Berberine, an isquinoline alkaloid, inhibits melanoma cancer cell migration by reducing the expressions of cyclooxygenase-2, prostaglandin E2 and prostaglandin E2 receptors

Tripti Singh1,1, Mudit Vaid1,1, Nandan Katiyar1, Samriti Sharma1 and Santosh K.Katiyar1,2,3

1Department of Dermatology, University of Alabama at Birmingham, Birmingham, AL 35294, USA, 2Birmingham Veterans Affairs Medical Center, Birmingham, AL 35294, USA and 3Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35294, USA

Introduction

Melanoma is the leading cause of death from skin disease due, in large part, to its propensity to metastasize. We have examined the effect of berberine, an isquinoline alkaloid, on human melanoma cancer cell migration and the molecular mechanisms underlying these effects using melanoma cell lines, A375 and Hs294. Using an in vitro cell migration assay, we show that overexpression of cyclooxygenase (COX)-2, its metabolite prostaglandin E2 (PGE2) and PGE2 receptors promote the migration of cells. We found that treatment of A375 and Hs294 cells with berberine resulted in concentration-dependent inhibition of migration of these cells, which was associated with a reduction in the levels of COX-2, PGE2 and PGE2 receptors (EP2 and EP4). Treatment of cells with celecoxib, a COX-2 inhibitor, or transient transfection of cells with COX-2 small interfering RNA, also inhibited cell migration. Treatment of the cells with 12-O-tetradecanoylphorbol-13-acetate (TPA), an inducer of COX-2 or PGE2, enhanced cell migration, whereas berberine inhibited TPA- or PGE2-promoted cell migration. Berberine reduced the basal levels as well as PGE2-stimulated expression levels of EP2 and EP4. Treatment of the cells with the EP4 agonist stimulated cell migration and berberine blocked EP4 agonist-induced cell migration activity. Moreover, berberine inhibited the activation of nuclear factor-kappa B (NF-kB), an upstream regulator of COX-2, in A375 cells, and treatment of cells with caffeic acid phenethyl ester, an inhibitor of NF-kB, inhibited cell migration. Together, these results indicate for the first time that berberine inhibits melanoma cell migration, an essential step in invasion and metastasis, by inhibition of COX-2, PGE2 and PGE2 receptors.

Abbreviations: COX, cyclooxygenase; NF-kB, nuclear factor-kappa B; PG, prostaglandin; PGE2, prostaglandin E2; siRNA, small interfering RNA; TPA, 12-O-tetradecanoylphorbol-13-acetate.

Materials and methods

Cell lines and cell culture conditions

The human melanoma cell lines, A375 and Hs294, were purchased from the American Type Culture Collection (Manassas, VA). The cell lines were cultured as monolayers in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 100 μg/ml penicillin and 100 μg/ml streptomycin and maintained in an incubator with 5% CO2 at 37°C. The berberine was dissolved in a small amount of ethanol, which was added to the complete cell culture medium (maximum concentration of ethanol, 0.1% [vol/vol] in media) prior to addition to subconfluent cells (60–70% confluent). Cells treated with ethanol only served as a vehicle control. To determine the effect of berberine on 12-O-tetradecanoylphorbol-13-acetate (TPA)- or PGE2-mediated effects, berberine was added in cell culture medium at least 30 min before the treatment of the cells with TPA, PGE2 or any other agent.

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**Results**

**Berberine inhibits human melanoma cancer cell migration in a concentration-dependent manner**

Molecular structure of berberine is shown in Figure 1A. We first determined whether treatment of A375 and Hs294 human melanoma cells with berberine inhibited their migration using Boyden chamber experiment repeated at least three times.

**Wound healing assay**

A375 cells were seeded in six-well plates and incubated overnight in starvation medium. Cell monolayers were wounded with a sterile 100 μl pipette tip, washed with starvation medium to remove detached cells from the plates. Cells were left either untreated or stimulated with the indicated doses of TPA and kept for 24 h in an incubator. After 24 h, medium was replaced with phosphate-buffered saline and cells were photographed using an Olympus BX41 microscope and digital camera.

**PGE2 immunoassay for quantitation of prostaglandin E2**

The analysis of PGE2 in cell homogenates was performed using the Cayman PGE2 Enzyme Immunoassay Kit (Cayman Chemicals) following the manufacturer’s instructions. Briefly, at the indicated time points, cells were harvested and homogenized in 100 mM phosphate buffer, pH 7.4, containing 1 mM ethylenediamine tetraacetic acid and 10 μM indomethacin using a homogenizer. Homogenates were centrifuged and the supernatants were collected and analyzed for PGE2 concentration according to the manufacturer’s instructions.

**NF-κB/p65 activity assay**

For quantitative analysis of NF-κB/p65 activity, the NF-κB TransAM Activity Assay Kit (Active Motif, Carlsbad, CA) was used following the manufacturer’s protocol. For this assay, the nuclear extracts of cells were prepared using the Nuclear Extraction Kit (Active Motif) according to the manufacturer’s instructions, and as performed previously (16). Absorbance was recorded at 450 nm using absorbance at 650 nm as the reference. The results are expressed as the percentage of the optical density of the control (non-berberine-treated) group.

**Preparation of cell lysates and western blot analysis**

Following treatment of melanoma cells for the indicated time periods with or without berberine or any other agent, the cells were harvested, washed with cold phosphate-buffered saline and lysed with ice-cold lysis buffer supplemented with protease inhibitors, as detailed previously (10,16). Equal amounts of proteins were resolved on 10% Tris–Glycine gels and transferred onto enhanced chemiluminescence reagents. To verify equal protein loading, the membrane was stripped and reprobed with anti-β-actin antibody.

**Statistical analysis**

For migration assays, the control and berberine, TPA or PGE2 treatment groups or combined treatment groups separately were compared using one-way analysis of variance followed by post hoc Tukey’s test. All quantitative data for cell migration are shown as mean ± SD per microscopic field. In each case, P < 0.05 was considered statistically significant.

**Cell migration assay**

The migration capacity of melanoma cancer cells was determined in vitro using Boyden Chambers (Neuroprobe, Inc., Gaithersburg, MD) in which the two chambers were separated with Millipore membranes (6.5 mm diameter filters, 8 μm pore size), as detailed previously (15). Briefly, melanoma cells (1.5 × 10⁴ cells/100 μl serum-reduced medium) were placed in the upper chamber of Boyden chambers; test agents were added alone, or in combination, to the upper (200 μl) chamber and the lower chamber contained the medium alone (150 μl). Chambers were assembled and kept in an incubator for 24 h. After incubation, cells from the upper surface of Millipore membranes were removed with gentle swabbing and the migrant cells on the lower surface of membranes were fixed and stained with crystal violet dye. Membranes were then washed with distilled water and mounted onto glass slides. The membranes were examined microscopically and cellular migration per sample was determined by counting the number of stained cells under microscopic field in at least — four to five randomly selected fields using an Olympus BX41 microscope. Data are presented as mean of the migrating cells ± SD per microscopic field per sample. Representative photomicrographs were obtained using a CoolSnap digital camera system fitted to an Olympus BX41 microscope. Each cell migration experiment was repeated at least three times.

**Fig. 1.** (A) Molecular structure of berberine. (B) Treatment of human melanoma cancer cells with berberine for 24 h inhibits migration of cells in a concentration-dependent manner. (C) The migrating cells were counted and the results expressed as the mean number of migratory cells ± SD per microscopic field. (D) Effect of berberine on the endogenous basal level of COX-2 in A375 and Hs294 cells. The levels of COX-2 were determined in cell lysates using western blot analysis. Significant inhibition by berberine versus non-berberine-treated controls, *P < 0.05; **P < 0.001.

**Chemicals and antibodies**

Purified berberine was purchased from Sigma Chemical Co. (St Louis, MO). Antibodies specific for COX-2, an enzyme immunoassay kit for PGE2 analysis and an EP4 agonist (PGE1 alcohol) were obtained from Cayman Chemicals (Ann Arbor, MI). The antibodies specific for EP1, EP2, EP3 and EP4 and their secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA). Celecoxib, PGE2 and TPA were purchased from Sigma Chemical Co. Boyden Chambers and polycarbonate membranes (8 μm pore size) were obtained from Neuroprobe (Gaithersburg, MD). The migration capacities of melanoma cells were determined using Boyden Chambers (Neuroprobe, Inc., Gaithersburg, MD) in which the two chambers were separated with Millipore membranes (6.5 mm diameter filters, 8 μm pore size), as detailed previously (15). Briefly, melanoma cells (1.5 × 10⁴ cells/100 μl serum-reduced medium) were placed in the upper chamber of Boyden chambers; test agents were added alone, or in combination, to the upper (200 μl) chamber and the lower chamber contained the medium alone (150 μl). Chambers were assembled and kept in an incubator for 24 h. After incubation, cells from the upper surface of Millipore membranes were removed with gentle swabbing and the migrant cells on the lower surface of membranes were fixed and stained with crystal violet dye. Membranes were then washed with distilled water and mounted onto glass slides. The membranes were examined microscopically and cellular migration per sample was determined by counting the number of stained cells under microscopic field in at least — four to five randomly selected fields using an Olympus BX41 microscope. Data are presented as mean of the migrating cells ± SD per microscopic field per sample. Representative photomicrographs were obtained using a CoolSnap digital camera system fitted to an Olympus BX41 microscope. Each cell migration experiment was repeated at least three times.
cell migration assays. Preliminary screening experiments were performed to determine the effects of lower concentrations of berberine (micrometers) that did not induce cell death. As shown in Figure 1B, relative to untreated control cells, treatment with berberine at concentrations of 0, 5, 10 and 20 μM reduced the migratory capacity of A375 and Hs294 cells in a concentration-dependent manner. The density of the migrating cells on the membrane after staining with crystal violet is shown in Figure 1B, and the numbers of migrating cells per microscopic field are summarized in Figure 1C. The cell migration was inhibited by 17–69% (P < 0.01–0.001) in A375 cells and by 27–74% (P < 0.01–0.001) in Hs294 cells in a concentration-dependent manner after treatment with berberine for 24 h. A similar but comparative higher inhibitory effect was observed at the 48 h time point. To confirm that the inhibition of cancer cell migration by berberine was a direct effect on migration and not due to a reduction in cell viability, a trypan blue assay was performed using cells that were treated identically to those used in the migration assays. Treatment of A375 and Hs294 cells with various concentrations of berberine (0, 5, 10 and 20 μM) for 24 h had no significant effect on cell viability, change in cell morphology and cell death (data not shown). Similar experiments were also conducted with normal human epidermal melanocytes (Life Sciences, Carlsbad, CA). Under identical in vitro conditions, cell migration was not observed in normal human epidermal melanocytes (data not shown).

The inhibitory effect of berberine on cell migration is associated with the inhibition of endogenous COX-2 expression and PGE2 production

To determine whether the inhibitory effect of berberine on the migration of the cells is associated with inhibition of basal COX-2 expression, we determined the levels of COX-2 in lysates of cells from the various treatment groups using western blot analysis. As shown in Figure 1D, treatment of A375 and Hs294 cells with berberine reduced the levels of COX-2 expression in a concentration-dependent manner as compared with the expression in untreated controls. As the COX-2 metabolite, PGE2, has been implicated in COX-2-mediated effects; we determined the levels of PGE2 in the berberine-treated cells. Our results revealed that treatment with berberine for 24 h resulted in significant inhibition of PGE2 production in both A375 (15–75%, P < 0.01–0.001) and Hs294 (16–63%, P < 0.01–0.001) cells in a dose-dependent manner (Figure 2A), suggesting that berberine-induced reduction in PGE2 production is associated with an inhibitory effect of the berberine on COX-2 in these cells.

Celecoxib, a COX-2 inhibitor, inhibits melanoma cell migration

This experiment was performed to determine whether the inhibitory effect of berberine on melanoma cell migration is mediated through its inhibitory effect on COX-2 expression. For this purpose, equal numbers of A375 and Hs294 cells were subjected to the cell migration assay after treatment with various concentrations of celecoxib (0, 5, 10, 20 μM) for 24 h. As shown in Figure 2B, treatment of the cells with celecoxib resulted in a dose-dependent reduction in the cell migration capacity of melanoma cells as compared with non-celecoxib-treated controls (P < 0.01–0.001). These data suggested that the inhibition of constitutive levels of COX-2 expression in the presence of celecoxib resulted in inhibition of melanoma cancer cell migration.

siRNA knockdown of COX-2 leads to reduction of melanoma cell migration

We further verified the role of COX-2 in cell migration through siRNA knockdown of COX-2 in the melanoma cells and examined whether it would lead to the inhibition of the cell migration in these cells. The transfection of A375 and Hs294 cells with COX-2 siRNA resulted in significant reduction of cell migration in A375 (85%, P < 0.001) and Hs294 (86%, P < 0.001) cells after 24 h as compared with the migration of control siRNA-transfected A375 and Hs294 cells (Figure 2C). We also confirmed using western blot analysis that COX-2 siRNA transfection of A375 and Hs294 cells resulted in marked reduction in the levels of COX-2 protein (>80%) in these cells (Figure 2D). As the inhibitory effect of berberine on the migration of A375 and Hs294 cell lines was very similar, the subsequent studies were performed using A375 cells.

Berberine inhibits TPA-induced cell migration of melanoma cells

Treatment of skin with TPA stimulates the levels of COX-2 expression (17,18); therefore, we determined the effects of TPA on the melanoma cell migration. As shown in Figure 3A, treatment of A375 cells with TPA for 24 h resulted in significantly enhanced cell migration (P < 0.01, P < 0.001) that was dose dependent. Similar observations were also noted when wound healing assay was performed (Figure 3B). To determine whether berberine inhibits TPA-induced cell migration in human melanoma cells, A375 cells were treated with TPA (40 ng/ml) with and without the treatment of berberine for 24 h. The treatment of A375 cells with berberine resulted in a dose-dependent inhibition of TPA-induced cell migration (Figure 3C). A summary of the cell migration data for the various treatment groups is provided in Figure 3D. Treatment of berberine at the doses of 10 and 20 μM inhibited TPA-induced cell migration by 50% (P < 0.01) and >100% (P < 0.001), respectively. To verify whether this inhibition of cell migration by berberine is mediated through the inhibition of TPA-induced COX-2 expression, cell lysates were prepared and subjected to western blot analysis to estimate the levels of COX-2 expression. We found that treatment of A375 cells with TPA for 24 h resulted in higher expression of COX-2 as compared with the expression in cells that were not treated with TPA (Figure 3E). Pretreatment of A375 cells with
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To assess the role of the PGE$_2$ receptors on the cell migration of melanoma cells and the therapeutic effect of berberine, A375 and Hs294 melanoma cells were treated with an EP4 agonist (PGE$_1$ alcohol) for 24 h with or without addition of berberine. As shown in Figure 5C, treatment of A375 and Hs294 cells with the EP4 agonist resulted in significant enhancement of cellular migration (76 and 95%, respectively) due to downregulation of COX-2 and PGE$_2$ receptors in melanoma cells.

Berberine decreases the levels of PGE$_2$ receptors in melanoma cells

As it has been shown that PGE$_2$ manifests its biological activity via four known G-protein-coupled receptors (i.e. EP1–EP4), we determined the effect of berberine on the basal levels of PGE$_2$ receptors in melanoma cells. Western blot analysis revealed that treatment of A375 cells with berberine (0, 5, 10 and 20 µM) for 24 h resulted in a dose-dependent reduction in the levels of EP2 and EP4 (Figure 5A). A similar inhibitory effect on EP1 and EP3 was observed but was less prominent than the effect on EP2 and EP4 (data not shown).

In further experiments, we tested whether berberine inhibits the PGE$_2$ induction of higher levels of the EP2 and EP4 receptors in melanoma cells. For this purpose, the A375 cells were treated with PGE$_2$ (10 µM) with and without the treatment of berberine (10 and 20 µM) for 24 h, then cells were harvested and cell lysates prepared. As shown in Figure 5B, western blot analysis indicated that the treatment of cells with PGE$_2$ enhanced the levels of both EP2 and EP4 and that berberine inhibited the PGE$_2$-induced increase in the levels of EP2 and EP4.
that treatment of berberine resulted in the downregulation of IKK activity of NF-κB and its translocation to the nucleus in a dose-dependent manner (Figure 6A). The results also indicated the migratory potential of melanoma cells. Many human cancers experience the death of their disease within 2 years (22). Therefore, innovative strategies are required to be developed for the prevention of the invasive or the migratory potential of melanoma cells. Human cancers express elevated levels of COX-2, an enzyme responsible for the biosynthesis of PGs. COX-2 overexpression and abundant production of PGE2 have a role in cell migration and that berberine inhibits the migration of melanoma cells, at least in part, by inhibiting the levels of PGE2 receptors EP2 and EP4, an enzyme responsible for the bio-

![Fig. 5. (A) Treatment of A375 cells with berberine for 24 h decreases the expression levels of PGE2 receptors EP2 and EP4 in a concentration-dependent manner. The cells were harvested 24 h after the treatment and cell lysates were prepared and subjected to western blot analysis, as detailed in Materials and Methods. (B) Berberine decreases PGE2-enhanced expression levels of EP2 and EP4. Cell lysates were subjected to western blot analysis to determine the levels of EP2 and EP4 receptors using EP2- and EP4-specific antibodies. (C) Berberine inhibits EP4 agonist (25 μM)-induced cell migration of A375 and Hs294 cells. A375 and Hs294 cells were treated with berberine (10 and 20 μM) for at least 1 h prior to addition of the EP4 agonist. Cell migration was assessed 24 h after the berberine treatment, and results are expressed as a mean number of migratory cells ± SD/microscopic field from two separate experiments. Significant difference versus EP4 agonist alone-treated group. *P < 0.05, **P < 0.001.

![Fig. 6. Treatment of A375 cells with berberine decreases the basal expression levels of NF-κB and IKKα while inhibiting the degradation of IκBα. (A) After 24 h treatment with various concentrations of berberine, the cells were harvested and cytosolic and nuclear fractions were prepared and subjected to the analysis of NF-κB, IKKα and IκBα using western blot analysis. Representative blot is shown from three independent experiments with identical observations. (B) The activity of NF-κB in the nuclear fraction of cells after treatment with and without berberine for 24 h was measured using NF-κB/p65-specific activity assay kit, n = 3. Activity of NF-κB is expressed in terms of percent of control (non-berberine-treated) group. Significant decrease versus control: *P < 0.01, **P < 0.001. (C) Treatment of A375 cells with caffeic acid phenethyl ester (CAPE), an inhibitor of NF-κB, for 24 h inhibits cell migration in a concentration-dependent manner. Data on cell migration capacity are summarized as the mean number of migratory cells ± SD per microscopic field. Significant inhibition versus non-CAPE-treated cells: **P < 0.001.

COX-2 is a downstream target gene of NF-κB, therefore we assessed whether berberine also affects the basal levels of proteins of NF-κB family in melanoma cells. For this purpose, A375 cells were treated with various concentrations of berberine (0, 5.0, 10.0 and 20.0 μM) for 24 h, and thereafter cells were harvested and cell lysates prepared. The results of western blot analysis revealed that treatment of cells with berberine downregulated the transcription of NF-κB/p65 from the cytosol to the nucleus in a dose-dependent manner (Figure 6A). The activity of NF-κB was also significantly reduced (15–80%, P < 0.01 and P < 0.001) after the treatment of cells with berberine in a concentration-dependent manner (Figure 6B). The results also indicated that treatment of berberine resulted in the downregulation of IKKα and degradation of IκBα (Figure 6A), which leads to the inactivation of NF-κB and its translocation to the nucleus. To check whether
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PGs, and particularly PGE₂, have been linked with tumor progression, invasion and metastasis (23). Because of its important role in tumor invasion and metastasis, COX-2 is not a new target but a promising target for cancer therapy (7,24); therefore, the search of potential COX-2 as well as PGE₂ inhibitors for the prevention or treatment of melanoma may prove to be an important strategy.

The significant findings in the present study are that the treatment of melanoma cells with berberine for 24 h inhibits cell migration in a dose-dependent manner and that is associated with the inhibition of COX-2 expression and PGE₂ production. Based on our observation, cells will go under apoptosis or cell death if melanoma cells are treated with berberine for > 24 h time period. Under these conditions, cell migration will decrease, and this reduction in cell migration could be due to cell death and not because of changes in migrating behavior of cells. In our study, cell death or apoptosis is not a reason of berberine-caused inhibition of melanoma cell migration. The melanoma cells overexpress COX-2, and the inhibition of COX-2 by berberine may contribute to the inhibition of cell migration of these cells. This concept is supported by the evidence that treatment of the melanoma cells with celecoxib, a potent COX-2 inhibitor, resulted in a reduction in cell migration. Similar effects were also noted when the melanoma cancer cells, A375 and Hs294, were transfected with COX-2 siRNA. It has been shown that TPA activates COX-2 expression, and we found that treatment of melanoma cells with TPA enhances cell migration and that this TPA-induced cell migration was blocked by the treatment of cells with berberine. These observations support the evidence that inhibition of melanoma cell migration by berberine requires the inhibition of COX-2 expression. It is well known that PGE₂ exerts its biologic functions through four G-protein-coupled receptors, EP1, EP2, EP3 and EP4 (7,19,25), that can stimulate epithelial cell growth, invasion potential and cellular survival signals (26–28). It has been shown very recently that PGE₂ promotes lung cancer cell migration and that this effect is mediated through activation of PGE₂ receptors (29). Because PGE₂ is a major prostaglandin associated with skin tumor promotion, progression and invasion (23), we assessed the possible involvement of the PGE₂ receptors in berberine-induced inhibition of melanoma cell migration.

We observed that A375 and Hs294 cells overexpress the PGE₂ receptors, EP2 and EP4, and that the expression of EP2 and EP4 was decreased when cells were treated with berberine in vitro. These data suggest that inhibition of the EP2 and EP4 levels by berberine may contribute to the inhibition of cell migration. The inhibitory effect of berberine on melanoma cancer cell migration through the inhibitory effect on EP2 or EP4 was further confirmed by treating the cells with an EP4 agonist. We found that the treatment of A375 and Hs294 cells with the EP4 agonist resulted in enhancement of cell migration, and that EP4 agonist-induced cell migration was inhibited by the treatment of cells with berberine. This observation supports the concept that inhibition of PGE₂ receptors by berberine may have contributed to the inhibition of melanoma cancer cell migration. These findings also demonstrate the feasibility of using berberine as an alternative to COX-2 inhibitors, which show toxicity in some patients, given the fact that COX-2 remains an attractive cancer target. Since berberine acts by inhibiting expression of both COX-2 and EP receptors, this could be more effective since it targets both ligand (PGE₂) and receptor (EP). Similar to berberine, other phytochemicals also have been examined for their effect on cell migration. Punathil et al. (30) have reported that treatment of non-small-cell lung cancer cells with proanthocyanidins resulted in inhibition of cell migration following the inhibition of nitric oxide and guanylate cyclase pathways. Another dietary polyphenol, (-)-epigallocatechin-3-gallate from green tea, has been shown to inhibit mammary cancer cell migration through the inhibition of nitric oxide synthase and guanylate cyclase. Biochem. Biophys. Res. Commun., 375, 162–167.

As COX-2 is a downstream target of NF-kB pathway, we further checked the effect of berberine on the basal levels of NF-kB in melanoma cells and found that treatment of melanoma cells with berberine downregulated the basal levels of proteins of NF-kB pathway in a dose-dependent manner. Treatment of melanoma cells with caffeic acid phenethyl ester, an inhibitor of NF-kB, resulted in an inhibitory effect on cell migration. These observations further support the concept that the inhibitory effect of berberine on melanoma cell migration is mediated, at least in part, through the downregulation of COX-2, PGE₂ and PGE₂ receptors expressions. However, it remains possible that downregulation of other NF-kB target genes could also contribute to the inhibition of cell migration.

In summary, the results from this study have identified for the first time that berberine inhibits the migration of melanoma cancer cells through the inhibitory effect on endogenous COX-2 overexpression and successive downregulation of PGE₂ and PGE₂ receptors. More mechanism-based studies are therefore needed to develop berberine as a pharmacologically safe agent alone, or in combination with other anti-metastatic drugs, for the treatment of metastatic melanoma in humans.

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