Obtusilactone B from Machilus Thunbergii Targets Barrier-to-Autointegration Factor to Treat Cancer


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ABSTRACT

Targeting specific molecules is a promising cancer treatment because certain types of cancer cells are dependent on specific oncogenes. This strategy led to the development of therapeutics that use monoclonal antibodies or small-molecule inhibitors. However, the continued development of novel molecular targeting inhibitors is required to target the various oncogenes associated with the diverse types and stages of cancer. Obtusilactone B is a butanolide derivative purified from Machilus thunbergii. In this study, we show that obtusilactone B functions as a small-molecule inhibitor that causes abnormal nuclear envelope dynamics and inhibits growth by suppressing vaccinia-related kinase 1 (VRK1)–mediated phosphorylation of barrier-to-autointegration factor (BAF). BAF is important in maintaining lamin integrity, which is closely associated with diseases that include cancer. Specific binding of obtusilactone B to BAF suppressed VRK1-mediated BAF phosphorylation and the subsequent dissociation of the nuclear envelope from DNA that allows cells to progress through the cell cycle. Obtusilactone B potently induced tumor cell death in vitro, indicating that specific targeting of BAF to block cell cycle progression can be an effective anticancer strategy. Our results demonstrate that targeting a major constituent of the nuclear envelope may be a novel and promising alternative approach to cancer treatment.

Introduction

Targeted cancer therapy and personalized cancer therapeutics are aimed at a particular protein, or even its polymorphic subtypes, rather than common cellular factors shared by all proliferating cell types. This strategy is now anticipated to be effective against many kinds of tumors. In light of targeted cancer therapy concepts, determining the appropriate candidate oncogene to target with small molecules or monoclonal antibodies is very important. To date, most drugs in this category have been developed against oncogenic receptor tyrosine kinases or mitotic cellular kinases.

Because the rapid proliferation associated with the mutation of various growth factor receptors is an obvious cause of cancer, much effort has been spent developing drugs against these receptors. However, cell cycle–related proteins have recently drawn great attention from many researchers.

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ABBREVIATIONS: BAF, barrier-to-autointegration factor; EGFP, enhanced green fluorescent protein; EI-MS, electron ionization mass spectrometry; GST, glutathione S-transferase; IR, infrared; MTH, Machilus thunbergii Hexane fractions; SPR, surface plasmon resonance; VRK1, vaccinia-related kinase 1.
be disrupted to allow the cell to cycle from G₀ into the S phase and into the subsequent G₂/M phase. Once the link is disrupted, VRK1 phosphorylates BAF (Nichols et al., 2006; Lancaster et al., 2007), and the phosphorylated BAF releases DNA from the nuclear envelope; this release is followed by DNA synthesis, transcription factor binding, and DNA condensation (Haraguchi et al., 2007; Lancaster et al., 2007; Margalit et al., 2007). Furthermore, VRK1 is also involved in the G₁/S transition and the entry into mitosis (Kang et al., 2008; Lopez-Sanchez et al., 2009; Valbuena et al., 2008). Because of their prominent roles in tumorigenesis, we proposed the oncogene VRK1 and its cognate substrate BAF as novel targets of molecular medicine, and thus initiated a search for small-molecule inhibitors of VRK1 and BAF.

We acquired a natural herb library in which to search for small-molecule inhibitors of VRK1. This system has certain merits compared with synthetic or inorganic libraries. Natural products have played a significant role in drug discovery and specifically in the development of new anticancer agents; more than 79.8% of the anticancer drugs introduced from 1981 to 2008 were natural products, semisynthetic analogs, or synthetic compounds based on natural-product pharmacophores (Cragg et al., 2009).

To find inhibitory molecules of VRK1, we performed a high through-put screening of compounds derived from Machilus thunbergii Sieb. et Zucc. (Lauraceae), a deciduous tree of Korea and Japan. M. thunbergii has long been used as a traditional medicine in Korea, China, and Japan to treat various diseases, including edema, abdominal pain, and abdominal distension. Next, we characterized four butanolide derivatives whose hexane fractions prominently inhibited the phosphorylation of BAF by VRK1. Further characterization by surface plasmon resonance (SPR) detection and nuclear magnetic resonance titration of the most promising VRK1 inhibitor, obtusilactone B, demonstrated that the inhibitor specifically bound the substrate BAF. This interaction suppressed the phosphorylation of BAF by VRK1, thus arresting the cell cycle by causing defective mitotic nuclear envelope dynamics. We also showed preferential cytotoxicity of obtusilactone B toward tumor cells via cell viability assays.

Materials and Methods

NMR Titration of Recombinant BAF with Inhibitors

Binding modes of the small-molecule BAF inhibitors were examined by studying the two-dimensional 1H-15N heteronuclear single-quantum correlation spectroscopy spectrum of recombinant BAF protein before and after addition of the inhibitors. For this, the recombinant BAF protein was uniformly labeled using 15N-ammonium chloride and sodium bicarbonate (Bradley et al., 2005) and was used for titration studies using 1H-15N correlation spectrum of recombinant BAF protein before and after the addition of the ligand. A plot of chemical shifts versus the amino acids was studied according to the previously described method (Chakraborty et al., 2012).

Protein Ligand Docking Studies

The Genetic Optimization for Ligand Docking (GOLD) 5.0 (Cambridge Crystallographic Data Centre, Cambridge, UK) program (Verdonk et al., 2003) was used for docking studies. The crystal-structure BAF protein in complex with DNA (2BZF) (Bradley et al., 2005) was prepared for docking studies by deleting the heteroatoms and assigning the charges and potentials by Chemistry at Harvard Macromolecular Mechanics force field and energy minimized with a conjugate gradients algorithm in Discovery Studio 3.1 (Accelrys, San Diego, CA). The ligand compounds 1 and 3 were built with a three-dimensional sketcher, and energy was minimized with a smart minimizer algorithm module in Discovery Studio 3.1; thus, minimized molecules were used for docking studies. An active site radius of 10.0 Å around the DNA-binding region of BAF was used to define the active site point, and default docking parameters were used to dock compounds 1 and 3 into the DNA-binding site of BAF.

Plant Material and Purified Compound Identification

The stems of Machilus thunbergii were purchased from an oriental drug store in Pohang, Gyeongbuk, Korea, in July 2009. Plant material was identified by Yong-Ki Park (Department of Herballogy, College of Oriental Medicine, Dongguk University, Gyeongju, Korea. A voucher specimen (MT2009-01) is reserved at the Laboratory of Molecular Neurophysiology, POSTECH, Pohang, Korea. Optical rotation was measured on a JASCO P-1020 polarimeter (PerkinElmer, Waltham, MA). The infrared (IR) spectrum was obtained with a PerkinElmer Spectrum GX FT-IR spectrometer. Electron ionization mass spectroscopy (EI-MS) spectra were obtained with a WATERS Micromass Platform II mass spectrometer (Waters Corp., Milford, MA) or Agilent 7890A-5975C GC/MSD (Agilent Technologies, Santa Clara, CA). H NMR, 13C NMR, DEPT, COSY, HMBC, and HMQC NMR spectra were recorded on a Bruker DRX-500 FT-NMR spectrometer and a Bruker Avance 700 spectrometer. Chloroform-d, with metamethoxyisilane as the internal standard was purchased from Sigma-Aldrich (St. Louis, MO). Merck Silica Gel 60 (∼230–400 μm) and LiChrospher RP-18 (∼40–63 μm) (Darmstadt, Germany) were used for column chromatography. Analytical thin layer chromatography was performed on Merck Silica Gel 60 F-254 and Silica Gel 60 RP-18 F-254 thin layer chromatography plates. Spots were detected by UV light (254 and 365 nm), spraying with 10% H₂SO₄, and then heating on a hot plate.

NMR Characterization of Natural Compounds

Compounds 1 to 4 were characterized by comparison of their spectroscopic data, including NMR, MS, and IR, with literature values (Masatake Niwa, 1975; Hiroyuki Karikome, 1991; Anderson et al., 1992; Ruo et al., 2008).

Obtusilactone B (Compound 1). Compound 1, a yellowish oil, was composed of the following: IR (KBr, cm⁻¹): 3575, 3000, 1790, 1685; EI-MS m/z: 335 [M+H]+, 344 [M]+, 317 [M+H-O₂]⁺, 291 [M-43]⁻, 179, 177, 140, 135, 126, 123, 109, 97, 85, 83, 79, 70, 69, 67, 57, 55, 43, 41; 1H NMR (500 MHz, CDCl₃, δ): 6.69 (1H, td, J = 8.0, 2.0 Hz, H-1'), 5.35 (2H, m, H-7', 8'), 5.12 (1H, br s, H-4), 4.89 (1H, dd, J = 3.5, 2.0 Hz, H-6b), 4.68 (1H, dd, J = 1.0, 1.0 Hz, H-6a), 2.78 (2H, m, H-2'), 2.03 (2H, m, H-6'), 2.03 (2H, m, H-9'), 1.50 (2H, q, J = 7.0 Hz, H-3'), 0.90 (3H, br t, J = 7.0 Hz, H-16'); 13C NMR (125 MHz, CDCl₃, δ): 165.3 (C-2'), 152.7 (C-9'), 151.2 (C-1'), 130.2 (C-8'), 129.4 (C-7'), 126.9 (C-3), 90.2 (C-6), 68.8 (C-4), 31.9 (C-14), 29.8–28.9 (C-4', 5', 10', 13'), 28.6 (C-2'), 28.3 (C-9'), 27.2 (C-9'), 27.0 (C-6'), 26.7 (C-15'), 14.1 (C-16').

Secosubamolide A (Compound 2). Compound 2, a pale yellowish liquid, was composed of the following: IR (KBr, cm⁻¹): 3450, 1735, 1710; EI-MS m/z: 389 [M+H]+, 351 [M+H-O₂]⁺, 325 [M-43]⁻, 283, 265, 247, 191, 149, 125, 115, 97, 83, 69, 55; 1H NMR (500 MHz, CDCl₃, δ): 7.09 (1H, t, J = 7.5 Hz, H-3), 4.92 (1H, br s, H-1'), 3.75 (3H, s, OMe), 2.38 (2H, q, J = 7.5 Hz, H-4), 2.17 (3H, s, H-9'), 1.53 (2H, q, J = 7.5 Hz, H-5), 0.90 (3H, br t, J = 7.0 Hz, H-18'); 13C NMR (125 MHz, CDCl₃, δ): 206.3 (C-2'), 166.5 (C-1), 149.1 (C-3), 129.7 (C-2), 73.4 (C-1'), 52.0 (OMe), 31.9 (C-16), 29.2–29.3 (C-6'), 28.7 (C-5), 28.7 (C-4'), 24.8 (C-3'), 22.7 (C-17), 14.1 (C-18).
2-Octadecylidene-3-Hydroxy-4-Methylenebutanolide (Compound 3). Compound 3, a solid, was composed of the following: IR (KBr, cm⁻¹): 3584, 3350, 3020, 2935, 2860, 1780, 1700, 1680, 1665, 1465, 1270, 1205, 1145, 1060, 1015, 830, 815, 751, 739; EI-MS m/z: 356 [M+H]+, 341 [M+H-O]+, 321 [M+3-H]+, 140, 135, 126, 123, 109, 97, 95, 83, 81, 79, 70, 69, 67, 57, 55, 43, 41; 1H NMR (500 MHz, CDCl₃, δ): 7.05 (1H, td, J = 8.0, 2.0 Hz, H-1'), 5.22 (1H, brd, H-4'), 4.9 (1H, dd, J = 3.0, 2.0 Hz, H-6b), 4.70 (1H, dd, J = 3.0, 1.0 Hz, H-6a), 2.45 (2H, m, H-2'), 1.52 (2H, qu, J = 7.5 Hz, H-3'), 0.89 (3H, br t, J = 7.0 Hz, H-18'). 13C NMR (125 MHz, CDCl₃, δ): 166.8 (C-2'), 157.7 (C-5'), 150.1 (C-1'), 127.4 (C-3), 91.2 (C-6), 66.4 (C-4'), 31.9 (C-16'), 29.7~29.4 (C-2'-15'), 28.3 (C-3'), 22.7 (C-17'), 14.1 (C-18').

Linderanolide (Compound 4). Compound 4, crystals, was composed of the following: IR (KBr, cm⁻¹): 3395, 2920, 2850, 1779, 1770, 1671, 1429, 1033, 948, 863, 718, 691, 577, 365 [M+H]+, 336 [M]+, 319 [M+H-O]+, 293 [M+3-H]+, 140, 135, 126, 123, 109, 97, 95, 83, 81, 79, 70, 69, 67, 57, 55, 43, 41; 13C NMR (125 MHz, CDCl₃, δ): 152.0 (C-2'), 157.6 (C-5'), 151.3 (C-1'), 126.8 (C-3'), 90.3 (C-6'), 68.9 (C-4'), 31.9 (C-16'), 29.7~29.3 (C-4'-13'), 28.7 (C-2'), 28.3 (C-3'), 22.7 (C-15'), 14.1 (C-16').

Extraction and Isolation

The dried stems of Machilus thunbergii (20 kg) were extracted with n-hexane (40 L × 2) at room temperature. The extract was obtained by concentration under reduced pressure. The n-hexane extract (50 g from a total of 151 g) was chromatographed over silica gel (70~230 mesh, 7.5 × 12 cm) and eluted with a gradient of n-hexane-Ethyl Acetate (6:1, 4:1, 2:1, and 1:1) and acetone to produce 11 fractions (MTH5–MTH11). Fraction MTH5–6 (5.35 g) was subjected to a silica gel column chromatography (4.5 × 15 cm) and eluted with n-hexane-ethanol (7:1) to obtain 11 fractions (MTH5–6–1–MTH5–6–11). Fraction MTH5–6–4–5 (1.75 g) was purified by an octadecyl silica gel column chromatography (4 × 5 cm) and eluted with MeOH-H₂O (13:1) to ultimately produce compound 3 (80.0 mg). Fraction MTH7–8–1 (1.7 g) was subjected to a silica gel column chromatography (4.5 × 15 cm) and eluted with CHCl₃-acetone (100:1) to obtain 11 fractions (MTH7–8–1–MTH7–8–11). Fraction MTH7–8–4–3 (1.3 g) was applied to an octadecyl silica gel column chromatography (4 × 6 cm) and eluted with MeOH-H₂O (9:1, 10:1, and 12:1) to produce compound 1 (50 mg), compound 2 (31 mg), and compound 4 (35 mg).

Cell Culture, Transfection, and Flow Cytometry

A549 cells were cultured in an RPMI 1640 medium (Welgene, Daegu, Korea) containing 10% fetal bovine serum (HyClone Laboratories, Logan, UT) and 1% penicillin/streptomycin (Welgene) in a humidified 5% CO₂ incubator at 37°C. Transient transfection was performed using 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (Choi et al., 2008). For red fluorescent protein-tagged VRK1, DNA fragment coding for VRK1 was cloned into pDsRed-Monomer-N1 (Clontech Laboratories). In vitro kinase assay between recombinant VRK1 and BAF was performed as previously described (Kim et al., 2012). Antibody against lamin A/C (N-18) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Surface Plasma Resonance

The interaction between the BAF and obtusilactone B was analyzed by surface plasma resonance (SPR), using the Reichert SR7500DC system (Reichert Technologies, Depew, NY) and data collection software (SPR Autolink v1.1.8G; Reichert Technologies). BAF protein, the ligand, was covalently bound to a carboxymethyl dextran sensor chip (gold slide) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl and N-hydroxysuccinimide. The ligand was bound at densities of about 2400 resonance units (RU), where 1 RU = 1 pg/mm² on the sensor surface. Obtusilactone B and 2-octadecylidene-3-hydroxy-4-methylenebutanolide, the analytes, were injected over the sensor surface at the indicated concentrations diluted in 10 mM phosphate-buffered saline containing 1% dimethyl sulfoxide. RUs were recorded in real time before and during small-molecule injection (1 minute) and during washout (2 minute). Analysis of steady-state affinity was performed using Scrubber2 software (Biologic Software, Campbell, ACT, Australia) by selecting reference-subtracted curves. Recorded responses that included disturbances produced by air bubbles, aggregation, or precipitation of analytes were discarded. In the global approach, all of the samples, including both association and dissociation steps, were fit at the same time using a sum of squared residuals over every data point.

Dye Conjugation

Obtusilactone B was covalently conjugated with a red fluorescent dye, FPR552 (Bioacts, Incheon, Korea; λabs 551 nm, λem 570 nm). Ethanol-solubilized obtusilactone B and FPR552 were reacted in dimethylformamide, and then 0.1 M phosphate buffer (pH 12.0) was added. After 4 hours of incubation at room temperature, the FPR552-obtusilactone B conjugates were lyophilized and purified under an Agilent 1100 series high-pressure liquid chromatography system using a Jupiter 10u C18 300A (Phenomenex, Torrance, CA) 250 × 10 mm column.

Cell Viability Assay

Cell viability was assessed by measuring the activity of metabolizing 3-(4,5-dimethylisazol-2-yl)-2,5-diphenyltetrazolium bromide (Choi et al., 2008). A total of 1.0 × 10⁴ cells were seeded onto a 96-well plate and cultured up to a confluency of 90%. After treating the indicated concentration of inhibitors for 24 hours, cells were treated with 3-(4,5-dimethylisazol-2-yl)-2,5-diphenyltetrazolium bromide solution and cell viability was assessed.

Results

Small-Molecule Inhibitors of BAF Phosphorylation.

To find small-molecule inhibitors of VRK1, we analyzed hundreds of natural herb extracts prepared by extraction of Korean traditional herbs in 80% methanol (Supplemental Fig. 1). Among them, the crude extract of M. thunbergii substantially inhibited the autophosphorylation of VRK1. We next conducted in vitro kinase assays with M. thunbergii to find inhibitory molecules against VRK1 action by performing solvent-based extraction and purifying active subfractions (Fig. 1A). Certain fractions derived from hexane and butyl alcohol extraction completely inhibited the catalytic activity.
of VRK1 toward BAF. We further fractionated the hexane extract to purify the bioactive inhibitor molecule (Fig. 1B). We successfully isolated and characterized four bioactive butanolides: obtusilactone B (compound 1), secosubamolide A (compound 2), 2-octadecylidene-3-hydroxy-4-methylenebutanolide (compound 3), and linderanolide (compound 4). This study describes the first isolation of compounds 1, 2, and 4 from M. thunbergii (Fig. 1C).

Obtusilactone B Suppresses VRK1–Mediated Phosphorylation of BAF. VRK1 is a cell cycle–regulating protein kinase and acts on several related substrates that include p53, histone H3, BAF, and cAMP-response element binding protein (Nichols et al., 2006; Kang et al., 2007, 2008; Valbuena et al., 2008). Because BAF is one of the most important substrates of VRK during mitosis, during which BAF phosphorylation is required for DNA condensation and mitotic progression (Vlcek et al., 2001; Suzuki et al., 2010), we primarily analyzed whether our novel butanolide derivatives suppressed the enzymatic reaction of VRK1 toward BAF (Fig. 2A). Although two derivatives inhibited BAF phosphorylation, inhibition by obtusilactone B (compound 1) was the most pronounced. Next, we serially diluted obtusilactone B to estimate its median inhibitory concentration (Fig. 2B) and found that obtusilactone B strongly inhibited BAF phosphorylation by VRK1 in vitro, with a median inhibitory concentration of 0.8 μM. Interestingly, obtusilactone B inhibited VRK1 autophosphorylation very little, indicating that, rather than occupying the ATP-binding domain or another conserved domain, as many kinase inhibitors do, obtusilactone B may inhibit the interaction between VRK1 and BAF. We then tested other VRK1 substrates to confirm the inhibitory activity observed. To our surprise, obtusilactone B did not inhibit the catalytic activity of VRK1 toward histone H3 or casein peptide (Fig. 2, D and E), even though it inhibited BAF phosphorylation (Fig. 2C); this observation suggests that obtusilactone B specifically inhibits the interaction between VRK1 and BAF.

Obtusilactone B Specifically Interacts with BAF. To understand the molecular basis of the interaction between obtusilactone B and BAF suggested by the in vitro kinase assays, we studied the interaction between 15N-labeled recombinant BAF and obtusilactone B by nuclear magnetic resonance spectroscopy (Fig. 3A). Chemical shift perturbations observed in the heteronuclear single-quantum coherence spectrum of BAF upon addition of the ligands obtusilactone B and 2-octadecylidene-3-hydroxy-4-methylenebutanolide were analyzed and mapped on the surface of BAF (Fig. 3B). Obtusilactone B binds to BAF strongly, as indicated by dramatic chemical shift perturbations observed upon the addition of the ligand to the protein. In contrast, the inactive ligand 2-octadecylidene-3-hydroxy-4-methylenebutanolide binds to the protein weakly, as evidenced by smaller changes upon the addition of even high molar ratios of the inactive ligand (e.g., a 10:1 ratio of 2-octadecylidene-3-hydroxy-4-methylenebutanolide to BAF; data not shown). The residues whose backbone amide resonance was dramatically affected by the obtusilactone B interaction included Ser22, Ala24, Lys32, Gly31, and Glu35 (Fig. 3A). These residues are located near the protein’s helix-loop-helix region, which plays a major role in its interaction with double-stranded DNA molecules (Bradley et al., 2005). Other residues, such as Asp9, Glh73, and Asp76, also exhibited chemical shift perturbations in our study. An overlay of the heteronuclear single-quantum coherence spectrum of the free BAF protein and the bound protein (1:1 molar ratio of obtusilactone B to BAF) is presented in Supplemental Fig. 2.

In Silico Modeling and Analysis of the Binding Mode of Obtusilactone B. Obtusilactone B clearly bound near the DNA binding site of BAF (Bradley et al., 2005). The hexadec-7-enylidene bond of the obtusilactone B molecule seems to interact favorably with the glycine-rich region of BAF, forming tight van der Waal contacts with residues Gly21, Gly25, Gly29, and Gly31. The 4-hydroxy-5-methylene-dihydro-furan-2-1 moiety of obtusilactone B is stabilized by
hydrogen bonding with residue Glu28 and hydrophobic interactions with residues Val20, Gly25, Ile26, Val29, Leu30, Gly31, and Lys32 in the hydrophobic groove (Fig. 3D). In contrast, the same moiety of 2-octadecylidene-3-hydroxy-4-methylenebutanolide fails to form these interactions, and drifts away from the active pocket. The failure to interact with E28, the lack of unsaturated bonds, and a carbon chain that is two atoms longer are probable reasons for the inactivity of 2-octadecylidene-3-hydroxy-4-methylenebutanolide (Fig. 3D).

Taken together, our results suggest that obtusilactone B suppresses the VRK1-mediated phosphorylation of BAF specifically by binding near the DNA binding site of BAF. It is unclear whether this region is important in the interaction between VRK1 and BAF. However, the specific interaction between obtusilactone B and the N-terminal region of BAF, proximal to residues phosphorylated by VRK1 (Nichols et al., 2006), indicates the possible identification of obtusilactone B as a novel small-molecule inhibitor that targets a major nuclear envelope constituent.

**SPR Evidence for the Interaction of Obtusilactone B with BAF.** The interaction between BAF and obtusilactone B was further evidenced by SPR analysis and cytochemistry with fluorescent dye–conjugated obtusilactone B. SPR uses various biosensor systems to detect molecular interactions (Karlsson et al., 1991). We immobilized the BAF protein to a gold sensor chip slide and applied obtusilactone B to the slide. Sensor chip immobilized BAF protein was more sensitive to obtusilactone B than to 2-octadecylidene-3-hydroxy-4-methylenebutanolide (Fig. 3D). Calculated binding constants are presented in Table 1. As previous results suggest, obtusilactone B, the inhibitor of VRK1-mediated BAF phosphorylation, exhibited a higher binding affinity for BAF than did 2-octadecylidene-3-hydroxy-4-methylenebutanolide. Unreferenced raw data and responses of immobilized VRK1 protein are also presented in Supplemental Fig. 3. These results suggest that obtusilactone B specifically binds to BAF, but does not bind to VRK1.

Expression of enhanced green fluorescent protein-labeled BAF induced the localization of FPR552-conjugated obtusilactone B to
the nucleus, to which native BAF protein localizes (Fig. 4, B and C). In contrast, neither the unconjugated dye FPR552 nor the enhanced green fluorescent protein colocalized with each other. Taken together, our data suggest that obtusilactone B, a small-molecule inhibitor of BAF phosphorylation, specifically interacts with BAF near the protein’s DNA binding region, thereby inhibiting BAF phosphorylation by VRK1.

Physiologic Perturbations Induced by Obtusilactone B Treatment. As mentioned previously, BAF plays an important role in nuclear envelope structure (Vleck et al., 2001). The interactions and dynamics of nuclear membrane proteins must be tightly coordinated during many cellular processes, including cell cycle progression (Furukawa et al., 2003; Gorjanacz et al., 2007; Lancaster et al., 2007). Specifically, the interaction between chromatin and the nuclear envelope must be disrupted at the G1/S transition that occurs at the beginning of the cell cycle. At this stage, BAF is phosphorylated by VRK1, and next, chromatin is released from the nuclear envelope (Nichols et al., 2006; Lancaster et al., 2007). We visualized the mitotic nuclear envelope fragment by staining the nuclear lamin A/C of A549 cells (Fig. 5A) and found that the nuclear lamin A/C was dispersed into the cytosol at late anaphase. However, this release was completely abolished by treatment with obtusilactone B: compared with vehicle-treated cells, A549 cells grown in medium containing obtusilactone B could not release the nuclear envelope fragment containing lamin A/C from chromatids. This inability to release the fragment might be attributed to the inhibitory action of obtusilactone B in the phosphorylation of BAF by VRK1. To further analyze this cellular effect, we expressed VRK1 and BAF in concert with obtusilactone B treatment (Fig. 5B). As previously demonstrated (Nichols et al., 2006), coexpression of BAF and VRK1 induced their translocation into the cytosol, and this translocation was accompanied by DNA condensation. However, in obtusilactone B–treated cells, BAF could not translocate into the cytosol, but was trapped by the contorted nucleus despite the presence of VRK1. These results indicate that obtusilactone B blocked BAF phosphorylation by VRK1, resulting in dysfunctional cellular processes. Because the phosphorylation
of BAF is involved in cell cycle initiation, we quantitatively assessed the DNA content of obtusilactone B–treated cells by flow cytometry (Fig. 5, C and D). The A549 cell population treated with obtusilactone B included a significantly increased number of cells in the G1 phase, which possibly resulted from the defective DNA release from the nuclear envelope during the G1/S transition and the subsequent delay in cell cycle progression. This defect was successfully rescued by the introduction of enhanced green fluorescent protein (EGFP)–tagged BAF protein, suggesting that obtusilactone B–induced cell cycle arrest was induced by the silencing of endogenous BAF.

**Anticancer Activity of Obtusilactone B.** Because the suppression of BAF phosphorylation by VRK1 in obtusilactone...
B-treated cells appeared to trigger defective cell cycle progression, we next analyzed the antitumor activity of obtusilactone B. First, we performed a cell viability assay to provide evidence for the proposed molecular mechanism of interference with cell growth or viability. Obtusilactone B was moderately cytotoxic to A549 (Fig. 6A) and HeLa (Fig. 6B) cells. However, 2-octadecylidene-3-hydroxy-4-methylenebutanolide was significantly less cytotoxic than obtusilactone B. We assume that the difference in cytotoxicity is derived from the ability of obtusilactone B to specifically target endogenous proteins such as BAF.

We also analyzed the specificity of obtusilactone B against tumor cells versus normal cells. We prepared a mouse embryonic fibroblast derived from the C57BL/6 strain as a normal cell control. B16F10, a metastatic melanoma fibroblast line derived from the C57BL/6 strain, was prepared as the cancer cell control. As shown in Fig. 6C, obtusilactone B effectively killed metastatic melanoma cells. However, mouse embryonic fibroblast cells were quite resistant to obtusilactone B compared with melanoma cells. This result shows that obtusilactone B might target highly proliferative cancer cells with specificity. Obtusilactone B also effectively killed teratocarcinoma cells at very low concentrations approaching 1 μM (Fig. 6D).

The effect of obtusilactone B on aneuploidy was assessed by analyzing the molecule’s effect on a cell population with a DNA content more than 4 N (Fig. 6, E and F). Treatment with 10 μM obtusilactone B for 72 hours effectively induced cell death in the aneuploid cell population, but not in the euploid cell population. Together, these results suggest that the novel small-molecule inhibitor, obtusilactone B, preferentially targets oncogenic cells by blocking the cellular functions of BAF protein.

**Discussion**

Genomic DNA is attached to the nuclear envelope during the interphase via the interaction of the core nuclear envelope constituents lamin and BAF (Margalit et al., 2007). The interaction between DNA and the nuclear envelope is very...
important for nuclear integrity, and thus must be tightly regulated throughout the cell cycle. Progression of the cell cycle requires a disruption of the link between DNA and the nuclear envelope, which is achieved by VRK1-mediated BAF phosphorylation (Nichols et al., 2006). This process is very important for various cell functions; thus, Baf is well conserved from Drosophila melanogaster to Homo sapiens.

In this article, we report a small-molecule inhibitor, obtusilactone B, that targets BAF and inhibits VRK1-mediated phosphorylation. Obtusilactone B interacts with BAF specifically and suppresses subsequent VRK1-mediated BAF phosphorylation. Specific binding of obtusilactone B to cellular BAF causes an aberrant interaction between DNA and the nuclear envelope during the mitotic phase. We also demonstrated that obtusilactone B targets tumor cells and selectively kills aneuploid cells in vitro. However, more detailed molecular mechanistic studies should be performed to conclude that obtusilactone B has genuine specificity for cancer cells. Such studies could include a systematic approach to phosphor-profiling of BAF protein in various cancer tissues. Additional evidence including an animal experiment should also be obtained.

The nuclear envelope has not been a primary target in anticancer drug development. However, like other important...
cellular factors, nuclear envelope constituents play major roles in the regulation of proliferative gene expression and genome integrity (Chow et al., 2012). Among the nuclear envelope constituents, lamin is a key physiologic regulator, and the association of lamin dysfunction with various diseases has been frequently reported recently (Burke and Stewart, 2006; Gonzalez-Suarez et al., 2009; Chow et al., 2012). These reports suggest that effective targeting of nuclear envelope constituents awaits the advent of a novel cancer drug that targets such proteins as BAF. We posit that obtusilactone B specifically targets the core nuclear envelope constituent, BAF, and blocks abnormal cell division of tumor cells by inhibiting VRK1-mediated phosphorylation of BAF. A strategy targeting the action of VRK1 toward BAF might have potential therapeutic use in BAF-related abnormalities, including various cancers.

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