUNEL assay. It was found that the number of TUNEL-positive cells was greater in HL010183-treated tumors when compared to that of control (vehicle-treated) tumors (**Figure 3A**). The expression of anti-apoptotic Bcl2 and pro-apoptotic Bax was assessed by western blot analysis (**Figure 3B**). In HL010183-treated

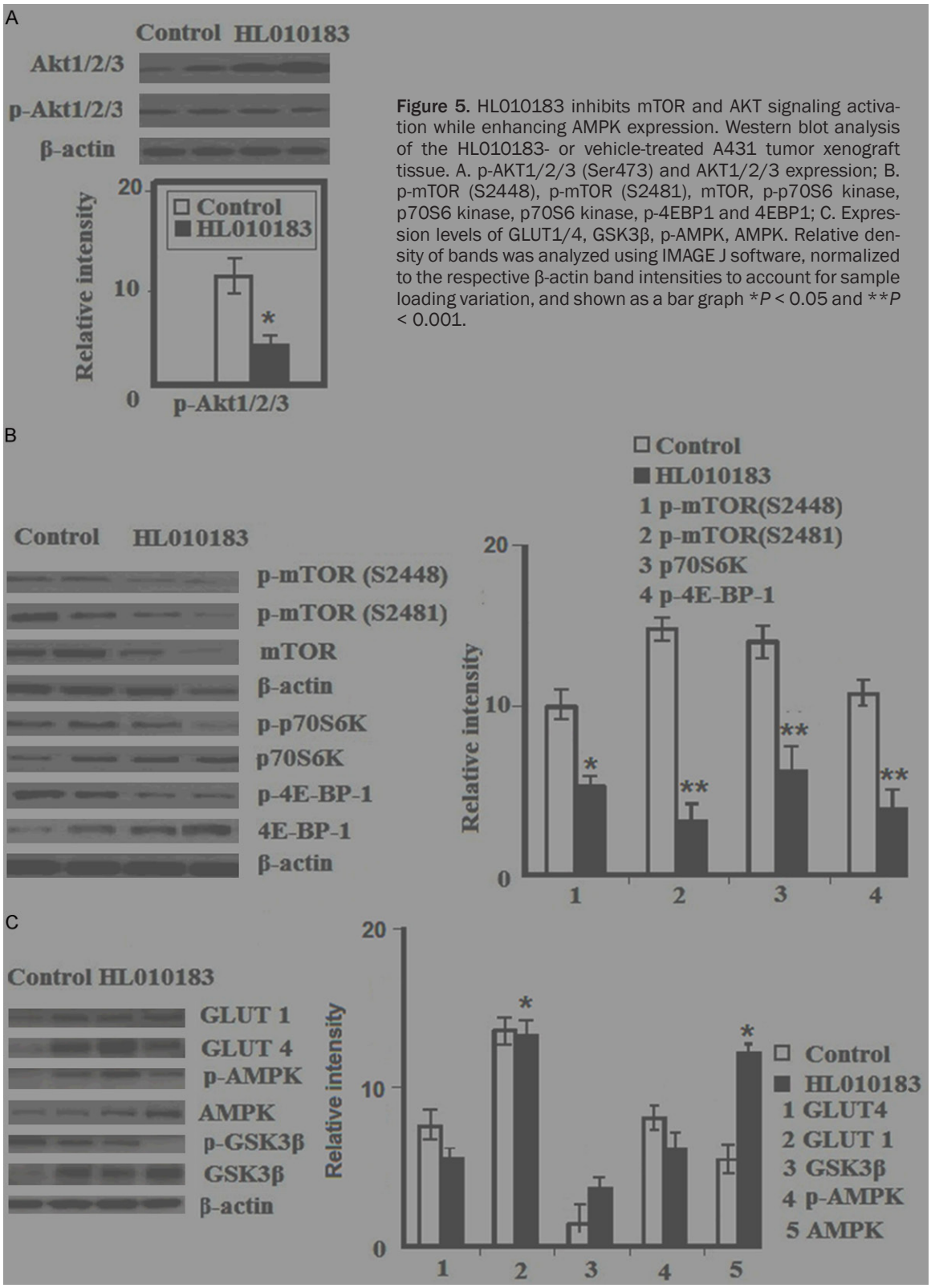

Figure 5. HL010183 inhibits mTOR and AKT signaling activation while enhancing AMPK expression. Western blot analysis of the HL010183- or vehicle-treated A431 tumor xenograft tissue. A. p-AKT1/2/3 (Ser473) and AKT1/2/3 expression; B. p-mTOR (S2448), p-mTOR (S2481), mTOR, p-p70S6 kinase, p70S6 kinase, p70S6 kinase, p-4EBP1 and 4EBP1; C. Expression levels of GLUT1/4, GSK3β, p-AMPK, AMPK. Relative density of bands was analyzed using IMAGE J software, normalized to the respective β-actin band intensities to account for sample loading variation, and shown as a bar graph $*P < 0.05$ and $**P < 0.001$.

tumors, the ratio of Bax: Bcl2 is detrimental to the live/dead signal following apoptotic stimuli was found to be significantly increased ($P < 0.001$) as shown in **Figure 3C**.

HL010183 targets NFkB and MAPK signaling pathways

It is known that NFkB is a transcription factor that can regulate both proliferation and apoptosis [14]. NFkB, when inactive, resides in the cytoplasm as a heterotrimeric complex comprised of p50/p52, p65 and inhibitory kappa B (IkB). This complex is disrupted upon phosphorylation of IkB through the activation of upstream kinases. Dissociated p-IkB is ubiquitinated and degrades, whereas the remaining heterodimeric are translocated to the nucleus to carry out its transcription functions [30]. In this work, detailed study was not performed to evaluate NFkB signaling but the phosphorylation status of IkBa and expression of NFkB transcription target proteins iNOS and COX-2 were determined. Reduction in NFkB activation was suggested from the observations of a significant decrease in the expression of p-IkBa with a concomitant increase in IkBa. In addition, a decrease in the expressions of iNOS and COX-2 was also supported the fact (**Figure 4A**). MAPKs (Mitogen-activated protein kinase) are serine/threonine kinases that involved in regulating different cellular responses, such as cell proliferation and apoptosis during the pathogenesis of skin cancer [31]. MAPK signaling cascade is also a target of NFkB signaling. The effects of HL010183 on the phosphorylation-dependent activation of ERK1/2 and p38 in A431 tumor xenografts are shown in **Figure 4B** and also the phosphorylation of ERK1/2 and p38 were reduced by 75.2% and 30.2%, respectively ($P < 0.05$) in the HL010183 treated group (**Figure 4B**).

Modulation of PI3k/Akt/mTOR signaling proteins

It is known that PI3K/Akt/mTOR signaling pathway is activated by physiologic sensors of nutrients, regulating metabolism and tumor growth [32]. Studies showed inducement of tumor cell proliferation and growth can be identified from the increased phosphorylation of mTOR (Ser-2448), p70S6K (Thr389), 4E-BP-1 (Ser65 and Thr37/46) and Akt (Thr308, Ser473) [33]. In this experiment, it was observed that HL010-183 treatment significantly reduced the phosphorylation of mTOR at S2448 and S2481, p70S6K, 4EBP1 and Akt at Ser473 (**Figure 5A**, **5B**) and left the expression of p-PI3k (p85), PI3k (p85) and PI3k (p110) unaltered (data not shown). Consistently, the phosphorylation of GSK3b was also found reduced. As shown in **Figure 5C**, it was observed that HL010183 treatment also activated AMPK, although no significant effects could be recognized on glucose regulatory GLUT1 and GLUT4 proteins.

Discussion

Insulin has proliferative and mitogenic effects promoting the development of cancer [34]. Indeed, hyperinsulinemia plays an important role in cancer proliferation. Metformin by decreasing circulating levels of insulin may ameliorate this negative effect of hyperinsulinemia in diabetic patients. Of note, a study indicated that metformin was associated with smaller cancer prevalence when compared with sulfonyl-urea derivatives which are insulin secretagogues [35]. Moreover, metformin treatment can lead to weight loss [36]. Indeed, a recent study described a weight reduction of 2.1 kg after 2 years of treatment (Diabetes Prevention Program Research Group, 2012). Obesity is a risk factor associated with cancer development. Therefore, metformin may help alleviate another cancer risk factor. Consistent with this notion, we found that the metmorfin derivative HL010183 could significantly reduce the growth of human epidermoid carcinoma A431 xenograft tumors. These responses are similar to those reported for other A431 xenograft tumors [29]. The inhibition in tumor growth was accompanied by an increase in Bax/Bcl2-regulated apoptosis signaling and inhibition of activated MAP kinase, ERK1/2 and p38 proteins. MAPK signaling is a target for metformin-mediated diminution of cancer cell growth. HL010183 exerted more potent inhibitory effects on the proliferation and invasiveness of Hs578T triple-negative breast carcinoma cells than metformin. HL010183 showed approximately 100-fold more potent effects compared to metformin. In a triple-negative breast cancer xenograft model, HL010183 showed a comparable degree of inhibitory effect on in vivo tumor growth at the 100 mg/kg dose to that of metformin at 500 mg/kg. Metformin-mediated decrease in IKBα phosphorylation, accompanied by diminished levels of iNOS and COX-2, suggested a role of metfomin in inhibiting the transcriptional activation of NFkB in SCCs as well as in the inhibition of inflammation regulatory protein expression. Donnini et al. reported a similar inhibition of iNOS and COX-2 associated with a reduction in SCC tumor growth [37].

mTOR plays a major role in carcinogenesis and its activation is linked with cancer progression and poor outcomes [38, 39]. Furthermore, as described above, metformin lowers hepatic gluconeogenesis in the absence of both AMPK and LKB-1 by reducing hepatic energy [40]. The observed results showed that HL010183 activated AMPK in A431 tumor xenografts were consistent with other studies suggesting that it acts by turning off mTOR activity. The importance of these signaling pathways in the pathogenesis of cutaneous neoplasm is clear from a number of studies. In many of tumor cell-types, the PI3k/Akt signaling pathway is known to promote cell proliferation, cell cycle progression and to reduce apoptosis [29]. In the skin, a mechanism by which Akt augments UVB-induced carcinogenesis involves mTOR activation [13]. The subunit composition of mTOR complex1 (mTORC1) regulates mRNA translation initiation and progression through p70S6 kinase 1 (p70S6K) and eIF4E-binding protein-1 (4E-BP1), thus controlling the rate of protein synthesis [41, 42]. The observations in this study showed that HL010183 diminished phosphorylation of Akt with a concomitant decrease in phosphorylated mTOR and their downstream substrates, suggested an inhibition of Akt/mTOR pathways in HL010183-mediated tumor suppression. In a human epidermal tumor analysis (actinic keratosis, Bowen's disease and SCCs), constitutive activation of the Akt/mTOR pathway was frequent [43]. Metformin has been shown to decrease the production of reactive oxygen species (ROS) in mouse embryonic fibroblasts independently of AMPK activation [44]. Gurumurthy et al. provided early evidence for the importance of LKB1 in skin carcinogenesis [45]. LKB1 is a central regulator of cell polarity and energy metabolism and acts via AMPK. Amornphimoltham et al. showed that rapamycin exerts remarkable antitumor activity in a chemically induced skin cancer model [46]. These studies demonstrate that rapamycin by causing a rapid decrease in the phosphorylation status of mTOR targets induces apoptotic death of cancer cells [46].

In conclusion, we showed that HL010183 reduced the growth of human cutaneous xenograft SCCs in nu/nu mice models which in turn is associated with the diminution in mTOR/Akt signaling pathway activation. This was also associated with enhancement in AMPK expression and the reduction in the expression of NFKB-mediated transcriptional target proteins, iNOS and COX-2 and cell cycle regulatory proteins. These studies suggested that HL010183 can be used as an effective drug for cutaneous cancer therapeutics.

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Disclosure of conflict of interest

None.

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