L-mimosine blocks cell proliferation via upregulation of B-cell translocation gene 1 and N-myc downstream regulated gene 1 in prostate carcinoma cells

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Running head: Hypoxia induces Btg2 and Ndrg1 gene expression

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L-mimosine, an iron chelator and a prolyl 4-hydroxylase inhibitor, blocks many cancer cells at the late G1 phase. B-cell translocation gene 2 (Btg2) regulates the G1/S transition phases of the cell cycle. N-myc downstream-regulated gene 1 (Ndrg1) is a differentiation-inducing gene upregulated by hypoxia. We evaluated the molecular mechanisms of L-mimosine on cell cycle modulation in PC-3 and LNCaP prostate carcinoma cells. The effect of L-mimosine on cell proliferation of prostate carcinoma cells was determined by the ^3H-thymidine incorporation and flow cytometry assays. L-mimosine arrested the cell cycle at the G1 phase in PC-3 cells and at S phase in LNCaP cells, thus attenuating cell proliferation. Immunoblot assays indicated that hypoxia and L-mimosine stabilized hypoxia-inducible factor-1α (HIF-1α) and induced Btg2 and Ndrg1 protein expression, but downregulated protein levels of cyclin A in both PC-3 and LNCaP cells. L-mimosine treatment decreased cyclin D1 protein in PC-3 cells, but not in LNCaP cells. Dimethyloxalylglycine, a pan-prolyl hydroxylase inhibitor, also induced Btg2 and Ndrg1 protein expression in LNCaP cells. The transient gene expression assay revealed that L-mimosine treatment or co-transfection with HIF-1α expression vector enhanced the promoter activities of Btg2 and Ndrg1 genes. Knockdown of HIF-1α attenuated the increasing protein levels of both Btg2
and Ndrg1 by hypoxia or L-mimosine in LNCaP cells. Our results indicated that hypoxia and L-mimosine modulated Btg2 and Ndrg1 at the transcriptional level, which is dependent on HIF-1α. L-mimosine enhanced expression of Btg2 and Ndrg1, which attenuated cell proliferation of the PC-3 and LNCaP prostate carcinoma cells.

Key words: hypoxia; HIF-1α; iron; chetomin; cell cycle
INTRODUCTION

Iron chelators with high antiproliferation activities upregulate the expression of growth-inhibitory and metastatic suppressor genes mediated by hypoxia-inducible factor-1α (HIF-1α)-dependent and independent mechanisms (16, 22). L-mimosine ((S)-α-Amino-β-[1-(3-hydroxy-4-oxopyridine)] propionic acid; C₈H₁₀N₂O₄), a plant amino acid, acts as an iron chelator and reversibly blocks mammalian cell proliferation at late G1 phase (5). An earlier study indicated that numbers of G0/G1 cells were elevated after incubation with L-mimosine in human prostate carcinoma DU145 cells (32). L-mimosine also acts as a prolyl 4-hydroxylase inhibitor and has similar effects on the hypoxic induction of HIF-1α protein in human and rodent cells (3, 31).

Expression of B-cell translocation gene 2 (Btg2) in cycling cells induced accumulation of growth-inhibitory forms of retinoblastoma protein and led to G1 cell cycle arrest (8). Immunohistochemistry and proliferative indices of selected human prostate peripheral zone lesions indicated that a reduction in, or loss of, Btg2 expression is associated with progression to malignancy (6). Additionally, forced overexpression of Btg2 attenuated cell proliferation in prostate carcinoma PC-3 cells, while thyroid hormone downregulated Btg2 gene expression in LNCaP cells (25). A
study using bone-marrow-derived mesenchymal stem cells revealed that hypoxia 
induced mesenchymal stem cell differentiation in neuron-like cells by increasing the 
expression of Btg2 and decreasing cyclin D1 expression (20).

N-myc downstream-regulated gene 1 (Ndrg1) plays an important role in both 
androgen-induced cell differentiation and inhibition of prostate cancer metastasis (19, 28). In vivo studies showed that expression of Ndrg1 was inversely associated with 
Gleason grading and overall survival rates of prostate cancer patients; moreover, 
Ndrg1 inhibited metastasis of prostate carcinoma PC-3 cells in vitro (1). Previous 
study indicated that iron chelators induced Ndrg1 gene expression via the HIF-1α 
pathway in several types of cancer cells (34).

The objectives of this study were to determine the regulatory effects of 
L-mimosine on HIF-1α and its association with gene expression of Btg2 and Ndrg1 in 
human prostate carcinoma cells.

MATERIALS AND METHODS

Cell culture and chemicals. Human prostate carcinoma LNCaP and PC-3 cells were 
obtained from the American Type Culture Collection (Manassas, VA) and were 
maintained as previously described (25). Cells were incubated under normoxia using a 
standard CO2 incubator at 37°C in a humidified atmosphere with 5% CO2 and 95%
room air (21% O₂) until the cells grew to 70%–80% confluence in RPMI-1640 medium with 10% fetal calf serum (FCS). On the day of hypoxic treatment (1% O₂, 5% CO₂ and 94% N₂), growth medium was switched to fresh medium with 10% FCS that had been pre-equilibrated in the hypoxic incubator (APM-30D, Astec, Fukuoka, Japan). Cells were then incubated in the hypoxic incubator for another 24 h.

L-mimosine, chetomin, dimethyloxalylglycine (DMOG), and ferric ammonium citrate (FAC) were purchased from Sigma Chemical Company (St. Louis, MO) and dissolved in the suggested solvent according to the manufacturer’s instructions. The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Protein Research (Rockford, IL). FCS was purchased from HyClone (Logan, Utah), and RPMI 1640 medium was purchased from GIBCO, Invitrogen Corporation (Grand Island, NY).

Cell proliferation assay with ³H-thymidine incorporation. Cell proliferation in response to L-mimosine was measured using a ³H-thymidine incorporation assay as previously described (12). In this assay, 1 × 10⁴ cells were cultured in each well of a 12-well plate in RPMI 1640 medium with 10% FCS and different concentrations (0–800 μM) of L-mimosine. After the required incubation periods (24 h and 48 h), 0.5 μCi/ml of ³H-thymidine was added to each well of the 12-well plate. The cells were
then incubated at 37°C in a humidified 5% CO₂ atmosphere for 4 h. Cells were then washed twice with cold phosphate buffered saline (PBS) and then with cold 5% trichloroacetic acid. Cells were lysed by adding 0.5 ml of 0.5 N NaOH. Then, 400 μl of the solubilized cell solution was mixed with 4 ml of scintillation cocktail and counted in a liquid scintillation analyzer (Packard BioScience, Meriden, CT). Each sample was tested in quadruplicate.

Flow cytometry. Cells were serum starved for 24 h and then cultured in RPMI 1640 medium with 10% FCS and with or without different concentrations (0–800 μM) of L-mimosine for another 24 h. The cells were collected, washed with cold PBS, and centrifuged. The pellet was resuspended in 300 μl of cold PBS and fixed in ethanol. The cells were centrifuged at 300 x g for 10 min, the fixative was discarded, and then the cells were washed with cold PBS. The cells were resuspended in 1 ml of PBS, and 50 μl of Triton X-100 and 50 μl of ribonuclease (10 mg/ml) were added and incubated for 1 h, 37°C. After further centrifugation, cells were resuspended in PBS, stained with propidium iodide, and incubated at 4°C overnight. Cell cycle analysis was performed using the FACS-Calibur cytometer and CellQuestPro software (BD Biosciences, San Jose, CA); the data were analyzed using ModFit LT Mac 3.0 software as previously described (26).
**Immunoblot assay.** Equal quantities of cell extract (40 μg) were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gels and analyzed using the Western lightning plus-Enhanced Chemiluminescence detection system (Perkin Elmer, Waltham, MA, USA) and were viewed using ChemiGenius image capture system (Syngene, Cambridge, UK). Polyclonal rabbit anti-human Btg2 serum was prepared as previously described (25). The blot membranes were probed with 1:500 HIF-1α (610958, BD Biosciences), 1:3000 diluted β-actin antiserum (I-19, Santa Cruz Biotechnology, Santa Cruz, CA), 1:100 diluted vascular endothelial growth factor (VEGF; A-20, Santa Cruz Biotechnology), 1:2000 diluted cyclin D1 (DCS6, Cell Signal, MA), 1:100 diluted cyclin A (C-19, Santa Cruz Biotechnology), 1:3000 diluted Ndrg1 antiserum (42-6200, Invitrogen, Carlsbad, CA), and 1:1500 diluted Btg2 antiserum. The intensities of the different bands were analyzed using the GeneTools program of ChemiGenius (Syngene).

**Real-time reverse transcription-polymerase chain reaction.** Total RNA from cells was isolated using Trizol reagent, and cDNA was synthesized as described previously (12). Real-time polymerase chain reaction (qPCR) was performed using the ABI StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA). FAM dye-labeled TaqMan MGB probes and PCR primers were purchased for human Ndrg1
(HS00608837-m1) and Btg2 (HS00198887-m1) from Applied Biosystems. For the
internal positive control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH; HS99999905-m1) was used with a FAM reporter dye-labeled TaqMan MGB probe. The amplification conditions were as follows: 40 cycles at 95°C for 15 sec and 60 °C for 1 min. Mean cycle threshold (Ct) values were calculated for GAPDH and the reporter gene using StepOne software v2.0 (Applied Biosystems). Ct values for Ndrg1 and Btg2 were normalized against the GAPDH control probe to calculate ΔCt values. All reactions were preformed in triplicate, and each experiment was conducted on at least three independent occasions.

HIF-1α expression vector. The human HIF-1α cDNA (MGC:10483) vector was purchased from Invitrogen. Human HIF-1α cDNA was linearized by cutting with Bam HI and Xba I and ligation with the overexpression vector pcDNA3 (Invitrogen) as previously described (3).

Knock-down HIF-1α. LNCaP cells were plated onto 6-well plates 1 day before transfection. The culture media were replaced with RPMI-1640 medium plus 10% FCS and 5 μg/ml polybrene (Santa Cruz Biotechnology) and then transduced with HIF-1α shRNA lentiviral particles (sc-35561-V; Santa Cruz Biotechnology) as described by manufacturer. Two days after transduction, the cells (LN-HIF-1αsi) were
selected by incubation with 10 μg/ml puromycin dihydrochloride for another 3 days.

The mock-transfected LNCaP cells (LN-COLsi) were transduced with control shRNA lentiviral particles (sc-10808-V, Santa Cruz Biotechnology) and were clonally selected in same manner as the LN-HIF-1αsi cells. Cells that expressed HIF-1α in resistant colonies were evaluated by aforementioned immunoblot assay.

**Reporter vectors.** The reporter vectors containing different fragments of the 5'-flanking region of the human Btg2 gene were cloned as previously described (25).

The DNA fragment containing the promoter of the Ndg1 gene (-4714 to +46) was cloned as previously described (26). The 5'-deletion of the human Ndg1 reporter vectors containing different DNA fragments were constructed by digesting with different restriction enzymes. Proper ligation and orientation of the reporter vectors were confirmed by extensive restriction mapping and sequencing.

**Transient transfection and reporter assay.** LNCaP cells or PC-3 cells were plated onto 24-well plates 1 day before transfection. Cells were transiently transfected using TransFast transfection reagent as previously described (27). For the transient co-transfection of HIF-1α experiments, cells were transfected with same amount of plasmid in each well by adding pcDNA3 vector to eliminate variable degrees of efficiency of the reporter activities. Reporter vector-transfected LNCaP or PC-3 cells
were then treated with or without L-mimosine in RPMI 1640 medium with 10% FCS for additional 24 h. The luciferase activity was adjusted for transfection efficiency using the normalization control plasmid pCMVSPORTβgal.

Statistical analysis. Results are expressed as means ± standard errors (SEs) of at least three independent replications of each experiment. Statistical significance was determined using one way ANOVA and Student’s *t* test using the SigmaStat program for Windows, version 2.03 (SPSS Inc, Chicago, IL).

**RESULTS**

To explore the antiproliferative effects of L-mimosine on prostate cancer cells, we compared and analyzed inhibitory effects and cell-cycle distributions in L-mimosine-treated cells. The $^3$H-thymidine incorporation assay revealed that inhibition of PC-3 cells growth occurred initially at 50 μM of L-mimosine, increasing as the dose increased. The 400 μM of L-mimosine significantly blocked 49% and 97% of $^3$H-thymidine incorporation in PC-3 cells after treatment with L-mimosine for 24 h and 48 h, respectively (Fig. 1A). In the flow cytometric analysis, 100–800 μM of L-mimosine induced a 24.4% increase in G1 arrest, together with a decrease in G2/M phase and S phase cells after 24 h of incubation (Fig. 1B). However, L-mimosine induced cell apoptosis in PC-3 cells after 48 h of incubation since 100–800 μM of
L-mimosine increased the sub-G1 fraction of cells by 5.9% to 20.2% (Fig. 1C). The immunoblotting assays showed that L-mimosine upregulated Btg2 and Ndrg1 protein levels, but downregulated protein levels of cyclin D1 and cyclin A (Fig. 1D). The quantitative analysis was done by determining the intensity of each band of different genes and β-actin from three independent experiments (Fig. 1E).

Similar antiproliferative results were also found in the L-mimosine-treated LNCaP cells. L-mimosine (400 μM) blocked about 30% of 3H-thymidine incorporation into LNCaP cells after 24 h or 48 h of incubation (Fig. 2A). However, results from flow cytometric analysis of LNCaP cells revealed that 800 μM of L-mimosine induced a 6.5% increase in S phase cell arrest together with a decrease in G2/M phase cells after 24 h of incubation (Fig. 2B). 800 μM of L-mimosine induced a 10.7% increase in S phase arrest after 48 h of incubation (Fig. 2C). Unlike those studies of PC-3 cells, L-mimosine did not induce cell apoptosis in LNCaP cells since the sub-G1 fraction of cells did not differ significantly among cells treated with 0–800 μM of L-mimosine after 24 h or 48 h of incubation (Fig. 2B and Fig. 2C).

Results of the immunoblotting assays showed that L-mimosine upregulated HIF-1α, Btg2, and Ndrg1 protein levels in LNCaP cells. However, L-mimosine treatments decreased the protein levels of cyclin A, but did not significantly affect the
protein expression of cyclin D1 ($P = 0.079$, Fig. 2D). The results of the quantitative analysis are presented in Fig. 2E.

Because we found that L-mimosine stabilized HIF-1α in both LNCaP and PC-3 cells, we further determined whether the genes induced by L-mimosine could also be induced by hypoxia. The immunoblotting assays revealed that hypoxia, in comparison to normoxia, significantly upregulated protein levels of HIF-1α in both LNCaP and PC-3 cells (Fig. 3A, left and Fig. 3B, left). The results of quantitative analysis indicated that hypoxia stabilized HIF-1α and upregulated gene expression of VEGF, Btg2, and Ndrg1 in both LNCaP and PC-3 cells (Fig. 3A, right and Fig. 3B, right). Hypoxic treatment downregulated protein expression of the cyclin A in both LNCaP and PC-3 cells. Interestingly, the quantitative results indicated cyclin D1 levels were downregulated by 51% after 24 h of hypoxic culture in comparison to PC-3 cells incubated under normoxia (Fig. 3B). However, we did not find the same phenomenon in the LNCaP cells. Hypoxic treatment increased minimally, but not significantly ($P = 0.647$) cyclin D1 levels in LNCaP cells (Fig. 3A).

The results of the immunoblotting assays showed that DMOG, a prolyl 4-hydroxylase inhibitor, at 100–500 μM stabilized HIF-1α and induced Ndrg1 and Btg2 gene expression in LNCaP cells (Fig. 3C, left). The results of the quantitative
analysis indicated that Btg2 and Ndrg1 protein levels were upregulated 2.4-fold and 3.8-fold, respectively, after treated with 500 μM of DMOG for 24 h (Fig. 3C, right).

The immunoblotting assay revealed that L-mimosine-induced increase in Btg2 gene expression was significantly blocked ($P < 0.01$) after co-treatment with ferric ammonium citrate (FAC), a cell-permeable iron donor (Fig. 4A). The results of reverse transcription (RT)-qPCR indicated that L-mimosine (400 μM) induced a 1.6-fold increase in Btg2 gene expression compared to the solvent-treated group. The transient gene expression assay showed similar results, suggesting that iron blocks the elevation of L-mimosine-induced Btg2 (Fig. 4B). Furthermore, transient gene expression assays indicated that transient overexpression of HIF-1α under normoxia induced Btg2 promoter activity (Fig. 4C). Induction of transient gene expression using a 5'-deletion assay indicated that HIF-1α increased Btg2 promoter activity is dependent on region -101 to -1 upstream of the translational initiation site of Btg2 gene (Fig. 4D). Results of immunoblotting assays and RT-qPCR showed hypoxia induced 1.5- and 2.3-fold increases in Btg2 gene expression, respectively, while co-treatment with chetomin did not significantly attenuate ($P = 0.714$) Btg2 gene expression by hypoxia (Fig. 4E).

Transient gene expression assays indicated that L-mimosine induced Ndrg1
promoter activity. L-mimosine (400 μM) induced a 2.09-fold increase in Ndrg1 promoter activity compared to the solvent-treated group (Fig. 5A). The results of the immunoblotting assay and RT-qPCR indicated that 400 μM L-mimosine increased Ndrg1 mRNA levels 1.99-fold compared to the solvent-treated group. Furthermore, this increase was significantly blocked \((P < 0.01)\) by co-treatment with FAC, indicating that iron blocks the elevation of L-mimosine-induced Ndrg1 (Fig. 5B). Similar results were found in the transient gene expression assays (Fig. 5C). Induction of transient gene expression using a 5'-deletion assay indicated that L-mimosine induced an increase in Ndrg1 promoter activity, which was dependent on the DNA fragment located at -4714 to -1319 upstream of the transcriptional initiation site of the Ndrg1 gene (Fig. 5D).

The results of RT-qPCR showed that hypoxia increased Ndrg1 mRNA levels 6.35-fold compared to cells cultured under normoxia; however, this increased effect was significantly attenuated \((P < 0.01)\) by co-treatment with chetomin (Fig. 6A). Similar results were found in the immunoblotting assays. Hypoxia increased Ndrg1 and VEGF protein levels, while chetomin blocked the increases (Fig. 6B). Transient gene expression assays revealed that transient overexpression of HIF-1α enhanced Ndrg1 promoter activity (Fig. 6C). Induction of transient gene expression using a
5'-deletion assay indicated that transient overexpression of HIF-1α increased Ndrg1 promoter activity, which was also dependent on region -4714 to -1319 upstream of the transcriptional initiation site of the Ndrg1 gene (Fig. 6D).

In order to determine whether the upregulation of Btg2 and Ndrg1 in prostate carcinoma cells by hypoxia or L-mimosine was HIF-1α-dependent, we transiently knocked down HIF-1α in LNCaP (LN-HIF-1αsi) cells. The immunoblotting assays revealed that hypoxia, in comparison to normoxia, significantly upregulated (P < 0.001) protein levels of Btg2 and Ndrg1 in mock-transfected LNCaP (LN-COLsi) cells. However, the increase in protein levels of both Btg2 and Ndrg1 by hypoxia was significantly attenuated (P < 0.001) when the HIF-1α gene was knocked-down from LNCaP (LN-HIF-1αsi) cells (Fig. 7A). Similar results were also found for L-mimosine treatment (Fig. 7B).

DISCUSSION

L-mimosine, a plant amino acid, reversibly blocks mammalian cell cycle at the late G1 phase (5). Earlier studies using prostate DU145 carcinoma cells revealed that numbers of G0/G1 cells were elevated after incubation with L-mimosine, resulting in upregulation of p27 (32). Our results indicated that L-mimosine inhibited PC-3 cell proliferation with increasing numbers of cells accumulating in the G1 phase of the
Moreover, L-mimosine induced cell apoptosis when PC-3 cells were treated with L-mimosine for long period of incubation (48 h). In consistent with previous studies, these results showed that L-mimosine exerted apoptotic activity in human pancreatic cancer and 937 leukemia cells (9, 35). With LNCaP cells, the numbers of S phase cells were elevated after incubation with L-mimosine. These results are in agreement with early studies indicating that L-mimosine reversibly arrested the cell cycle late in the G1 phase or at the beginning of the S-phase (10). Our flow cytometric analysis indicated that L-mimosine (800 μM) suppressed cell growth but did not exert apoptotic activity in human prostate carcinoma LNCaP cells in vitro. Results of our ³H-thymidine incorporation and flow cytometric assays indicated that LNCaP cells were more resistant to antiproliferation and apoptosis-inducing by L-mimosine in comparison to PC-3 cells.

Cyclin D1 serves as a key sensor and an integrator of extracellular signals, and promotes progression through G1-S phases of the cell cycle (7). Our in vitro study using PC-3 cells showed that hypoxia or L-mimosine treatment inhibited the expression of cyclin D1. This result is similar to the results of early studies that indicated L-mimosine blocked cell cycle progression and suppressed proliferation of H226 lung cancer cells and human breast cancer MDA-MB-453 cells by inhibition of
cyclin D1 expression (2, 14). Our results also are in agreement with results of other studies that indicated the inverse association between cyclin D1 and HIF-1α in A549 pulmonary cancer cells and bone-marrow-derived mesenchymal stem cells (20, 33). However, unlike in PC-3 cells, hypoxia or L-mimosine treatment increased cyclin D1 expression slightly but not significantly in LNCaP cells. In 3T3 cells, L-mimosine arrested cells in the G1 phase but did not affect the protein levels of cyclin D1 (29). Additionally, hypoxia enhanced cyclin D1 gene expression in human breast carcinoma MCF-7 cells (11). It appears that hypoxic effects on cyclin D1 expression are cell type dependent. It is worthy to note that LNCaP cells are p53-wild type cells and PC-3 cells are p53-null cells (27). p53 is a well-known regulator of the G1/S and G2/M check points (18). The difference in p53 expression between LNCaP and PC-3 cells could account for the differences in cyclin D1 expression and cell cycle arrest under hypoxic conditions. Our results also showed that L-mimosine treatment inhibited the expression of cyclin A in both PC-3 and LNCaP cells, which is consistent with results of other studies that indicated hypoxia or L-mimosine treatment downregulated the expression of cyclin A (21, 24). These results may also account for cell cycle arrest in S phase by L-mimosine in LNCaP cells. Induction of Btg2 led to G1 cell cycle arrest in several cell lines (8, 23, 25). Loss
of Btg2 expression is the earliest known indicator that a cell cycle regulator is lost in prostate carcinogenesis (6); nonetheless, the function and regulatory mechanisms of Btg2 in the human prostate are still unclear. In a previous study using stable Btg2-overexpressing PC-3 cells, we found that overexpression of Btg2 attenuated cell proliferation; moreover, Btg2 gene expression was downregulated by thyroid hormone in prostate carcinoma LNCaP cells (25). In the present study, upregulation of Btg2 gene expression by L-mimosine in PC-3 cells led to attenuated cell proliferation and cell cycle arrest in phase G1. The effect of L-mimosine on Btg2 in our study was mediated by iron depletion, since its modulation was reversed by adding iron. We also found that hypoxia enhanced Btg2 gene expression and HIF-1α enhanced the promoter activity of the Btg2 reporter vector. Our results are in agreement with another study in which hypoxia-induced increased Btg2 and decreased cyclin D1 expression in bone-marrow-derived mesenchymal stem cells (20). Induction of transient gene expression by 5′-deletion assay indicated that HIF-1α increased Btg2 promoter activity was dependent on the -101 to -1 region upstream of the translational initiation site of the Btg2 gene. Nonetheless, our RT-qPCR and immunoblot data showed that chetomin did not significantly affect hypoxia-induced Btg2 expression. Chetomin is a small molecule that disrupts the CH1 domain of p300, precluding its
interaction with HIF-1α, and thus, its subsequent transcriptional activity (15). Additionally, we did not find a conserved DNA sequence (5'-RCGTG-3') of HIF-1α response element (30) in the Btg2 promoter region (-297 to -1) when using simple sequence analysis (http://transfac.gbf.de/cgi-bin/matsearch). Taken together, we suggest that enhancement of Btg2 gene expression by L-mimosine occurs via an HIF-1α indirect pathway.

Previous study identified that induction of Ndrg1, which is essential for the G1 phase arrest, is an early event after L-mimosine treatment (4). Although L-mimosine is similar to other iron chelators inducing Ndrg1 gene expression in several cell lines (34), our study is the first to characterize L-mimosine induced Ndrg1 gene expression in prostate carcinoma cells. Moreover, we found that gene expression of Ndrg1 increased when LNCaP cells were treated with other prolyl hydroxylase inhibitors such as DMOG. The effect of L-mimosine on Ndrg1 in our study was mediated by iron depletion, because it was reversed by adding iron, which is in agreement with other studies (16, 17). RT-qPCR and immunoblotting assays showed that chetomin suppressed hypoxia-induced Ndrg1 expression. In combination with the transient gene expression assay, our data suggested that direct HIF-1α binding to the putative response element on the Ndrg1 genes is involved in the mediation of HIF-1α on
Ndrg1 gene expression. The actual mechanism responsible for the effect of HIF-1α on the Ndrg1 gene is still not well known. A previous study indicated that hypoxia-inducible transcription of the Ndrg1 gene is dependent on the activation of c-Jun/AP-1 transcription factor or Egr1/Sp1 binding site located -80 to -50 upstream of the transcriptional initiation site of the Ndrg1 gene (36). Our results suggest that induction of Ndrg1 gene expression by hypoxia is dependent on the binding of the HIF-1α on the putative HIF-1α response element located -4714 to -1319 bp upstream of the transcriptional initiation site of the Ndrg1 gene. Our results are in agreement with previous studies that determined the putative HIF-1α response element of the Ndrg1 gene based on bioinformatic analysis (4, 13). Moreover, our results (Fig. 7) indicated that the increasing of protein levels of both Btg2 and Ndrg1 by hypoxia is attenuated when the HIF-1α gene was knocked-down in LNCaP cells, suggesting that the upregulation of Btg2 and Ndrg1 in prostate carcinoma cells by hypoxia is dependent on HIF-1α.

In conclusion, the results suggest that L-mimosine treatment and hypoxia modulate the expression of Btg2 and Ndrg1 genes through different mechanisms. L-mimosine stabilizes HIF-1α protein and enhances expression of Btg2 and Ndrg1 at the transcriptional level, which attenuates cell proliferation of prostate carcinoma cells.
in vitro.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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Figure Legends

Fig. 1. Effect of L-mimosine on cell cycle distribution and cell proliferation in PC-3 cells. A: PC-3 cells were treated with varying concentrations of L-mimosine, as indicated, for 24 h (black circle) or 48 h (open circle) and cell proliferation was determined by the $^3$H-thymidine incorporation assay. Each point on the curve represents the mean percentage ± SE (n = 4) of $^3$H-thymidine incorporated into the cells relative to the control solvent-treated group (0 μM of L-mimosine treatment) ($^*P < 0.05$, $^{+}P < 0.01$). B: The cell-cycle distribution of PC-3 cells was analyzed by flow cytometry. The data shown in each bar chart are represents the mean percentage ± SE (n = 3) of cells in each phase of the cell cycle and compared to the control solvent-treated group ($^{*}P < 0.05$, $^{+}P < 0.01$). C: The sub-G1 fraction of PC-3 cells was analyzed by flow cytometry. The data shown in each bar chart represent the mean percentage ± SE (n = 3) of cells in the sub-G1 phase of the cell cycle ($^{+}P < 0.01$). D: PC-3 cells were treated with varying concentrations of L-mimosine for 24 h. Cells were lysed and the expressions of hypoxia-inducible factor-1α (HIF-1α), N-myc downstream regulation gene 1 (Ndrg1), cyclin D1, cyclin A, B-cell translocation gene 2 (Btg2), and β-actin were determined by immunoblot assay. E: The quantitative analysis was done by determining the intensity of each band for target genes and
β-actin from three independent experiments. Data are presented as the fold-induction (± SE, n = 3) of the relative density of the target gene/β-actin (± SE) in relation to the control solvent-treated group (*P < 0.05, †P < 0.01).

Fig. 2. Effect of L-mimosine on cell cycle distribution and cell proliferation in LNCaP cells. A: LNCaP cells were treated with varying concentrations of L-mimosine for 24 h (black circle) or 48 h (open circle) and cell proliferation was determined by the ³H-thymidine incorporation assay. Each point on the curve represents the mean percentage ± SE (n = 4) of ³H-thymidine incorporated into the cells relative to the control solvent-treated group (*P < 0.05, †P < 0.01). The cell-cycle distribution of LNCaP cells was analyzed after treatments L-mimosine 24 h (B) and 48 h (C) by flow cytometry. The data shown in each bar chart represent the mean percentage ± SE (n = 3) of cells in each phase of the cell cycle and compared to the control solvent-treated group (*P < 0.05). D: Immunoblotting for HIF-1α, Ndrg1, cyclin D1, cyclin A, Btg2, and β-actin using lysates from vary concentrations of L-mimosine-treated LNCaP cells. E: Quantitative results were analyzed by determining the intensity of each band for the target genes and β-actin from three independent experiments. Data are presented as the fold-induction (± SE, n = 3) of the relative density of the target gene/β-actin (± SE) in relation to the control
solvent-treated group (*$P < 0.05$, †$P < 0.01$).

Fig. 3. Hypoxia modulates gene expression of LNCaP and PC-3 cells. LNCaP (A) or PC-3 (B) cells were cultured under hypoxic (1% O$_2$) or normoxic (21% O$_2$) conditions for 24 h. The cells were then lysed and the expressions of HIF-1α, Ndr1, Btg2, vascular endothelial growth factor (VEGF), cyclin D1, cyclin A, and β-actin were determined by immunoblot assay. C: LNCaP cells were treated with 0–500 μM of dimethyloxalylglycine (DMOG) for 24 h. Cells were lysed and expression of HIF-1α, Ndr1, Btg2, and β-actin were determined by immunoblot assay. Quantitative results were analyzed by determining the intensity of each band for target genes and β-actin from three independent experiments. Data are presented as the fold-induction (± SE, n = 3) of the relative density of the target gene/β-actin (± SE) in relation to the control solvent-treated group (*$P < 0.05$, †$P < 0.01$).

Fig. 4. Hypoxia and L-mimosine modulate the gene expression of B-cell translocation gene 2 (Btg2) in prostate carcinoma cells. A: PC-3 cells were treated with 400 μM of L-mimosine and/or 100 μg/ml of ferric ammonium citrate for 24 h. Cells were then lysed and expression of Btg2 and β-actin were determined by the immunoblot assay (top) or RT-qPCR (bottom). Data are presented as the fold-induction (± SE, n = 3) of the relative mRNA levels in relation to the control
solvent-treated group (*, \textit{P} < 0.01; C, control solvent; L, L-mimosine; F, ferric ammonium citrate).  

\textit{B}: The Btg2 reporter vector-transfected PC-3 cells were treated with L-mimosine (400 \(\mu\)M) and/or ferric ammonium citrate (100 \(\mu\)g/ml) for 24 h. Data are presented as the mean percentage \(\pm\) SE (\(n = 6\)) of the luciferase activity in relation to the control solvent-treated group (*, \textit{P} < 0.01).  

\textit{C}: The Btg2 reporter vector-transfected LNCaP cells were co-transfected with HIF-1\(\alpha\) expression vector. Data are presented as the mean-percentage (\(\pm\) SE, \(n = 6\)) of the luciferase activity in relation to the mock-transfected group (\textit{P} < 0.01).  

\textit{D}: Nested deletion constructs of Btg2 reporter vectors were co-transfected with control expression vector (pcDNA3; white bars) or HIF-1\(\alpha\) expression vector (black bars) into LNCaP cells. Data are presented as the mean percentage \(\pm\) SE (\(n = 6\)) of the luciferase activity of the reporter vectors induced by the HIF-1\(\alpha\) expression vector relative to the luciferase activity associated with group that was co-transfected with control expression vector (\textit{P} < 0.01).  

\textit{E}: LNCaP cells were cultured under normoxic (N) or hypoxia (HP) conditions and treated with or without 50 nM of chetomin for 24 h. Cells were then lysed and expression of HIF-1\(\alpha\), Btg2 and \(\beta\)-actin were determined by immunoblot assay (\textit{top}) or RT-qPCR (\textit{bottom}). Data are presented as the fold-induction (\(\pm\) SE, \(n = 3\)) of the relative mRNA levels in relation to the group cultured under normoxic conditions (\textit{P} < 0.01).
Fig. 5. L-mimosine modulates the gene expression of N-myc downstream regulation gene 1 (Ndrg1) of prostate carcinoma cells. A: The Ndrg1 reporter vector-transfected LNCaP cells were treated with varying concentration of L-mimosine as indicated for 24 h. Data are presented as the mean percentage ± SE of the luciferase activity induced by the L-mimosine treatments relative to the control solvent-treated group (*$P < 0.05$, $^+P < 0.01$). Gene expression of Ndrg1 were determined by immunoblot assay (B, top), or RT-qPCR (B, bottom). Data are presented as the fold-induction (± SE, n = 3) of the relative mRNA levels in relation to the control solvent-treated group (*, $^+P < 0.01$) (C, control solvent; L, L-mimosine; F, ferric ammonium citrate). C: The Ndrg1 reporter vector-transfected PC-3 cells were treated with L-mimosine (400 μM) and/or ferric ammonium citrate (100 μg/ml) for 24 h. Data are presented as the mean percentage ± SE (n = 6) of the luciferase activity in relation to the control solvent-treated group (*, $^+P < 0.01$). PC-3 cells were treated with L-mimosine (400 μM) and/or ferric ammonium citrate (100 μg/ml) for 24 h. D: Luciferase activity of nested deletion constructs of Ndrg1 reporter vectors after treatment with control solvent (white bars) or 400 μM of L-mimosine (black bars). Data are expressed as the mean percentage ± SE (n = 6) of the Ndrg1 reporter activity.
induced by the L-mimosine treatments in relation to the control solvent-treated groups ($P < 0.01$).

Fig. 6. Hypoxia modulates the gene expression of N-myc downstream regulation gene 1 (Ndr1) of prostate carcinoma cells. LNCaP cells were cultured under normoxic (N) or hypoxic (HP) conditions and treated with or without 50 nM of chetomin for 24 h. Gene expression of Ndr1 was determined by RT-qPCR (A) and the expressions of HIF-1α, Ndr1, vascular endothelial growth factor (VEGF), and β-actin were determined by immunoblot assay (B). Data are presented as the fold-induction (± SE, n = 3) of the relative mRNA levels in relation to the group cultured under normoxic conditions (*, $P < 0.01$). C: The LNCaP cells were transient transfected with HIF-1α expression vector (LN-HIF-1α) or mocked expression vector (LN-DNA) for 36 h. Cells were then lysed and expression of HIF-1α and β-actin were determined by the immunoblot assay (top). The Ndr1 reporter vector-transfected LNCaP cells were co-transfected with and varying concentrations of HIF-1α expression vector. Data are presented as the mean percentage ± SE (n = 6) of the luciferase activity in relation to the mock-transfected group (bottom; *$P < 0.05$, $P < 0.01$). D: Luciferase activity of nested deletion constructs of Ndr1 reporter vectors co-transfected with control expression vector (pcDNA3; white bars) or HIF-1α
expression vector (black bars) into LNCaP cells. Data are presented as the mean percentage ± SE (n = 6) of the luciferase activity of the reporter vectors induced by the HIF-1α expression vector relative to the luciferase activity associated with group that was co-transfected with the control expression vector (\( ^+P < 0.01 \)).

Fig.7. HIF-1α knockdown attenuates the activation of hypoxia and L-mimosine on gene expression of N-myc downstream regulation gene 1 (Ndrg1) and B-cell translocation gene 2 (Btg2) of LNCaP cells. A: The mock-knockdown LNCaP cells (LN-COLsi) and HIF-1α-knockdown LNCaP cells (LN-HIF-1αsi) were cultured under normoxic (N) or hypoxic (HP) conditions for 24 h. B: LN-COLsi cells and LN-HIF-1αsi were treated without (-) or with (L) 400 μM of L-mimosine for 24 h. Cells were then lysed and expression of HIF-1α, Btg2, Ndrg1, and β-actin were determined by the immunoblot assay (left). Quantitative results were analyzed by determining the intensity of each band for the target genes (white bars, Btg2; black bars, Ndrg1; and dash line bars, HIF-1α) and β-actin from three independent experiments (right) (*\( P < 0.01 \), \( ^+P < 0.01 \)).