# Enhancement of hypoxia-induced apoptosis of human breast cancer cells via STAT5b by momilactone B

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**Abstract.** We have shown previously that hypoxia activates the cyclin D1 promoter via the Jak2/STAT5b pathway in breast cancer cells. Most solid tumors contain hypoxic components and overexpression of cyclin D1. The purpose of the present study was to investigate the molecular mechanism by which momilactone B exerts its inhibitory effects on breast cancer cells. Momilactone B, extracted from Korean rice hulls, suppressed hypoxia-induced increases in phospho-STAT5, STAT5b, cyclin D1, and cdk4 protein levels in human breast cancer cells. STAT5b expression was inhibited by siRNA experiments leading to decreased cyclin D1. The effects of momilactone B on cell growth and apoptosis-related gene expression were investigated in breast cancer cells under hypoxic conditions (2% $O_2$). Bax and p21 expression was found to be up-regulated, whereas ppRb and bcl-2 were down-regulated in momilactone B-treated cells under hypoxic conditions. However, the p53 protein level did not change. Flow cytometry with Annexin-FITC staining showed that the number of apoptotic cells increased in hypoxic cells treated with momilactone B compared with untreated hypoxic cells. Furthermore, caspase activity increased upon treatment with momilactone B under hypoxic conditions. These results indicate that momilactone B inhibits the growth of breast cancer cells, regulates the expression of apoptosis-related genes, and induces apoptosis through STAT5b and a caspase-3 dependent pathway. We suggest that momilactone B accelerates hypoxia-induced apoptosis of human breast cancer cells through STAT5b, and may represent an effective chemo-preventive or therapeutic agent against breast cancer.

## Introduction

Hypoxia, a common effect of solid tumor growth in breast and other cancers, induces a cascade of molecular pathways including angiogenesis, glycolysis, and various cell cycle control proteins, and prolonged hypoxia can induce cell death (1,2). Disordered perfusion and unregulated growth of cancer cells results in a low regional oxygen supply within solid tumors (3,4). The low level of oxygen in human breast and other cancers has been documented with micro-electrodes and is considered a therapeutic problem because it makes solid tumors resistant to radiation and chemotherapy (5). Cells adapt to hypoxic stress via cell-specific protective mechanisms such as cell cycle arrest, reduced energy-dependent protein synthesis, and selective gene induction (6,7).

Signal transducer and activator of transcription 5 (STAT5) regulates growth, differentiation, and survival of mammary and hematopoietic cells. STAT5 may act as a mediator in hypoxia- or DFO (desferrioxamine)-mediated gene expression in mammary epithelial cells (8). Our previous studies showed that STAT5b regulates the transactivation of cyclin D1 and insulin-like growth factor-1 (IGF-1) upon hypoxia stimulation in solid tumor cells (2,9). These results argue that DFO or hypoxia is a critical stimulator for the activation and phosphorylation of STAT proteins in mammary epithelial cells (HC11) and human breast cancer cells (MCF-7) (10). We

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*Abbreviations:* CDK4, cyclin-dependent kinase 4; DFO, desferrioxamin; EMSA, electrophoretic mobility shift assay; Jak2, Janus kinase 2; STAT5b, signal transducer and activator of transcription 5b

*Key words:* hypoxia, momilactone B, STAT5b, apoptosis, human breast cancer cells

have confirmed that hypoxia or DFO (as a hypoxia inducer) specifically activates STAT5b during hypoxia-stimulated cyclin D1 transactivation in animal and human breast cancer cells (2).

STATs are thought of as having 'yin and yang'-type properties, whereby, depending on the type of cellular stressor, either cell-death or cell-survival pathways are activated (11,12). Dominant-negative STAT5 inhibits the growth of T47D-derived tumors in nude mice via the induction of apoptosis (13). Induction of apoptosis and inhibition of tumor cell proliferation have been used as markers for the evaluation of phytochemical anti-cancer activities (14), and many chemotherapy agents exert their effects by these mechanisms. An imbalance between cell proliferation and apoptosis has been implicated in breast cancer development (15,16).

Cancer chemotherapy has gradually improved with the development of novel antitumor drugs (17). However, many therapeutic anticancer agents exhibit intrinsic and potent cytotoxic activities against normal cells (18,19). Rice is one of the principal cereals in Asia, some countries of Africa, and Latin America (20). Rice hulls are an agricultural byproduct that poses a very serious problem for the global environment (21). Momilactone B was originally isolated from rice hulls as a growth inhibitor involved in seed dormancy and was later found in rice leaves and straw as a phytoalexin (22,23). Recent reports have revealed that momilactone B may represent a novel therapeutic agent to induce cell death in solid tumor cells (17).

We recently reported that hypoxia activates the cyclin D1 promoter via the Jak2/STAT5b pathway in breast cancer cells (2). The purpose of the present study was to investigate the molecular mechanism by which momilactone B exerts its inhibitory effects on breast cancer cells. We predicted that specific blocking of hypoxia-stimulated STAT5b in human breast cancer cells under hypoxic conditions by momilactone B should enhance solid cancer cell apoptosis. Thus, specific inhibition of STAT5b could be another strategy for blocking tumor growth.

*In vitro* treatment of breast cancer cells under hypoxic conditions with momilactone B induced growth arrest and apoptosis in conjunction with the blockade of the constitutively active Jak-STAT signaling pathway. These results indicate that momilactone B inhibits the growth of breast cancer cells, regulates the expression of apoptosis-related genes, and induces apoptosis through a caspase-3-dependent pathway.

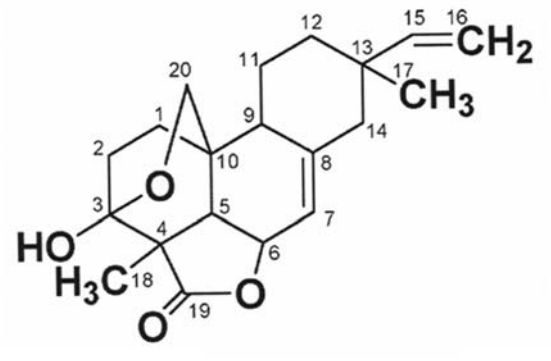



Figure 1. Structure of momilactone B. Rice hulls, from which momilactone B is extracted, comprise 20% of the rice grain kernel and, similar to other plant biomass, contains a high percentage of organic substances. It is therefore recognized as a potential source of energy and organic chemicals.

## Materials and methods

*Materials*. Momilactone B was kindly provided by Dr Il Min Chung (Konkuk University, Korea, Fig. 1). L-15 and RPMI-1640 cell growth media were purchased from Gibco-BRL (Grand Island, NY). The cyclin D1 antibody (MS-210-P1) was purchased from Neomarkers (Fremont, CA). Phospho-STAT5, STAT5b, p21, p53, cdk4, bcl-2, and bax antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-Rb (Ser 795) and cleaved caspase-3 antibodies were from Cell Signaling Technology (CST, Beverly, MA). The pRb antibody was purchased from BD Transduction Laboratories (BD Bioscience, Palo Alto, CA). The FLICA apoptosis detection kit (caspase activity assay) was from Immunochemistry Technologies (Bloomington, MN). The anti-actin antibody, fetal bovine serum (FBS), Hoechst 33258, Annexin V-FITC apoptosis detection kit, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). The electrophoretic mobility shift assay (EMSA) kit was purchased from Promega Corp. (Madison, WI). FuGene 6 transfection reagent was from Roche (Basel, Switzerland). The HRP-conjugated donkey anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, enhanced chemi-luminescence (ECL) detection kit, and [$\gamma$-$^{32}$P]ATP were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The Coomassie protein assay kit and Restore™ Western Blot Stripping Buffer were purchased from Pierce (Rockford, IL). The oligonucleotide probes for EMSA analysis were synthesized by Bioneer (Daejeon, Korea).

*Cell culture*. MCF-7 and T47D human breast cancer cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 2 mM glutamine, and 100 U/ml penicillin and streptomycin at 37˚C in 5% $CO_2$. At the initiation of each experiment, cells were resuspended in the appropriate medium at a density of $2.5x10^5$ cells/ml. For hypoxic conditions, the cells were placed in airtight chambers (NuAire, Plymouth, MN) that were flushed with a 5% carbon dioxide/95% nitrogen mixture until the oxygen concentration was 2%.

*Cytotoxicity determined by MTT assay for cell viability*. Cell viability was assayed by measuring blue formazan that was metabolized from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial dehydrogenase, which is active only in live cells. One day before drug application, cells were seeded in 96-well flat-bottomed microtiter plates (3000-5000 cells/well). Cells were incubated for 24 h with various concentrations of momilactone B under normoxic or hypoxic condition. Twenty microliters of MTT (5 mg/ml) was added to each well and incubated for 4 h at 37˚C. The formazan product was dissolved by adding 200 $\mu$l dimethylsulfoxide (DMSO) to each well, and the plates were read at 550 nm. All measurements were performed in

triplicate, and each experiment was repeated at least three times.

*Detection of apoptosis*. For DNA fragmentation analysis, T47D cells were treated with normoxia, hypoxia, or hypoxia plus momilactone B. Treated cells were collected and resuspended in 0.5 ml cold lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% Triton X-100) for 30 min. Supernatants were treated with proteinase K (20 $\mu$g/ml) and RNase A (10 $\mu$g/ml), and DNA was extracted with phenol/chloroform and precipitated with absolute ethanol. The concentration of DNA was measured with a spectrophotometer, and 5 $\mu$g DNA was fractionated by electrophoresis on a 1.8% agarose gel containing ethidium bromide. For staining of apoptotic bodies by Hoechst 33258, T47D cells were cultured in RPMI-1640 complete medium on a chamber slide ($1x10^4$ cells/ml). After incubation, the culture medium was removed, and the cells were fixed by 4% formaldehyde for 20 min. The fixing solution was then removed, and cells were washed three times. Cells were stained by Hoechst 33258 stain solution (0.5 $\mu$g/ml in PBS) at room temperature for 30 min. After staining, the solution was removed, the cells were washed three times with PBS, and one drop of mounting solution was added before application of a cover slip. Apoptotic cells exhibited blue, peripherally clumped or fragmented chromatin by fluorescence microscopy.

*Apoptosis analysis by flow cytometry*. Fluorescein-conjugated Annexin V (Annexin V-FITC) was used to quantitatively determine the percentage of cells undergoing apoptosis. Treated cells were washed twice with cold PBS and then resuspended in binding buffer at a concentration of $1x10^6$ cells/ml. Five microliters of Annexin V-FITC and 10 $\mu$l of propidium iodide were added to suspended cells. After incubation for 15 min at room temperature in the dark, the percentage of apoptotic cells was analyzed by flow cytometry (Becton-Dickinson FACScan, San Jose, CA).

*Western blot analysis*. Cells were lysed in whole lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, and 1% Triton X-100) containing protease and phosphatase inhibitors (1 mM PMSF, 2 $\mu$g/ml leupeptin, 4 $\mu$g/ml aprotinin, and 1 $\mu$g /ml pepstatin), and protein concentrations were determined using the Coomassie protein assay (Bradford). An equivalent amount of protein extract from each sample was electrophoresed by 8-12% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked for 1 h with 5% non-fat milk in T-TBS buffer (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1X Tween-20) and incubated overnight at 4˚C with primary antibodies. Membranes were then washed three times in T-TBS and incubated with the corresponding secondary antibody, anti-mouse or -rabbit IgG HRP-conjugate (1:1000 dilution), in T-TBS with 5% non-fat milk for 1 h under agitation at room temperature. After washing three times in T-TBS, the membranes were developed by using the enhanced chemiluminescence (ECL) PLUS kit.

*Oligonucleotide labeling and electrophoretic mobility shift assay (EMSA)*. The GAS1 probe consisted of a synthetic double-stranded oligonucleotide corresponding to the sequence 5'-CGTGGAGTTCTTGGAAATGCGCC-3'. The double-stranded DNA probe was end-labeled using $T_4$ polynucleotide kinase and [$\gamma$-$^{32}$P]ATP. EMSAs were performed as described previously (8).

*RNA interference*. The siRNA constructs for STAT5b siRNA (pKD-STAT5b-v1) and negative control (pKD-NegCon-v1) were purchased from Upstate (Lake Placid, NY) (9). The siRNA target sequence is 21 nucleotides in length and was designed using a highly advanced search algorithm. The sequence contains perfect complementarity to the STAT5b gene target but contains minimal or no homology to other sequences within the genome. The siRNA target sequence was utilized to design the DNA oligonucleotides that were cloned into the pKD vector (Upstate). MCF-7 cells into 35-mm culture dishes were transfected with STAT5b siRNA (pKD-STAT5b-v1, 1 $\mu$g) or negative control (pKD-Negcon-v1, 1 $\mu$g) using the FuGene6 (Roche Applied Science) according to the manufacturer's recommendation.

*Caspase activity assay*. T47D cells under hypoxic conditions were treated with various concentrations of momilactone B for 24 h. The cells were harvested and washed twice with PBS buffer. Caspase activity was measured by the FLICA (Fluorochrome Inhibitor of Caspases) apoptosis detection kit according to the procedure described by the manufacturer. Briefly, cells were labeled with the green fluorescent-labeled inhibitor FAM-VAD-FMK for 1 h, washed, and analyzed. Caspase activity was detected using a BD FACScan flow cytometer.

## Results

*Cytotoxicity of momilactone B in human breast cancer cells*. To determine the effect of momilactone B on cell survival, a human breast cancer cell line (T47D) was exposed to various concentrations of momilactone B (25, 50, or 100 $\mu$M) for 24 h under hypoxic conditions. The number of momilactone B-treated cells in the logarithmic phase of growth was compared with that of control cells (hypoxia-treated cells). Cell growth was inhibited by ~35% with 25 $\mu$M momilactone B and by ~50% with 50 $\mu$M momilactone B (Fig. 2A). As compared with hypoxia or normoxia plus 50 $\mu$M momilactone B, hypoxia plus 50 $\mu$M momilactone B-treated cells resulted in a marked decrease in cell number after 12 h (Fig. 2B). On the other hand, normoxia plus 50 $\mu$M momilactone B-treated cells did not change. Thus, momilactone B treatment substantially decreased the viability of T47D cells in a dose- and time-dependent manner.

*Momilactone B promotes hypoxia-induced apoptosis in human breast cancer cells*. Human breast cancer cell death under hypoxic conditions was evaluated by several techniques to determine whether this death was due to apoptosis. Likewise, to determine if momilactone B-induced cytotoxicity is mediated through apoptosis, several assays were performed. DNA fragmentation analysis of hypoxia-plus momilactone B-treated T47D cells showed the laddering pattern characteristic of apoptosis (Fig. 3A). These results were confirmed by the observation of apoptotic bodies in hypoxia-treated

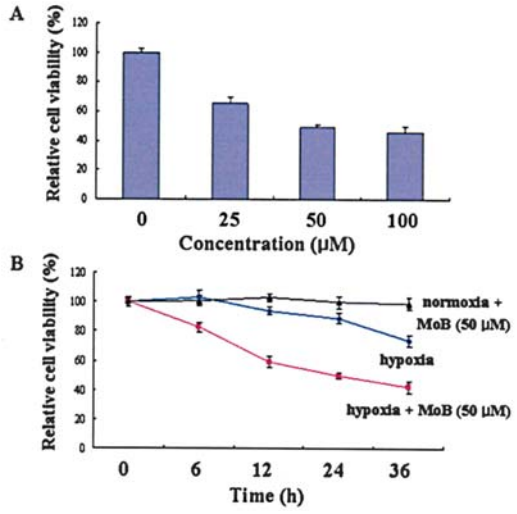
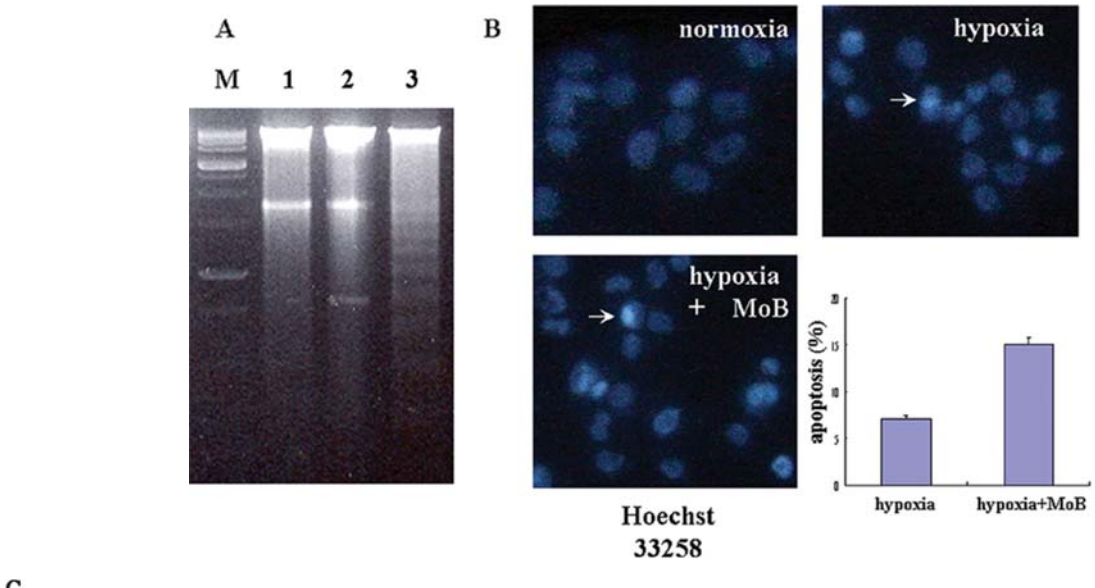



Figure 2. Effect of momilactone B on viability of T47D cells under hypoxic conditions. (A) Effect of momilactone B concentration on the relative number of T47D cells. Cells were cultured in 96-well dishes for 24 h and then treated with various concentrations of momilactone B for 24 h. (B) Time course effects of momilactone B on the growth of T47D cells. Cells were cultured and exposed to 50 $\mu$M momilactone B under normoxic or hypoxic condition for the indicated times. MoB, momilactone B. The error bars represent the standard deviation from three experiments.

T47D cells using Hoechst stain. Apoptotic cells exhibited blue, peripherally clumped or fragmented chromatin, as indicated by arrows in Fig. 3B. Apoptotic bodies were observed in T47D cells treated with 50 $\mu$M momilactone B under hypoxic conditions. When T47D cells were treated with hypoxia for 24 h, apoptosis was induced. We next investigated whether momilactone B promotes this hypoxia-induced apoptosis. After hypoxia treatment, the percentage of apoptotic cells increased to 15% by Annexin-PI staining, and this percentage was markedly increased to 34% when cells were treated with 50 $\mu$M momilactone B (Fig. 3C). These results indicate that momilactone B accelerates hypoxia-induced apoptosis in human breast cancer cells.

*Momilactone B inhibits the expression of hypoxia-related proteins in human breast cancer cells*. In our previous studies, we found that hypoxia activates the cyclin D1 promoter via the Jak2/STAT5b pathway in breast cancer cells. To determine the effect of momilactone B on the Jak2/STAT5b pathway, the levels of various proteins regulated by hypoxia in human breast cancer cells were examined. MCF-7 cells were treated with 0, 25, 50, and 100 $\mu$M momilactone B under hypoxic conditions (2% $O_2$) for 24 h. As shown in



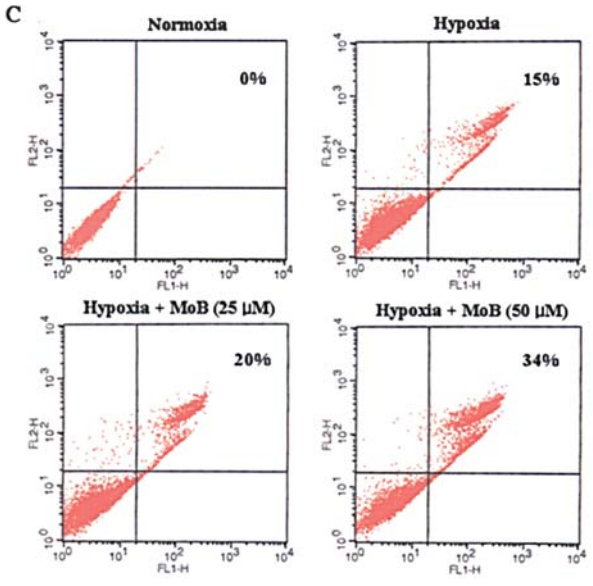




Figure 3. Hypoxia induces apoptosis, and momilactone B promotes hypoxia-induced apoptosis in T47D cells. (A) DNA was isolated, and ladder formation was examined by 1.8% agarose gel electrophoresis. Lane M, DNA ladder; lane 1, normoxia; lane 2, hypoxia; lane 3, hypoxia plus momilactone B. (B) T47D cells were treated with normoxia, hypoxia, or hypoxia plus momilactone B (50 $\mu$M) before Hoechst staining and analysis of nuclei. Apoptotic cells exhibited blue, peripherally clumped or fragmented chromatin, as indicated by the arrows. MoB, momilactone B. Data are representative of three independent experiments. The error bars represent the standard deviation from three experiments. (C) T47D cells were treated with normoxia (control), hypoxia, or hypoxia plus 50 $\mu$M momilactone B. Treated cells were analyzed by flow cytometry using Annexin V-FITC as described in Materials and methods, and the percentage of cells in apoptosis was determined. The x-axis represents the fluorescence intensity of Annexin V-FITC, and the y-axis represents the intensity of propidium iodide. MoB, momilactone B. Data are representative of three independent experiments.

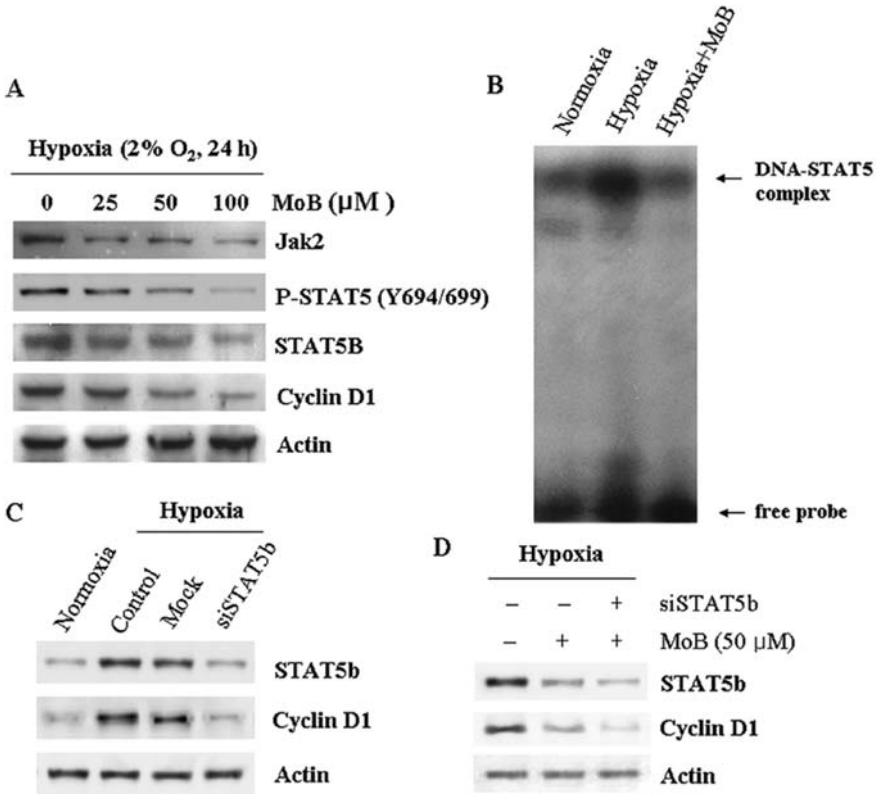



Figure 4. Effects of momilactone B on Jak2/STAT5b pathway-related proteins activated by hypoxia. (A) Momilactone B inhibits expression of the Jak2/STAT5b pathway-related proteins regulated by hypoxia. MCF-7 cells were treated with momilactone B concentration (0, 25, 50, and 100 $\mu$M) under hypoxic condition (2% $O_2$) for 24 h. Protein extracts (20 $\mu$g) were separated by 12% SDS-PAGE, and Western blot analyses were performed as described in Materials and methods. ß-actin was used as a control for protein loading. (B) Momilactone B suppresses the hypoxia-induced binding of STAT5 to the cyclin D1-GAS1 site. MCF-7 cells were treated with normoxia (control), hypoxia, or hypoxia plus 50 $\mu$M momilactone B, and STAT5 DNA binding was detected by EMSA. Nuclear extracts were incubated with [$^{32}$P]-labeled probe specific for the GAS1 site. The resulting complexes were electrophoresed on a 6% non-denaturing gel. MoB, momilactone B. Shown is a representative gel from three independent experiments. (C) MCF-7 cells were treated with mock (pKD-NegCon) or siSTAT5b (pKD-siSTAT5b) under hypoxic conditions. Cells were harvested after 24 h for assay of STAT5b, cyclin D1 and ß-actin by immunoblotting. (D) MCF-7 cells were treated with 50 $\mu$M momilactone B or 50 $\mu$M momilactone B with transfected siSTAT5b (pKD-siSTAT5b) under hypoxic conditions. Cells were harvested after 24 h for assay of STAT5b, cyclin D1 and ß-actin by immunoblotting.

Fig. 4A, the protein levels of Jak2, phospho-STAT5, STAT5b, and cyclin D1, which are increased by hypoxia, were dose-dependently down-regulated by momilactone B. These results indicate that the growth inhibitor momilactone B regulates the cyclin D1 promoter via the Jak2/STAT5b pathway in hypoxic human breast cancer cells.

*Momilactone B suppresses the hypoxia-induced binding of STAT5 to the cyclin D1-GAS1 site*. Activated STATs form dimers, translocate to the nucleus, bind to specific response elements in the promoters of target genes, and transcriptionally activate these genes. Under hypoxic conditions, STAT5b recognizes the GAS sequence in the promoter of cyclin D1. Hypoxia increases STAT5b DNA-binding activity in human breast cancer cells (2). To test the hypothesis that momilactone B inhibits STAT5b, we examined the binding of STAT5b to the GAS1 promoter under hypoxic conditions. As shown in Fig. 4B, MCF-7 cells were treated with normoxia (control), hypoxia, or hypoxia plus 50 $\mu$M momilactone B. The results show that momilactone B suppressed hypoxia-induced STAT5 DNA-binding activity in human breast cancer cells.

*The increase of cyclin D1 expression by hypoxia and reduction of cyclin D1 expression by STAT5b knock-down*. STAT5b protein levels were monitored by Western blotting from negative control and STAT5b knock-down cells 24 h following siRNA transfection. As a control, ß-actin expression was shown to be unaffected following STAT5b knock-down at the protein level. Treatment of MCF-7 cells with siRNA-STAT5b resulted in efficient and specific inhibition of STAT5b transcripts. And, the siRNA-STAT5b reduced high significantly the cyclin D1 expression in MCF-7 cells (Fig. 4C). Thus, cyclin D1 is a target gene of hypoxia signal transduction and the regulation in cyclin D1 protein level is likely to be the result of hypoxia activation of STAT5b. In addition, the expression of STAT5b and cyclin D1 by treatment of 50 $\mu$M momilactone B with siRNA-STAT5b were more reduced than by treatment of only 50 $\mu$M momilactone B without siRNA-STAT5b under hypoxic condition of MCF-7 cells (Fig. 4D).

*Momilactone B affects the expression of apoptosis-related proteins in human breast cancer cells under hypoxic conditions*. Immunoblot analyses were performed to determine the levels of different proteins controlling cell proliferation and/or apoptosis in MCF-7 and T47D cells (Fig. 5), focusing on the expression of bcl-2 and bax proteins. Bcl-2 and bax are homologous proteins that have opposing effects on cell life and death, with bcl-2 serving to prolong cell survival and

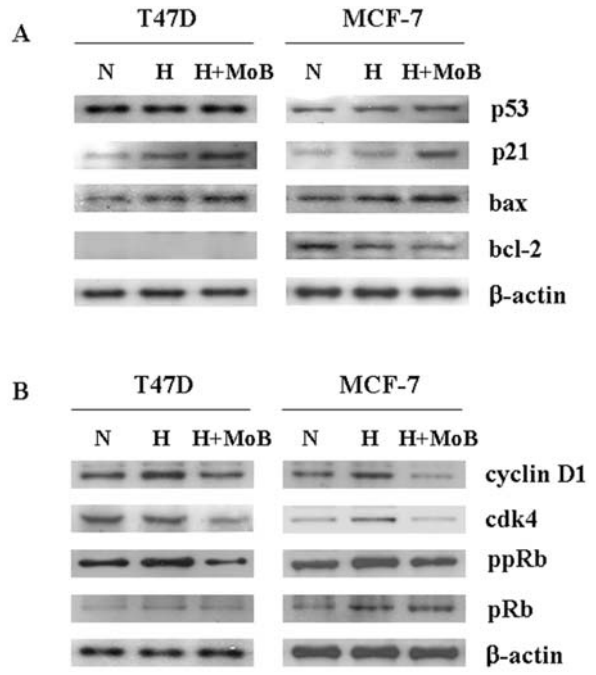



Figure 5. Momilactone B affects the expression of apoptosis-related proteins in MCF-7 and T47D cells under hypoxic conditions. Hypoxia treated cells were treated with momilactone B (50 $\mu$M) for different periods of time. The protein extracts (20 $\mu$g) were separated by 12% SDS-PAGE, and Western blot analyses were performed as described in Materials and methods. ß-actin was used as a control for protein loading. N, normoxia; H, hypoxia; MoB, momi-lactone B. Shown are representative blots from three independent experiments.

bax acting as an accelerator of apoptosis. Bcl-2 expression was slightly down-regulated by the addition of 50 $\mu$M momilactone B when cells were exposed to hypoxic conditions for 24 h in only MCF-7 cells. In contrast, the expression of bax was up-regulated by momilactone B treatment (Fig. 5A). Phosphorylation of pRb is required for cell cycle progression, and changes in the phosphorylation status of pRb are observed with apoptosis. Therefore, the phosphorylation status of pRb in T47D and MCF-7 cells was examined following treatment with 50 $\mu$M momilactone B under hypoxic conditions. T47D cells showed a dramatic loss of hyperphosphorylated forms of pRb with momilactone B treatment, whereas no detectable change in pRb phosphorylation was observed in MCF-7 cells (Fig. 5B). The loss of the hyperphosphorylated forms of pRb coincided with the induction of apoptosis.

*Momilactone B induces caspase-dependent cell death of human breast cancer cells under hypoxic conditions*. As shown in Fig. 3, momilactone B induced apoptosis under hypoxic conditions. To determine whether caspases are involved in this hypoxia-induced apoptosis, caspase activity in human breast cancer cells was analyzed using a potent caspase inhibitor, FAM-VAD-FMK. Compared with control (normoxia) cells, caspase activity increased by 16% in hypoxia-treated cells, and this percentage dose-dependently increased to 33% when cells were treated with 50 $\mu$M momilactone B under hypoxic conditions (Fig. 6A). Hypoxia-induced apoptosis was evaluated further by measurement

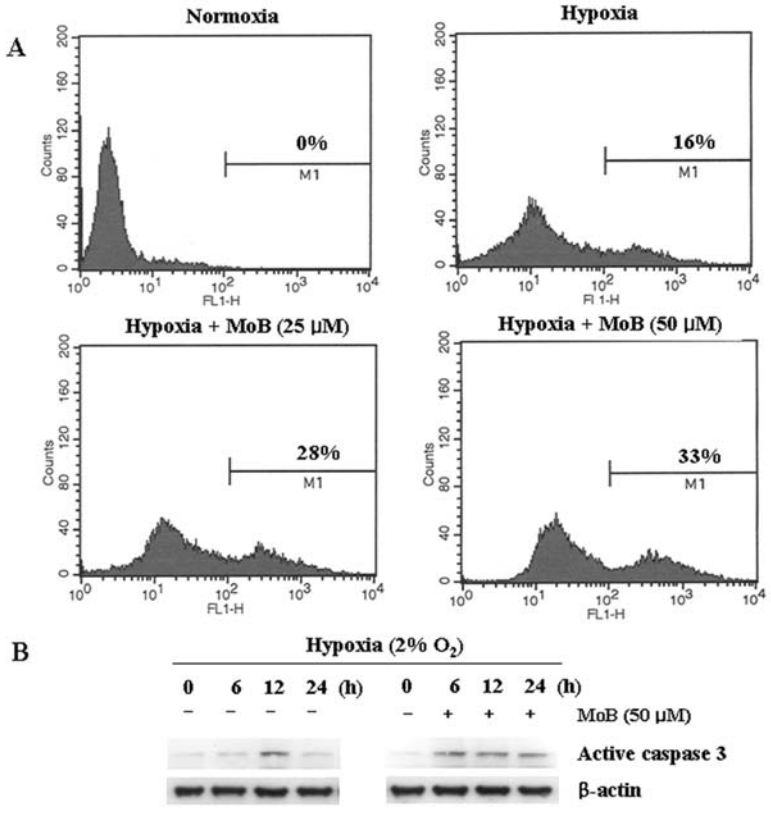



Figure 6. Momilactone B induces caspase-dependent cell death of breast cancer cells under hypoxic conditions. (A) T47D cells were treated with normoxia (control), hypoxia, or hypoxia plus 50 $\mu$M momilactone B. Treated cells were labeled with the green fluorescent-labeled inhibitor FAM-VAD-FMK for 1 h, washed, and analyzed. Caspase activity was detected using a BD FACScan flow cytometer. (B) Whole cell lysates were prepared from MCF-7 and T47D cells. A total of 20 $\mu$g cell lysate was separated on 12% SDS-PAGE, transferred to nitrocellulose, and probed with cleaved caspase-3 antibody. Then, the blot was stripped and reprobed with the anti-actin antibody. Caspase-3 was not detected in MCF-7 cells (not shown). Data are representative of three independent experiments.

of the cysteine protease caspase-3, which is essential for apoptotic death in mammalian cells. It was established previously that MCF-7 cells do not express a functional caspase-3. Consistent with those results, we did not detect caspase-3 expression in MCF-7 cells, but a 17-kDa band likely corresponding to active caspase-3 was detected in the T47D cell line. The caspase-3-like activity was markedly increased 12 h after hypoxia treatment (Fig. 6B). Treatment with 50 $\mu$M momilactone B under hypoxic conditions for 6 h caused an even greater increase in caspase-3-like activity. These results indicate that apoptosis induced by momilactone B under hypoxic conditions is dependent upon caspase activation.

## Discussion

The present study demonstrates that momilactone B accelerates hypoxia-induced apoptosis in human breast cancer cells through STAT5b. When STAT5b was inhibited by momilactone B in human breast cancer cells under hypoxic conditions, the levels of cyclin D1, CDK4, phospho-pRb, caspase-3, and bcl-2 (only in MCF-7 cells) were markedly reduced, and the levels of bax and p21 were increased. Momilactone B suppressed STAT5 DNA-binding activity induced by hypoxia in human breast cancer cells. siRNA-STAT5b assay showed the ability to knock-down at >70% of the cyclin D1 expression by immunoblotting. Thus, the inhibition of STAT5 DNA binding activity is likely to be through STAT5b inhibition that momilactone B induces apoptosis. These results support the feasibility of utilizing momilactone B to induce apoptosis in certain STAT5-activated human breast cancers.

Several constitutively activated STAT proteins have been observed to contribute directly to oncogenesis by stimulating cell proliferation and preventing apoptosis in various cancers (24). STAT5 participates in oncogenesis by up-regulating genes encoding apoptosis inhibitors and cell cycle regulators, including bcl-xL, cyclin D1, and p21 (13,25). In addition, cyclin D1 has been shown to be regulated by STAT5b (26). Moreover, we have shown previously that STAT5b may mediate transcriptional activation of cyclin D1 after hypoxic stimulation in human breast cancer cells (2) pRb phosphorylation is a critical step in the G1-to-S phase transition. Phosphorylation of pRb is controlled primarily by complexes of D cyclins associated with cdk4/6 and later by cyclin E associated with cdk2 (27,28). The present study shows that momilactone B's inhibitory effect on cell cycle progression under hypoxic conditions is due to rapid down-regulation of cyclin D1 and cdk4 expression, which in turn decreases pRb hyperphosphorylation. Treatment of human breast cancer cells with momilactone B under hypoxic conditions led to reduced pRb phosphorylation on serine 795, a site that is known to be phosphorylated by the cyclin D1/cdk4 complex.

Cell death due to hypoxia is a major concern in various clinical entities such as ischemic diseases. Until recently, cell death by hypoxia was generally thought to be manifested as necrosis (29). In contrast, recent biochemical observations have suggested the possibility of hypoxia-induced apoptosis. Hypoxia treatment induces endothelial cell death through the induction of apoptosis, consistent with previous findings (30,31). This study has shown that hypoxia induces apoptosis in human breast cancer cells, and the apoptosis is promoted by momilactone B.

Rice hulls, from which momilactone B is extracted, have recently been reported to contain antioxidant substances that protect the rice seed from oxidative stress (17). As a potential chemopreventive or therapeutic agent against breast cancer, momilactone B has several advantages. The rice hull comprises 20% of the rice grain kernel and, similar to other plant biomass, contains a high percentage of organic substances. It is therefore recognized as a potential source of energy and organic chemicals (21).

Momilactone B in combination with hypoxia stimulation showed a cytotoxic effect in cultured human breast cancer cells, and flow cytometry analysis using Annexin V-PI staining implicated apoptosis as the cell death mechanism. In addition, observations of DNA laddering and apoptotic bodies confirmed that momilactone B induces apoptosis under hypoxic conditions. The activation of caspases is a key regulator of apoptosis. Tumor cell apoptosis was associated with caspase protein expression and caspase-3 activity. The pro-caspase protein was in its inactive form when the cell was in the resting state, and the protein expression was decreasing to convert to the active form of caspase-3 in the process of apoptosis (32,33). Caspase-3 activation is known to occur under hypoxic conditions in various cells, including cardiomyocytes (34). In the present study, caspase activity and active caspase-3 protein expression were increased by momilactone B treatment under hypoxic conditions, demonstrating that momilactone B caused cell death through caspase-3-dependent apoptosis. Moreover, we explored factors upstream of the caspase pathway. During apoptosis, the intra-cytosolic balance of members of the antiapoptotic bcl-2 family of proteins is critical for maintaining the integrity of the mitochondrial membrane (35,36). Bcl-2 heterodimerizes *in vivo* with its conserved, proapoptotic homolog bax, which is also known to prevent bax oligomerization and its insertion into the mitochondrial membrane (37,38). Immunoblot analyses demonstrated that exposure of hypoxic cells to momilactone B increased the level of bax and reduced the level of bcl-2 (only in MCF-7 cells).

The cyclin-dependent kinase inhibitor p21 was reported to be a STAT5-regulated gene in the p53-deficient CMK human megakaryoblastic leukemia cell line. p21 is induced by both p53-dependent and p53-independent mechanisms following stress, and induction of p21 may cause cell cycle arrest (39). Our data showed that the inhibition of STAT5b by momilactone B resulted in marked expression of p21 in T47D and MCF-7 cells. However, the level of p53 protein did not change (expressed as mutant form in T47D and as wild-type in MCF-7 cells). Thus, momilactone B-induced apoptosis under hypoxic conditions occurred independently of the p53 protein level in human breast cancer cells.

This study showed that hypoxia-induced increases in STAT5b and cyclin D1 were down-regulated by momilactone B. In human breast cancer cells under hypoxic conditions, growth inhibitory and apoptotic processes, activation of caspase-3 activity, apoptotic body formation, and increased apoptosis were characteristics of the cell death effect induced by momilactone B. The apoptosis pathway of human breast cancer cells induced by momilactone B under

hypoxic conditions was mediated by STAT5b and caspase-3 but not by p53. Thus, momilactone B may be potentially effective chemopreventive or therapeutic agent for breast cancer. However, further *in vivo* studies are needed to establish the mechanism of momilactone B as an anti-breast cancer agent.

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