Dykellic Acid Inhibits Cell Migration and Tube Formation by RhoA-GTP Expression

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Dykellic acid, a novel factor initially identified from the culture broth of *Westerdykella multispora* F50733, has been shown to inhibit matrix metalloprotease 9 activity, caspase-3 activity, B cell proliferation and LPS-induced IgM production, suggesting that this factor may have anti-cancer effects. In an effort to further address the possible anti-tumoral effects of dykellic acid, we used wound healing, invasion and RhoA-GTP assays to examine the effects of dykellic acid on cell migration, invasion and angiogenesis. Our results revealed that dykellic acid dose-dependently inhibits B16 cell migration and motility, and inhibits HUVEC tube formation. Western blot analysis of the active form of RhoA (RhoA-GTP) showed that dykellic acid treatment decreased the levels of RhoA-GTP. These findings collectively suggest that dykellic acid may have both anti-metastatic and anti-angiogenic acitivites, and provides the first evidence for the involvement of RhoA in dykellic acid-induced effects.

Key words dykellic acid; RhoA; RhoA-GTP; angiogenesis; cell migration; invasion

Dykellic acid ($C_{14}H_{16}O_4$) is a novel compound initially isolated from the culture broth of *Westerdykella multispora* F50733.[1,2] This multi-functional agent has been shown to inhibit matrix metalloproteinase 9 (MMP9) activity in the extra-cellular matrix (ECM) *via* binding of the NF-κB transcription factor at the MMP9 promoter,[3] suggesting dykellic acid can play a role in anti-invasion and/or anti-angiogenesis as well as in caspase-3 inhibition.[4]

Cell motility, and particularly cell migration, is critical to tumor metastasis.[5,6] The signal transduction pathways responsible for mediating cell motility appear to involve various nuclear transcription factors, as well as cytoskeletal changes that alter interactions among membrane- and extracellular matrix (ECM)-bound proteins (*e.g.* RhoA, cdc42 and Rac), which mediate cellular and membrane morphology by regulating the polymerization and depolymerization of the actin cytoskeleton.[7] Interestingly, one of the Rho family proteins, RhoA, is also involved in the formation of new blood vessels (angiogenesis) *via* vascular endothelial growth factor (VEGF).[8—10] As both cell migration and angiogenesis are central to the process of cancer metastasis, the Rho family proteins are considered strong molecular targets for inhibiting the spread of cancer.[11] We previously showed that dykellic acid inhibits drug-induced apoptosis *via* a caspase-3-like protease-suppressing mechanism.[4] However, no previous work has directly examined the effects of dykellic acid on cell motility, or the possible signaling molecules involved in these effects.

Here, we report for the first time that dykellic acid decreases cell migration, invasion and tube formation, and that these effects may involve RhoA. Our findings, in combination with the previous reports, strongly suggest that dykellic acid should be considered a good candidate for development as an anti-tumoral therapeutic agent, and provide the first insight into possible signaling partners of dykellic acid.

MATERIALS AND METHODS

Cell Culture Mouse melanoma cell line B16-F1 (B16; Catalog # CRL-6323) and human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). The B16 cells were cultured in RPMI 1640 supplemented with 10% FBS, while the HUVECs were cultured in EGM-2 medium (BD Bioscience, Lake Placid, NJ, U.S.A.). The B16 cells were maintained in RPMI 1640 medium and subcultured by trypsinization every 3—4 d. The HUVECs were subcultured every 3—4 d and were used for experiments at passages 2—7.

Wound Healing Assay The strips of thin tape (2 mm× 2 cm; 3M, Seoul, Korea) were attached to the bottom of each well of 6-well plates (Greiner, Frickenhausen, Germany), and B16 cells were plated at 1×10^7 cells/well and allowed to attach for 3—5 h at 37 °C in a 5% CO_2 atmosphere. The tape strips were then removed, creating linear wounds. The plates were photographed and incubated as above with media containing a concentration of dykellic acid (50 μM). The plates were photographed at 16, 24 and 40 h and the exact wound width was calculated by a microluler (http://www.eeob.iastate.edu/faculty/DrewsC/htdocs/microruler-links.htm).[12]

Invasion Assay Transwell plates (pore size 8 μm; Costar, NY, U.S.A.) were loaded with 100 μl Matrigel (BD Biosciences, NJ, U.S.A.), which was allowed to solidify at 37 °C for 2 h and then coated with 10 μl fibronectin (200 μg/ml). The plates were loaded with B16 cells suspended in 10% FBS (1×10^5 cells/well), the samples were exposed to dykellic acid at a final concentration of 0 or 50 μM, and the plates were incubated at 37 °C in 5% CO_2 for 24 h. The migrated cells were fixed with methanol, stained with hematoxylin, and counted under a microscope.[13]

Tube Formation Assay HUVECs (2×10^4 cells/well) were dispensed to Matrigel-coated 24-well plates in 0.5 ml of EGM-2 containing various concentrations of dykellic acid (0

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or 50 μM), and incubated for 24 h. The cells were then visualized by microscopy and tube formation was scored by counting the tube formed or not.[14]

Western Blot Analysis RhoA activity was assessed using a RhoA-GTP Kit (Pierce, Rockford, IL, U.S.A.), according to the manufacturer's instructions. Briefly, B16 cells (1×10^6 cells) were centrifuged at 16000×***g***. The supernatant (cell lysate) was incubated for 1 h at 4 °C with the provided GST-Rhotekin-RBD, and then 25 μl/lane was loaded onto a SwellGel immobilized glutathione disk (Pierce, Rockford, IL, U.S.A.) The glutathione disk-bound proteins were solubilized with 2×SDS [125 mM Tris–HCl, pH 6.8, 2% glycerol, 4% SDS (w/v), 0.05% mercaptoethanol, and 0.05% bromophenol blue], eluted from the disk, resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with Tween (0.1%)/TBS and incubated with the primary antibody (Pierce, Rockford, IL, U.S.A.). The results were detected with an HRP-conjugated secondary antibody (Pierce, Rockford, IL, U.S.A.) and visualized with an ECL detection system (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).[12]

Statistical Analysis Data were expressed as the mean±standard deviation of the mean values. Statistical significance was determined by Student–Newman–Keuls method for independent means, using the Sigma Plot program. The critical level for significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Dykellic acid, a novel compound initially identified from a culture broth of *Westerdykella multispora* F50733, has been shown to inhibit MMP9 activation and caspase-3 protease activity, and also appears to have immunosuppressive effects.[2—4] Because MMP9 and caspase-3 are associated with cancer promotion, we question whether dykellic acid affect cell motility and invasion, two major hallmarks of angiogenesis.[15]

Therefore, we first examined the motility of dykellic acid-treated B16 cells. We used wound healing and invasion assays to test the *in vitro* effect of dykellic acid on cell migration, which is an important facet of cell motility, particularly in relation to cancer. Our wound healing assay revealed that while untreated control monolayers showed complete wound healing within 48 h, monolayers treated with 50 μM dykellic acid showed clear wound width, suggesting that dykellic acid inhibit cell migration *in vitro* in a dose-dependent fashion (Fig. 1A). Interestingly, PMA-treated monolayers showed also strong cell growth inhibitory pattern as control did, sug-

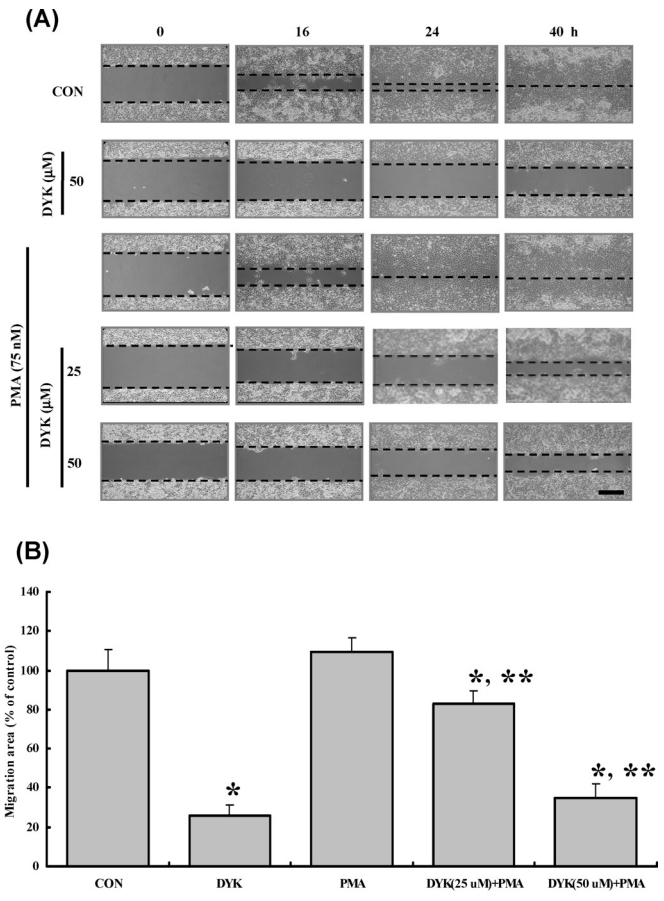

Fig. 1. Dykellic Acid Inhibits Cell Migration *in Vitro*

(A) Confluent monolayers of B16 cells were pretreated with or without 50 μM dykellic acid. The monolayers were then wounded and stimulated with 75 nM PMA or left untreated (untreated). The plates were photographed 0, 16, 24 and 40 h post-wounding. Bar=200 μm. (B) Quantification of the wound healing. Cell migration was quantified by counting the wound width 24 h after the plates were treated with or without PMA. Values are means±S.D. from 5 cultures each in duplicate experiments. * Significant difference from control, $p<0.05$. ** Significant difference from PMA alonel, $p<0.05$.

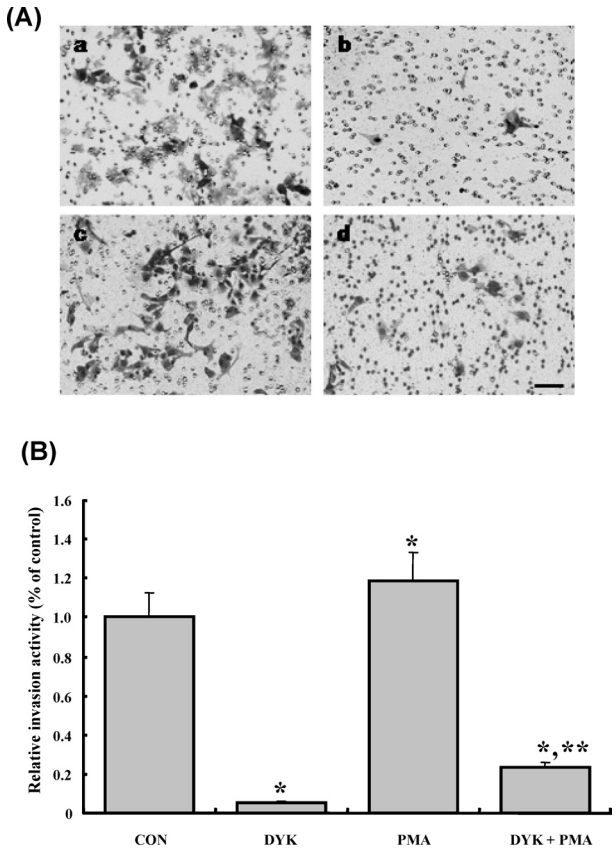

Fig. 2. Dykellic Acid Inhibits Cell Invasion *in Vitro*

Cell invasion was assayed in Matrigel-coated Transwell chambers. (A) Invasion was compared among B16 cells exposed to no treatment (control; a), 50 μM dykellic acid (b), 75 nM PMA (c), and both dykellic acid and PMA (d). Representative fields of migrated cells were photographed. Bar=200 μm. (B) Quantification of the invasion activity. Cell migration was quantified 24 h after the cells were exposed to no treatment (control; CON), dykellic acid (DYK), PMA (PMA) or both dykellic acid and PMA (DYK+PMA). Migrated cells were counted from five randomly selected microscopic fields, and the results are given as the average per field±S.D. of 3 independent experiments. * Significant difference from control, $p<0.05$. ** Significant difference from PMA alonel, $p<0.05$.

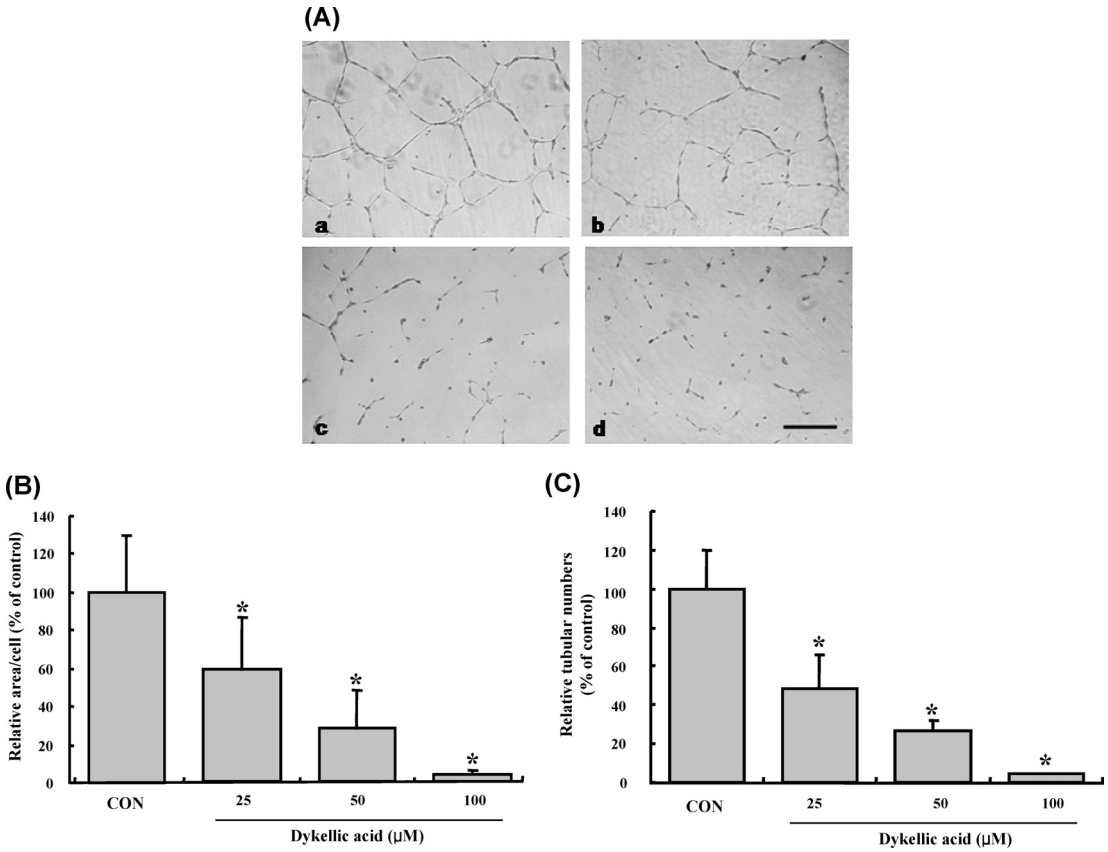

Fig. 3. Dykellic Acid Inhibits HUVEC Tube Formation on Matrigel

(A) HUVECs were plated at 2×10^4 cells/well in Matrigel-coated 24-well plates, and then exposed to 0 (a), 25 (b), 50 (c) or 100 (d) μM dykellic acid. After 24 h, the culture supernatants were removed and the cells were fixed with 10% formalin. (B, C) The areas (B) and numbers (C) of the formed tubes were calculated. Bar=20 μm. The results are given as the average per field±S.D. of 3 independent experiments. * Significant difference from control (dykellic acid 0 μM), $p<0.05$.

gesting that dykellic acid inhibited cell migration activity with or without cell proliferation inducer. Similarly, our invasion assay revealed that 50 μM dykellic acid inhibited invasive activity by more than 80% *versus* the control group (Fig. 2). These results indicate that dykellic acid dose-dependently inhibits wound healing and inhibits invasion *in vitro*.

Next, we examined the effect of dykellic acid on angiogenesis, which is a second critical component of metastasis. HUVECs were treated with and without various concentrations of dykellic acid, and tube formation was assessed in terms of tube size and number. Our results revealed that dykellic acid dose-dependently inhibited HUVEC tube formation *in vitro* at 0 to 100 μM (Fig. 3). The results revealed that the untreated controls showed the highest levels of proliferation, indicating that endothelial cell growth was not restricted by contact inhibition under our experimental conditions.[7] Collectively, these findings indicate that dykellic acid inhibits cell migration, cell invasiveness and tube formation *in vitro*, suggesting that this factor might function as an anti-tumoral agent *in vivo* by decreasing metastasis and angiogenesis.

Lastly, we investigated the effect of dykellic acid on the levels of its potential signaling partner, RhoA. Signal transduction during angiogenesis is fairly well understood. Many transcription factors (*e.g.* EST-1, c-Fos and c-jun) and migration-related proteins are associated with gene expression in endothelial cells (ECs) during angiogenesis.[16] VEGF acts as a central regulator, while many morphological changes are overseen by members of the Rho protein family of GTPases. These GTPases mediate cell contractility by organizing actin filaments into stress fibers,[17,18] thereby influencing the motility and migration of ECs, as well as the formation of new blood vessels. Therefore, we examined the effect of dykellic acid on RhoA-GTP levels in B16 cells. Our Western blot analysis revealed that treatment of cells with 50 μM dykellic acid inhibited RhoA-GTP activity to approximately 50% (Figs. 4A, B), suggesting that dykellic acid have potential to decrease the active form of RhoA rather than the activity level *per se*. Not only other GTPases (such as Cdc42, Rac1), but also cancer-related molecules (CD44, Timp-1, Timp-2, Paxillin, Src, and MMP-14) did not change in its expression level by dykellic acid (data not shown), although the precise up- and down-stream signal transduction pathway need to be understood.

In sum, we herein show for the first time that dykellic acid dose-dependently inhibits B16 cell migration and motility, and inhibits HUVEC tube formation *in vitro*, resulting in decreasing the levels of RhoA-GTP. These findings, in combination with previous reports indicating dykellic acid inhibits not only caspase-3-like protease activation, but also PMA-induced increase in MMP-9 expression, suggest that dykellic acid should be considered a promising candidate for future development as a possible anti-cancer agent.

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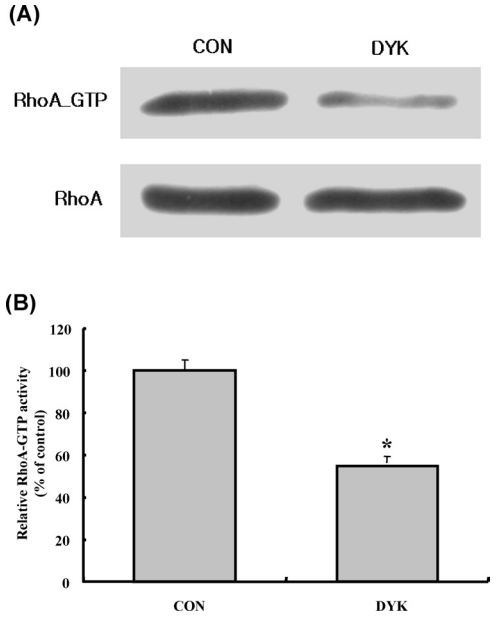

Fig. 4. Dykellic Acid Inhibits RhoA-GTP Expression

(A) B16 cells were stimulated for 1 h with (DYK) or without (CON) 50 μM dykellic acid, and active RhoA (RhoA-GTP) was affinity precipitated and quantified by immunoblot analysis. (B) Quantification of RhoA (RhoA-GTP) activity. The results are given as the average per field±S.D. of 2 independent experiments. * Significant difference from control, $p<0.05$.

through Grape Research Projects Group on anticancer *in vitro* assays.

REFERENCES

1) Lee H. J., Chun H. K., Chung M. C., Lee C. H., Rhee J. S., Kho Y. H., *J. Antibiot.*, **53**, 78—80 (2000).
2) Han S. B., Lee H. J., Kho Y. H., Jeon Y. J., Lee S. H., Kim H. C., Kim H. M., *J. Antibiot.*, **54**, 840—843 (2001).
3) Woo J. H., Park J. W., Lee S. H., Kim Y. H., Lee I. K., Gabrielson E., Lee S. H., Lee H. J., Kho Y. H., Kwon T. K., *Cancer Res.*, **63**, 3430—3434 (2003).
4) Lee S. H., Youk E. S., Lee H. J., Kho Y. H., Kim H. M., Kim S. U., *Biochem. Biophys. Res. Commun.*, **302**, 539—544 (2003).
5) Yamaguchi H., Wyckoff J., Condeelis J., *Curr. Opin. Cell. Biol.*, **17**, 559—564 (2005).
6) Stamenkovic I., *J. Pathol.*, **200**, 448—464 (2003).
7) Cascone I., Giraudo E., Caccavari F., Napione L., Bertotti E., Collard J. G., Serini G., Bussolino F., *J. Biol. Chem.*, **278**, 50702—50713 (2003).
8) Hoang M. V., Whelan M. C., Senger D. R., *Proc. Natl. Acad. Sci. U.S.A.*, **101**, 1874—1879 (2004).
9) van Nieuw Amerongen G. P., Koolwijk P., Versteilen A., van Hinsbergh V. W., *Arterioscler. Thromb. Vasc. Biol.*, **23**, 211—217 (2003).
10) Wickstrom S. A., Alitalo K., Keski-Oja J., *J. Biol. Chem.*, **278**, 37895—37901 (2003).
11) Lin M., van Golen K. L., *Breast Cancer Res. Treat.*, **84**, 49—60 (2004).
12) Heo J. C., Park J. Y., Lee J. M., Kwon T. K., Kim S. U., Chung S. K., Lee S. H., *J. Ethnopharmacol.*, **102**, 10—14 (2005).
13) Wey J. S., Fan F., Gray M. J., Bauer T. W., McCarty M. F., Somcio R., Liu W., Evans D. B., Wu Y., Hicklin D. J., Ellis L. M., *Cancer*, **104**, 427—438 (2005).
14) Cascone I., Giraudo E., Caccavari F., Napione L., Bertotti E., Collard J. G., Serini G., Bussolino F., *J. Biol. Chem.*, **278**, 50702—50713 (2003).
15) Lin V. C., Ng E. H., Aw S. E., Tan M. G., Ng E. H., Bay B. H., *Mol. Endocrinol.*, **14**, 348—358 (2000).
16) Potente M., Urbich C., Sasaki K. I., Hofmann W. K., Heeschen C., Aicher A., Kollipara R., Depinho R. A., Zeiher A. M., Dimmeler S., *J. Clin. Invest.*, **115**, 2382—2392 (2005).
17) Abecassis I., Olofsson B., Schmid M., Zalcman G., Karniguian A., *Exp. Cell Res.*, **291**, 363—376 (2003).
18) Gingras D., Lamy S., Beliveau R., *Biochem. J.*, **348**, 273—280 (2000).