

Dykellic Acid Inhibits Phorbol Myristate Acetate-induced Matrix Metalloproteinase-9 Expression by Inhibiting Nuclear Factor κ B Transcriptional Activity¹

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ABSTRACT

Proteolytic degradation of the extracellular matrix and tumor metastasis correlate with expression of endopeptidases known as matrix metalloproteinases (MMPs). Expression of MMPs is regulated by cytokines and signal transduction pathways, including those activated by phorbol myristate acetate. We found that dykellic acid, a fungal metabolite, significantly inhibits the phorbol myristate acetate-induced increase in MMP-9 expression and activity. These effects of dykellic acid are time- and dose-dependent, and correlate with decreased MMP-9 promoter activity and mRNA expression. Whereas this compound does not affect DNA binding activity of nuclear factor κ B (NF κ B), dykellic acid does inhibit transactivation of NF κ B. These data demonstrate a role for NF κ B in the regulation of MMP-9 expression and the ability of dykellic acid to suppress this action of NF κ B.

INTRODUCTION

The MMPs³ are a family of >20 zinc-dependent endoproteases that are capable of degrading almost all of the components of the extracellular matrix (1, 2). MMPs can be divided into four categories based on substrate preference: collagenases, gelatinases, stromelysins, and membrane-associated MMPs. Among human MMPs reported previously, gelatinase-A (MMP-2) and gelatinase-B (MMP-9) are key enzymes for degrading type IV collagen, which is a major component of the basement membrane (3, 4). Expression levels of MMP-2 and MMP-9 are associated with tumor metastasis for various human cancers (5, 6). Recent studies show a positive correlation between expression of MMP-9 and tumor metastasis for colorectal cancer and for several types of epithelial cancer (7, 8), thus suggesting an important functional role for these proteinases in the metastasis process. The mechanisms of MMP-9 gene activation in human cancer cells are not well defined. It is known that the human MMP-9 promoter contains *cis*-acting regulatory elements and transcription factors including AP-1 (–533 bp, –79 bp), NF κ B (–600 bp), and Sp1 (–588 bp), which participate in the regulation of the MMP-9 gene (6). MMPs are synthesized as inactive precursors and are activated by proteolytic cleavage (9–11). Therefore, the regulation of MMPs occurs at three levels: gene expression, proenzyme processing, and inhibition of enzymatic activity.

The present study is based on a finding that dykellic acid suppresses MMP-9 gene transcription. Dykellic acid, a novel fungal metabolite, was first isolated from the culture filtrate of *Westerdykella multispora*

F50733 and has not been described previously to have this effect (12, 13). Extending these initial findings, we investigated the molecular mechanism by which dykellic acid inhibits MMP-9 expression.

MATERIALS AND METHODS

Cells and Materials. Human Caski cells were obtained from the American Type Culture Collection (Rockville, MD). The culture medium used throughout these experiments was DMEM, containing 10% FCS, 20 mM HEPES buffer, and 100 μ g/ml gentamicin. Dykellic acid was identified and isolated from *W. multispora* F50733 (12, 13). Anti-inhibitor of NF κ B and anti-NF κ B antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Lipofectamine reagent was from Life Technologies, Inc. (Rockville, MA). Luciferase assay and β -galactosidase assay systems were from Promega (Madison, WI).

Western Blot Analysis. Cellular lysates were prepared by suspending 1×10^6 cells in 100 μ l of lysis buffer [137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM MgCl₂, 0.1% Triton X-100, 25 mM 4-morpholinepropanesulfonic acid, 100 μ M phenylmethylsulfonyl fluoride, and 20 μ M leupeptin, adjusted to (pH 7.2)]. The cells were disrupted by sonication and extracted at 4°C for 30 min. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA). Detection of specific proteins was carried out with an enhanced chemiluminescence Western blotting kit following the manufacturer's instructions. Densitometric measurements of the bands in Western blot analysis were performed using digitalized scientific software program UN-SCAN-IT purchased from Silk Scientific Corporation (Orem, UT).

Gelatin Substrate Gel Zymography. To determine the effect of dykellic acid on PMA-induced MMP-9 activity, cells were treated with various concentrations of dykellic acid in the presence of 75 nM PMA and MMP-9 expression was evaluated by zymography. Zymography was performed by the procedure described by Overall *et al.* (14) with minor modification. The human cell lines were suspended in their respective medium containing 10% fetal bovine serum and plated at 8×10^5 cells/35 mm² dish. Dishes were incubated until ~80% confluent, the medium was aspirated, and then fresh serum-free medium was added to each dish, with and without dykellic acid. Supernatants were collected after incubation for 24 h. Proteins were subjected to SDS-PAGE in 10% polyacrylamide gels that were copolymerized with 1 mg/ml of gelatin. After electrophoresis, the gels were washed several times in 2.5% Triton X-100 for 1 h at room temperature to remove the SDS, then incubated for 24–48 h at 37°C in buffer containing 5 mM CaCl₂ and 1 μ M ZnCl₂. The gels were stained with Coomassie blue (0.25%) for 30 min, and then destained for 1 h in a solution of acetic acid and methanol. The proteolytic activity was evidenced as clear bands (zones of gelatin degradation) against the blue background of stained gelatin.

Cloning of Human MMP-9 Promoters. A 0.7 kb segment at the 5'-flanking region of the human MMP-9 gene was amplified by PCR using specific primers from the human MMP-9 gene (GenBank accession no. D110051): 5'-ACATTGCCCCGAGCTCCTGAAG (forward/*Sac*I) and 5'-AGGGGCTGCCAGAAGCTTATGGT (reverse/*Hind*III). The pGL2-Basic vector containing a polyadenylation signal upstream from the luciferase gene was used to construct expression vectors by subcloning PCR-amplified DNA of MMP-9 promoter into the *Sac*I/*Hind*III site of the pGL2-Basic vector. Point mutations of the AP-1 and NF κ B binding sites to the MMP-9 promoter were generated by a two-step PCR method using the following primers: AP-1-1 (5'-CTGACCCTGAGTCAGCACTTG to 5'-CTGACCCTGAGTTGGCACTTG), AP-1-2 (GAAGCTGAGTCAAAGAAGGCT to 5'-GAAGCTGAGT-

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³ The abbreviations used are: MMP, matrix metalloproteinase; NF κ B, nuclear factor κ B; PMA, phorbol myristate acetate; AP, activator protein; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcription-PCR; PDTC, pyrrolidine dithiocarbamate; WT, wild-type.

Table 1 Primer sequences used for RT-PCR of MMPs, TIMPs,^a and MT1-MMP expressions

Genes	Sequences	PCR product (bp)
MMP-2		447
Sense	5'-ACAAAGAGTGGCAGCAA-3'	
Antisense	5'-CACGAGCAAAGGCATCATCC-3'	
MMP-9		590
Sense	5'-CACTGTCCACCCCTCAGAGC-3'	
Antisense	5'-GCCACTTGTCCGGCATAAGG-3'	
TIMP-1		400
Sense	5'-GGGGACACCAGAAGTCAACCAGA-3'	
Antisense	3'-CTTTTCAGAGCCTTGGAGGAGCT-3'	
TIMP-2		590
Sense	5'-TGCAGCTGCTCCCGGTGCAC-3'	
Antisense	5'-TTATGGGTCTCGATGTGCAG-3'	
MT1-MMP		550
Sense	5'-CCCTATGCCTACATCCGTGA-3'	
Antisense	5'-TCCATCCATCACTTGGTTAT-3'	
GAPDH		300
Sense	5'-CGGACTCAACGGATTGGTCTGAT-3'	
Antisense	5'-AGCCTTCTCCATGGTGGTGAAGA-3'	

^a TIMP, tissue inhibitor of metalloproteinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

method. After transfection, cells were cultured in 10% FCS medium with vehicle (DMSO) or drugs for 24 h. Luciferase and β -galactosidase activities were assayed according to the manufacturer's protocol (Promega). Luciferase activity was normalized for β -galactosidase activity in cell lysate and expressed as an average of three independent experiments.

Nuclear Extract Preparation and EMSA. Preparation of nuclear extracts from control or drug-treated cells was carried out as described previously (16). The following oligonucleotide 5'-AGTTGAGGGGACTTCCAGGC corresponding to the NF κ B site was used as probe. The reaction mixture for EMSA contained 20 mM Tris-HCl (pH 7.6), 1 mM DTT, 2 mM MgCl₂, 1 mM EDTA, 10% glycerol, 1% NP40, 1 μ g of poly(deoxyinosinic-deoxycytidylic acid) and 5 μ g of nuclear proteins. Nonlabeled WT oligonucleotide was added into the reaction mixture and incubated for 10 min at room temperature. ³²P-labeled probe DNA (300,000 cpm) was added, and the binding reaction was allowed to proceed for another 20 min. Mixtures were resolved on 8% polyacrylamide gels at 150 V for 4 h. Gels were dried and subjected to autoradiography.

RNA Isolation and RT-PCR. To determine whether the reduced amounts of MMP-9 activity were a result of decreased levels of mRNA encoding this collagenase, we compared the levels of MMP-9 in Caski cells, which were treated with or without various concentrations of dykkellic acid in the presence of 75 nM PMA. MMP-9 mRNA expression was determined by RT-PCR. Total

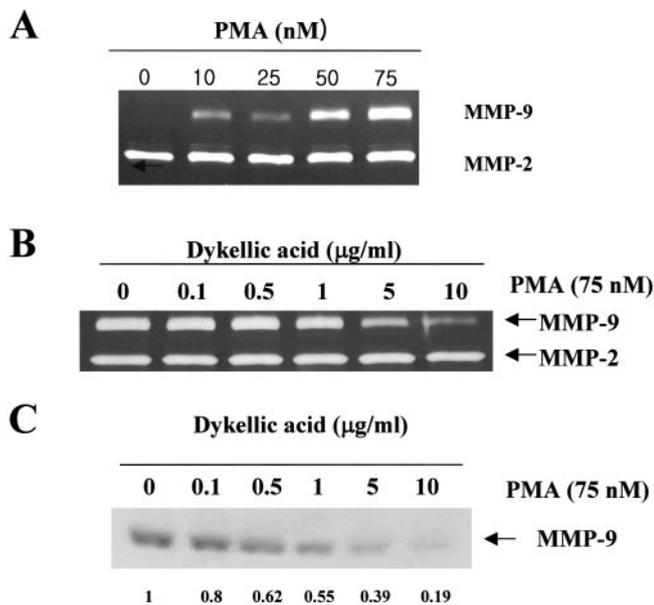


Fig. 1. Effect of dykkellic acid on MMP-9 activity. *A*, Caski cells were treated with various concentrations of PMA. Conditional medium was collected after 24 h, and gelatin zymography was performed. *B*, Caski cells were treated with various concentrations of dykkellic acid in the presence of PMA (75 nM). Conditional medium was collected after 24 h followed by gelatin zymography. *C*, conditional medium was collected and concentrated. MMP-9 was identified by Western blot analysis using anti-MMP-9 antibody. The MMP-9 levels shown are representative of three independent experiments. The values below the figure represent change in protein expression of bands.

TGAAGAAGGCT), and NF κ B (CCAGTGGAAATCCCCAGCCT to CC-CAGTGGAAATGGCCAGCCT). *Kpn*I and *Hind*III sites were included in PCR products so that after *Kpn*I and *Hind*III digestion of the PCR products they could be subcloned into the pGL2-Basic *Kpn*I/*Hind*III site. Clones representing each point mutation were sequenced to ensure the accuracy of the PCR amplification procedure.

Plasmids, Transfections, and Luciferase Gene Assays. AP-1 and NF κ B reporter constructs were purchased from Clontech (Palo Alto, CA). Expression plasmids GAL4p65TA1 and GAL4p65TA1+TA2, which use the Rous sarcoma virus promoter to drive expression of a chimeric protein with the GAL4 DNA binding domain (amino acids 1–147) fused to the p65 transactivating domains, were generated by Schmitz and Baeuerle (15) and obtained from Albert Baldwin (University of North Carolina, Chapel Hill, NC). In brief, cells were plated onto six-well plates at a density of 5×10^5 cells/well and grown overnight. Cells were cotransfected with 2 μ g of various plasmid constructs and 1 μ g of the pCMV- β -galactosidase plasmid for 5 h by the Lipofectamine

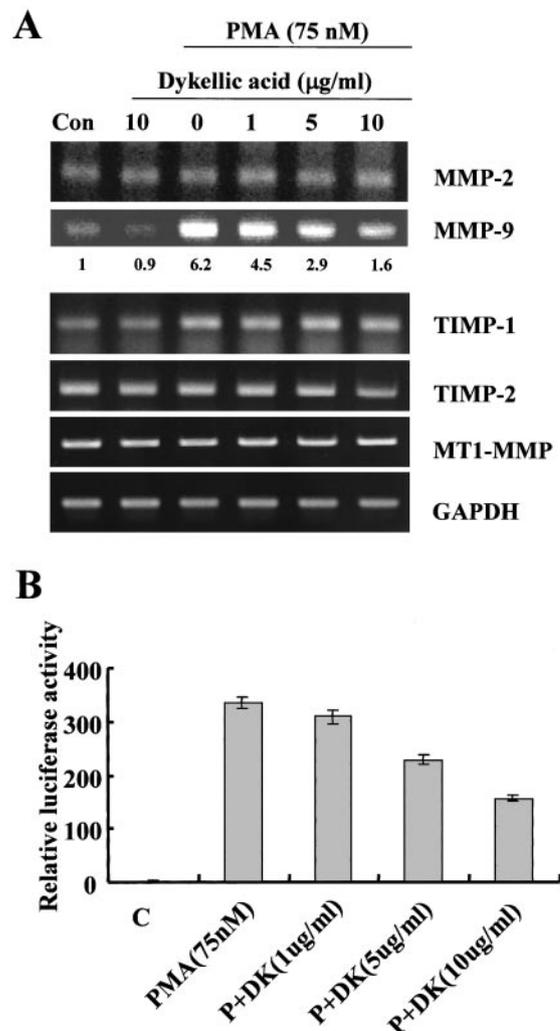


Fig. 2. Repression of PMA-induced MMP-9 transcription and promoter activity by dykkellic acid. *A*, Caski cells were treated with or without various concentrations of dykkellic acid in the presence of PMA (75 nM). Total RNA was isolated, and RT-PCR analysis was performed. The MMP-9 mRNA levels shown are representative of three independent experiments. The values below the figure represent change in mRNA expression of the bands normalized to GAPDH. *B*, WT-MMP-9 promoter-containing reporter vector was transfected and treated with various concentrations of dykkellic acid in the absence or presence of PMA (75 nM). The cells were lysed and luciferase activity measured. Data represent the mean of at least three independent experiments; bars, \pm SD.

cellular RNA was extracted from Caski cells using the TRIzol reagent (Life Technologies, Inc.). A cDNA was synthesized from 2 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). PCR primers are described in Table 1. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

Invasion Assay. Five $\times 10^4$ cells/chamber were used for each invasion assay. Invasion assays were performed using modified Boyden chambers with polycarbonate nucleopore membrane (Corning, Corning, NY). Precoated filters (6.5 mm in diameter, 8 μm pore-size, Matrigel 100 $\mu\text{g}/\text{cm}^2$) were rehydrated with 250 μl of medium, and 5 $\times 10^4$ cells in 200 μl medium with or without dykellic acid in the presence of PMA were seeded into the upper part of each chamber. After incubation for 24 h at 37°C, nonmigratory cells on the upper surface of the filter were wiped with a cotton swab, and migrated cells on the lower surface of the filter were fixed and stained with 0.125% Coomassie Blue in a methanol:acetic acid:water mixture (45:10:45 v/v/v). Random fields were counted under a light microscope.

RESULTS

Dykellic Acid Inhibits MMP-9 Activity. Caski cells, a human cervical cancer cell line that releases basal levels of MMP-9 when cultured in serum-free medium, were treated with various concentrations of PMA for 20 h. Whereas the level of MMP-2 expression is not significantly altered by PMA, PMA induces the expression and secretion of large amounts of latent MMP-9 as determined by gelatin zymography (Fig. 1A). The expression and secretion of MMP-9 is induced by PMA in a dose-dependent manner, with a dramatic increase in MMP-9 activity after treatment with 75 nM PMA. As shown in Fig. 1B, dykellic acid decreases PMA-induced MMP-9 activity in a dose-dependent manner, whereas the activity of a control metalloproteinase, of which the size was identical to the M_r 72,000 type IV

collagenase MMP-2 (17), is not reduced in dykellic acid-treated cells. We assayed the conditioned medium for MMP-9 by Western blotting to exclude the presence of a physiological inhibitor such as a tissue inhibitor of metalloproteinase family members (Ref. 5; Fig. 1C). Similar to zymography data, expression levels of MMP-9 protein gradually decrease in a dose-dependent manner indicating that the reduced MMP-9 enzyme activity is because of smaller amounts of the protein.

Dykellic Acid Inhibits Transcription of MMP-9. Treatment of Caski cells with dykellic acid also induces a decrease in the levels of PMA-stimulated MMP-9 mRNA (Fig. 2A). RT-PCR shows that steady-state MMP-9 mRNA levels are lower in dykellic acid-treated cells compared with cells not treated with dykellic acid. The effect of dykellic acid on MMP-9 expression was additionally investigated using HEK293 cells transiently transfected with a luciferase reporter gene linked to the 0.7-kb fragment of MMP-9 promoter sequence. As shown in Fig. 2B, luciferase gene expression is activated up to 23-fold in these cells treated with PMA compared with untreated cells. Treatment of cells with dykellic acid (10 $\mu\text{g}/\text{ml}$) decreases PMA-mediated luciferase activity up to 2-fold, indicating that the 0.7-kb fragment of the MMP-9 promoter contains dykellic acid response elements.

Effects of Dykellic Acid on NF κ B and AP-1 Activities. AP-1 and NF κ B point-mutated MMP-9 promoters show a diminished response to treatment with PMA, compared with the constructs made using a WT MMP-9 promoter (Fig. 3, A and B). In subsequent experiments, cells were transiently transfected with reporter vectors that included either the AP-1 or NF κ B binding sites. Luciferase activity in the cells with the NF κ B construct is significantly reduced by treatment with dykellic acid, whereas luciferase activity in cells with the AP-1

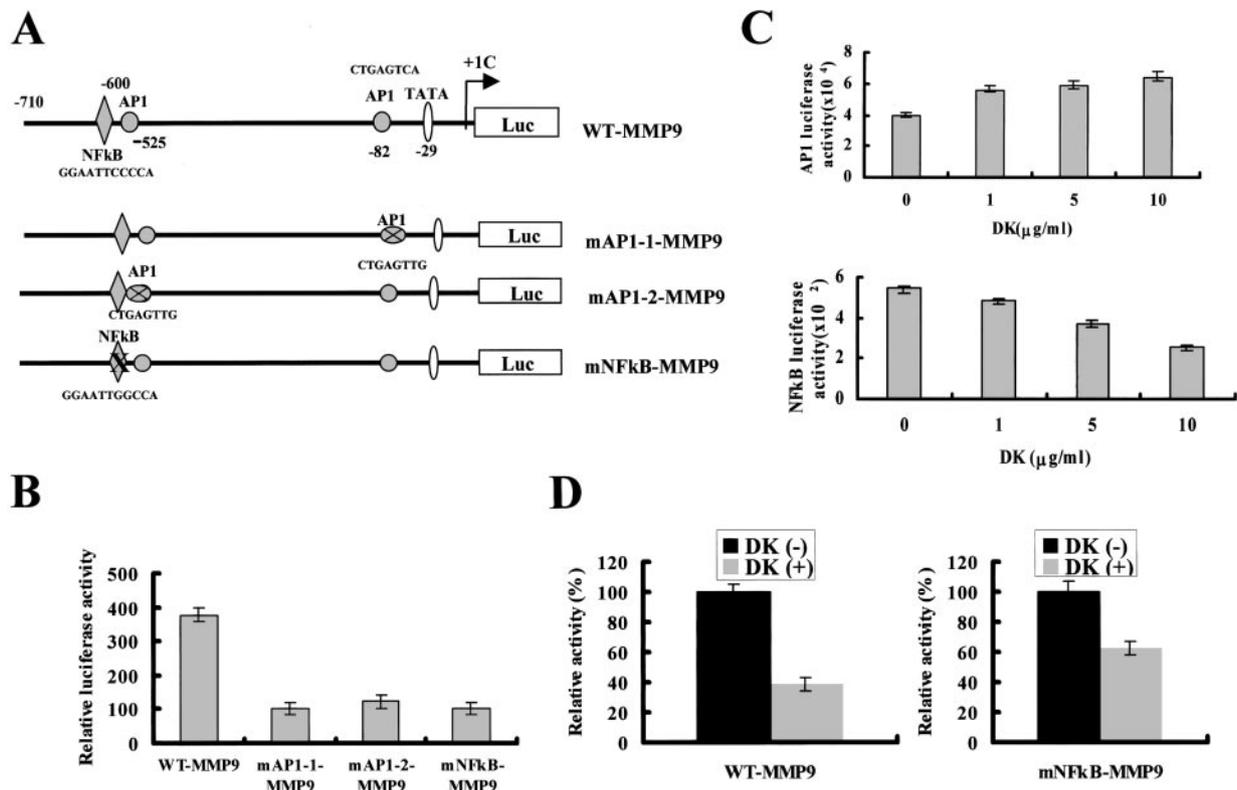


Fig. 3. Effects of dykellic acid on the activities of AP-1 and NF κ B. A, schematic structure of MMP-9 promoter constructs used for testing luciferase activity. Mutations were introduced into the NF κ B or AP-1 binding sites of WT-MMP-9 by 2-bp changes. B, WT or mutant MMP-9 promoters were transfected and treated with 75 nM PMA for 24 h, and luciferase activity measured. C, to elucidate the effects of dykellic acid on AP-1 and NF κ B activities, a reporter vector that has AP-1 (top panel) or NF κ B (bottom panel)-binding sites was transfected. The cells were treated with or without various concentrations of dykellic acid in presence of PMA (75 nM). Luciferase activity was measured. D, WT-MMP9 or mNF κ B-MMP9 plasmid was transfected, and treated with or without dykellic acid in presence of PMA (75 nM). Luciferase activity was measured. Data represent the mean of at least three independent experiments; bars, \pm SD.

construct is slightly increased by treatment with dykellic acid (Fig. 3C). As shown in Fig. 3D, dykellic acid-mediated MMP-9 promoter inhibition measured by dykellic acid-driven luciferase activity is significantly greater in the WT MMP-9 construct than in the construct with the NF κ B-mutated MMP-9 promoter.

Dykellic Acid Does Not Inhibit DNA Binding of NF κ B, But Inhibits Activation through NF κ B Transactivation Domains TA1 and TA2. To determine whether dykellic acid inhibits activation of NF κ B through the inhibition of DNA binding of NF κ B, we examined the effect of dykellic acid on PMA-induced binding of NF κ B by EMSA. Dykellic acid did not affect the intensity of the NF κ B-DNA complex induced by PMA or its migration in Caski cells (data not shown). We investigated whether dykellic acid inhibits transcriptional activation through a chimeric transcription factor in which the transactivating domain of p65 is fused to the yeast GAL4 DNA binding domain. Schmitz and Baeuerle (15) identified that the COOH-terminal portion of NF κ B p65 contains two transactivation domains, TA1 and TA2, and showed that chimeric fusion proteins with the TA1 (p65 amino acids 522–551) or TA1+TA2 (p65 amino acids 286–521) domains fused to the DNA binding domain of GAL4 (amino acids 1–147) confer PMA-inducible transcriptional activation onto a GAL4 reporter gene (15). We transiently transfected HEK293 cells with a GAL4 response element-luciferase reporter construct together with expression constructs encoding either the GAL4 DNA binding domain alone (GAL4DB), GAL4DB-p65 TA1, or GAL4DB-p65

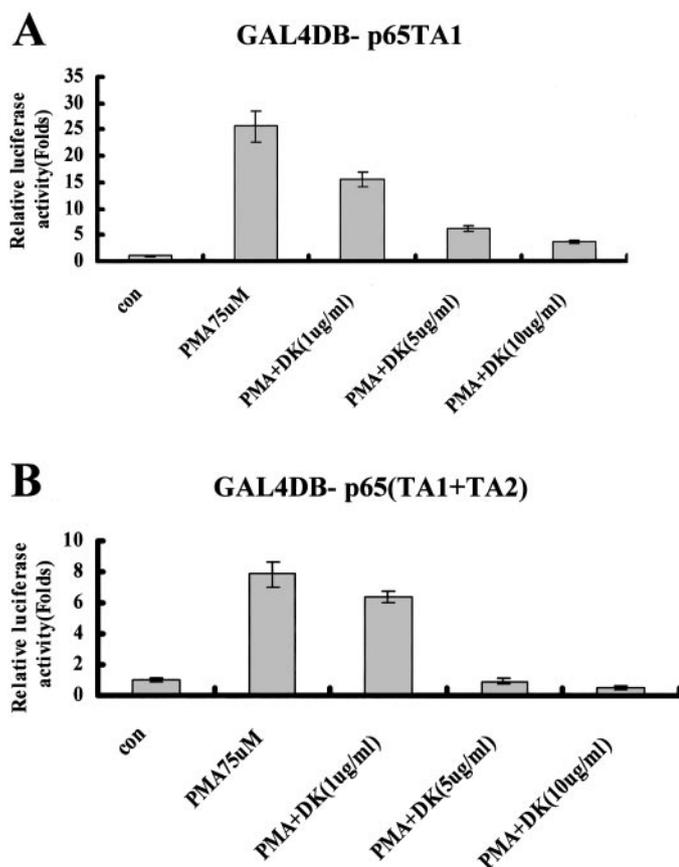


Fig. 4. Dykellic acid inhibits transcriptional activity of p65. HEK293 cells were transiently transfected with 1 μ g of GAL4RE luciferase reporter plasmid and 1 μ g of expression plasmids for either GAL4DB-p65 TA1 (A) or GAL4DB-p65 (TA1+TA2; B). The cells were treated with or without the indicated concentrations of dykellic acid in the absence or presence of PMA (75 nM). The mean normalized luciferase activity of GAL4DB-p65 (TA1+TA2) in untreated PMA cells was 500-fold higher than that of GAL4DB-p65 TA1. Data represent the mean of at least three independent experiments; bars, \pm SD.

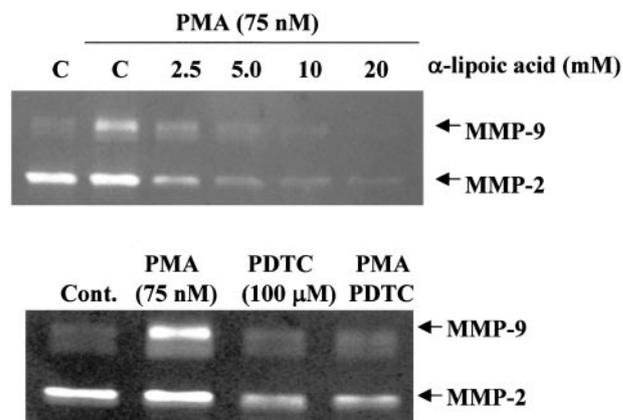


Fig. 5. Effect of α -lipoic acid and PDTC on MMP-9 activity. Caski cells were treated with various concentrations of α -lipoic acid in the presence or absence of PMA (75 nM). Conditional medium was collected 24 h after treatment followed by gelatin zymography. Caski cells were treated with vehicle, PMA (75 nM), PDTC (100 μ M), and PMA plus PDTC. Conditional medium was collected after 24 h followed by gelatin zymography.

(TA1+TA2) followed by subsequent stimulation with increasing doses of dykellic acid. The expression construct for GAL4DB alone shows no transactivation of the GAL4 response element (data not shown). Constitutive transcription by the chimeric transcription factor, GAL4DB-p65 TA1, is enhanced \sim 25-fold after stimulation with PMA, and the enhancement of transcription is inhibited by dykellic acid (Fig. 4A). We observed a similar pattern of regulation using the chimeric transcription factor GAL4DB-p65 (TA1+TA2). Constitutive transcription is enhanced \sim 8-fold after stimulation with PMA, and the PMA enhancement of transcription is inhibited by dykellic acid (Fig. 4B).

Inhibition of PMA-induced MMP-9 Activation by Other NF κ B Inhibitors. To examine effects of other well-known NF κ B inhibitors on PMA-induced MMP-9 activation, Caski cells were treated with α -lipoic acid, and PDTC in the presence of 75 nM PMA and MMP-9 expression was evaluated by zymography. As shown in Fig. 5, interestingly, α -lipoic acid and PDTC decrease MMP-2 and MMP-9 activity in a dose-dependent manner. However, PDTC and α -lipoic acid have no specific inactivation of MMP-9 activity (Fig. 5). Taken together, these data suggest that dykellic acid is unique in its ability to inhibit NF κ B activation and suppress MMP-9 gene expression.

Effect of Dykellic Acid on *in Vitro* Invasion of Caski Cells. Treatment of cells with 10 μ g/ml dykellic acid neither induced morphological changes nor inhibited the growth of Caski cells (data not shown). In the *in vitro* invasion assay, as shown in Fig. 6, dykellic acid inhibited migration by $>$ 40%. Therefore, the effect of dykellic acid on *in vitro* invasion inhibition was correlated well with its effect on MMP-9 inhibition.

DISCUSSION

The expression of proteases such as MMP-9 is regulated by diverse growth factors, cytokines, and xenobiotics such as PMA. Studies have shown that the mechanism responsible for PMA-mediated responses may involve direct alteration of transcription factors, but these mechanisms are not completely understood. Small molecular weight inhibitors that target the pathways that regulate MMP-9 expression could improve our understanding of these pathways and potentially be of clinical utility for the treatment of cancer. Using a cervical cancer cell line, our study demonstrates the ability of dykellic acid to reduce the expression of MMP-9.

We also investigated the molecular mechanism by which dykellic acid inhibits PMA-mediated expression of MMP-9 using AP-1 and

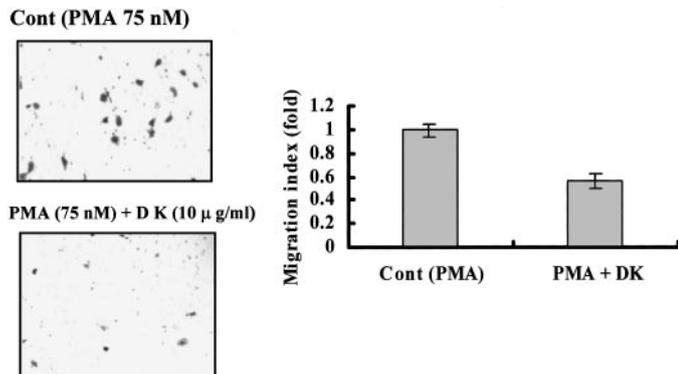


Fig. 6. Effect of dykellic acid on Matrigel invasion by Caski cells. For invasion assay, the lower and upper parts of Transwells were coated with Matrigel. Caski cells cultured in the presence or absence of either PMA or dykellic acid at indicated concentrations were placed in the upper well. Invasiveness of the cells was determined by measuring ability to pass through a layer of Matrigel-coated filter. After 24 h, cells on the bottom side of the filter were fixed, stained, and counted as described under "Materials and Methods." Data represent the mean of at least three independent experiments; bars, \pm SD.

NF κ B reporter constructs and found that NF κ B activity, but not AP-1 activity, is significantly reduced by treatment with dykellic acid. Thus, dykellic acid suppresses expression of MMP-9 via inhibition of NF κ B transactivation. To our knowledge, this is the first report of a small molecule that inhibits transcriptional activation by NF κ B. Whereas our study is unique in the demonstration of a small molecule inhibitor of NF κ B having an effect on the MMP-9 promoter, a previous study demonstrated the stimulation of the MMP-9 promoter by the activation of the AP-1 motif at -79 bp, and either tumor necrosis factor α or PMA activation of NF κ B and Sp1 binding sites at -600 bp and -558 bp, respectively (9). We show that NF κ B is a crucial transactivator for MMP-9 gene expression, as PMA-driven luciferase activity is reduced significantly in both NF κ B and AP-1 mutant promoters.

Our experiments have also clarified the mechanism by which dykellic acid controls NF κ B transcriptional activation. NF κ B signaling involves stimulation-induced degradation of cytoplasmic inhibitor of NF κ B (18), releasing p65 for translocation from the cytoplasm into the nucleus, where p65 interacts with p50 and binds specifically to the NF κ B target DNA sequence. After specific binding to DNA, transcriptional activation of NF κ B is regulated through phosphorylation of p65 at several distinct sites (19, 20). We found that dykellic acid does not affect the DNA binding of NF κ B, but it does block transactivation of NF κ B. We extended our analysis of the inhibition of NF κ B transcriptional activation by demonstrating that dykellic acid inhibits the PMA-induced activation of chimeric transcription factors containing the NF κ B p65 TA1 and TA2 transactivation domains. Thus, our data indicate that dykellic acid-mediated MMP-9 transcriptional down-regulation critically depends on intact NF κ B binding sites within the MMP-9 promoter region.

Dykellic acid is a low molecular weight compound, which selectively targets NF κ B transactivation without affecting AP-1 during

suppression of PMA-induced MMP-9 expression. This compound will undoubtedly be a useful tool for laboratory investigations of NF κ B activity. Furthermore, considering the strong evidence supporting a role for NF κ B in PMA-induced MMP-9 up-regulation, additional studies determining the potential efficacy of dykellic acid in inhibiting invasion are warranted.

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