A Phthalide with in Vitro Growth Inhibitory Activity from an *Oidiodendron* Strain

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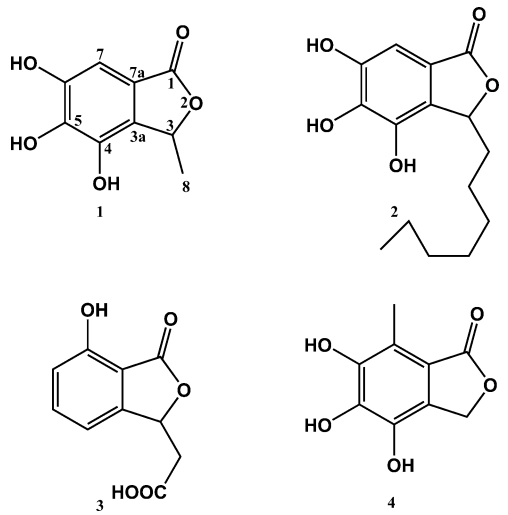
A screening campaign was implemented utilizing capillary electrophoresis as a primary assay to discover binders to the cancer target Akt1 from a crude natural extract library. Fungal extracts with binding activities were characterized for biochemical inhibition of Akt1 to phosphorylate the downstream substrate protein Bad. One of the crude extracts with bioactivity selected for isolation and structure elucidation from fermentation of the fungal culture *Oidiodendron* sp. F01895 yielded a new trihydroxy phthalide (**1**). The structure of **1** was determined by a combination of 1D and 2D NMR spectroscopic data along with high-resolution mass spectrometric data. Compound **1** displays inhibition of Akt1 biochemical activity in vitro and confers growth inhibition on some cancer-derived cell lines in culture.

Natural extracts derived from fungi are a proven source of bioactive compounds with therapeutic indications against a wide range of diseases and infections.[1,2] In particular, the fungal genus *Oidiodendron* has been shown to make secondary metabolites that display antibacterial,[3] antiviral,[4] anti-inflammatory,[5] and anticancer capacities.[6−8] For example, a dilactone (corresponding to LL-Z1271) from *Oidiodendron* has been shown to inhibit the growth of multiple cancer cell lines in vitro.[6] Also, *Oidiodendron truncatum* has been shown to produce the compound PR-1350 or clerocidin, a diterpenoid with antitumor capacities due to its targeting of DNA topoisomerase II.[7,8]

Akt1 is a key component of biochemical pathways that control apoptosis, or programmed cell death, and is important in the etiology of many different types of cancer. As cells grow and divide, they accumulate genetic alterations (mutations). Under normal cellular growth, these cells may respond to either external stimuli (stress, cytokines, cell−cell contact, etc.) or internal signaling resulting in induction of programmed cell death mechanisms. Under conditions in which Akt1 is highly active, such as when the PTEN tumor suppressor gene is mutated, leading to a constitutive activation of the PTEN-PI3K-Akt pathway, cells exhibit unregulated growth and the inability to undergo apoptosis.[9−11] Thus, cells carrying genetic damage are allowed to proliferate in an uncontrolled fashion.

Additionally, many chemotherapeutic agents operate through the induction of apoptosis. Thus, the constitutive activation of Akt1-directed pathways leads not only to uncontrolled growth but also to an increase in drug-resistance. An inhibitor to the PI3K-Akt1 axis would be able to stop the unregulated growth and restore programmed cell death pathway operation to these cells.[12] The potential for Akt1 inhibitors to be used in combinational approaches with existing therapeutics would hold great promise for improved patient care.[13,14]

The MeOH crude extract of the fungus *Oidiodendron* sp. F01895 exhibited activity against Akt1. The methanolic extract was absorbed onto HP20, eluted, and then solvent-partitioned with methyl ethyl ketone (MEK) and H_2O. The organic soluble fraction was then separated by successive chromatographic procedures to afford active compound **1** (4 mg). The UV absorptions at 224 and 278 nm were attributed to lactone and phenolic moieties. The molecular formula for compound **1** was established as $C_9H_8O_5$ on the basis of high-resolution mass spectrometry and NMR data, requiring the compound to have six degrees of unsaturation.



The ^1H and ^{13}C NMR spectra (see Experimental Section) revealed the presence of a CH_3 (δ_C 19.3), an oxygenic CH (δ_C 75.5), three phenolic hydroxyl groups (δ_H 9.31, 9.39, 9.89; exchangeable), a pentasubstituted benzenoid ring (δ_H 6.71), and an ester group (δ_C 170.2). This accounted for five degrees of unsaturation, and the additional degree of unsaturation required a ring moiety. Gradient $^1H-^1H$ COSY revealed only one spin system in which CH_3 (C-8) was coupled with oxygenated methine (C-3). The somewhat deshielded resonance of this lactone ring methine proton (δ_H 5.49) in the ^1H NMR spectrum suggested attachment to the aromatic ring. HMBC correlation of a methine proton (δ_H 6.7) with an ester carbonyl at C-1 and sp^2 carbon of C-7a supported the attachment of C-1 to C-7a. Also, observing ^{13}C resonances (δ 139.6, 139.9, 147.4) for an aromatic ring and upfield olefinic ^1H and ^{13}C NMR signals, H-7 (δ_H 6.71) and C-7 (δ_C 101.5), suggested connection of C-7a to a carbonyl C-1 in a lactone ring. Thus, compound

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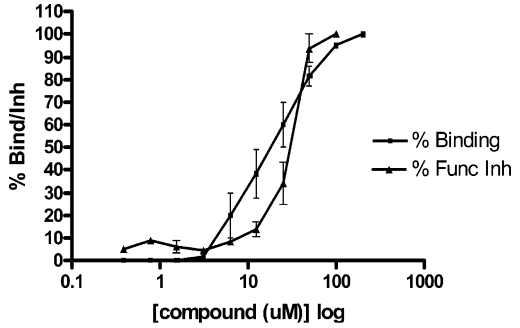

Figure 1. Dose−response for compound **1** binding to and functional inhibition of Akt1.

1 was assigned the structure 4,5,6-trihydroxy-3-methylphthalide. Compound **1** is closely related to metabolites cytosporone E (**2**) previously isolated from endophytic fungi *Cytospora* sp. and *Diaporthe* sp.[15] and isoochracinic acid (**3**) previously isolated from filamentous fungi *Alternaria kikuchiana*[16] and *Cladosporium* sp.[17]

A structural isomer, 4,5,6-trihydroxy-7-methylphthalide (**4**), was isolated by Koenig[18] from the marine fungus *Epicoccum*. The methyl group is on the 7-position of the phthalide ring instead of the 3-position. We confirmed the final structure assignment of our isolated compound by unambiguous synthesis;[19] the spectral data of the synthesized compound is identical to that of our isolated compound: 4,5,6-trihydroxy-3-methylphthalide.

No optical activity was observed for compound **1**, consistent with its isolation as a racemic mixture, $[\alpha_d] = 0$. Compound **3** has been reported to be optically active[16,17] with $[\alpha_d] = -5$; however, the data from compound **1** are more consistent with compound **2**, which was previously shown to be isolated as a racemic mixture as well.[20]

Compound **1** was titrated in an ACE binding assay against labeled Akt1 target. The IC_{50} of compound **1** for Akt1 was determined from a mean of three experiments to be 19.7 μM (±3.98 μM SEM) (Figure 1). An IC_{50} for functional inhibition of compound **1** for Akt1-directed phosphorylation of its substrate protein Bad was determined from a mean of three experiments to be 30.4 μM (±5.22 μM SEM) (Figure 1).

Compound **1** was tested for growth inhibitory properties against human cancer-derived cell lines. Ten cell lines were tested at Cetek Corporation and 40 cell lines tested through the Developmental Therapeutics Program at the National Cancer Institute. While the majority of cell lines tested showed no growth inhibition in the presence of compound **1** at 100 μM, several did respond to compound **1** with GI_{50}'s in the 20−80 μM range. This indicates a level of selectivity to the action of the compound, and not that the compound is generally toxic or inhibitory indiscriminately to all cell lines tested. Representative data are indicated in Table 1.

Interestingly, IC_{50}'s of 20 and 24 μM were observed in the human T cell leukemia Jurkat cell line[21] and the human breast cancer cell line BT-549,[22] respectively, both of which lack PTEN tumor suppressor activity. This lack of PTEN function confers a selective growth advantage to cells particularly resistant to apoptotic processes. Additionally, the U937 human lymphoma cell line has been shown to have a frameshift mutation in one of the copies of the gene, leading to a truncated aberrant transcript.[23,24] This hemizygous deletion may also confer a selective growth advantage to these cells that may be abrogated partially by administration of compound **1**. In support of this hypothesis, growth inhibition of U937 cells treated with compound **1** occurs with an IC_{50} of 60 uM.

Table 1. Growth Inhibition of Compound **1** against Selected Human Cell Lines

cell line	origin	GI_{50} (μM)
RPMI-8226	leukemia	67
Jurkat[a]	leukemia	20
K-562	leukemia	>100
NCI-H23	non small cell lung cancer	87
EKVX	non small cell lung cancer	>100
SNB-75	central nervous system cancer	60
U251	central nervous system cancer	>100
SK-MEL-28	melanoma	37
M14	melanoma	>100
OVCAR-5	ovarian cancer	74
OVCAR-8	ovarian cancer	82
OVCAR-3	ovarian cancer	>100
MDA-MB-435	breast cancer	85
BT-549[a]	breast cancer	24
MCF7	breast cancer	>100
U937[b]	lymphoma	60
HepG2	hepatocellular cancer	>100

[a] Known to be functionally devoid of PTEN activity. [b] Known to have one functional copy and one nonfunctional copy of PTEN.

Experimental Section

General Experimental Procedures. ^1H and ^{13}C NMR data were obtained at room temperature on a Bruker Avance 500 MHz spectrometer operating at 500.13 MHz for proton and at 125.77 MHz for carbon 13. The solvent (DMSO) was used as internal reference (δ_H 2.50 for ^1H and δ_C 39.5 for ^{13}C). LC-MS experiments were performed using an Agilent 1100 HPLC system and a Zorbax Eclipse XDB C18 reverse-phase HPLC column, 2 × 100 mm (Agilent Technologies, Palo Alto, CA), with high-resolution mass spectrometric analysis conducted on a PE Sciex QStar Pulsar-i quadrupole-time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA). Mass spectrometric data were acquired in positive-ion mode using a TurboSpray ion source. Internal calibration was used for optimal mass accuracy and was achieved by infusing a 2-point calibrant solution, a mixture of cesium iodide (Cs^+ = *m/z* 132.9049) and verapamil ($M + H^+$ = *m/z* 455.2904), during LC analysis. LC solvent A was HPLC grade H_2O with 0.1% formic acid, and solvent B was HPLC grade acetonitrile with 0.1% formic acid. The gradient program ranged from 5% to 50% B over 30 min, at a flow rate of 200 μL/min. A 20:1 postcolumn split delivered an effective flow rate of 10 μL/min into the ESI source. UV data were obtained via Agilent 1100 series instrumentation equipped with a diode array detector (DAD).

Protein Labeling. Recombinant human Akt1 expressed via baculovirus (Upstate Biotechnology, Lake Placid, NY) was sulfhydryl-labeled. Protein was buffer-exchanged into 30 mM HEPES/NaOH pH 7.5, 20 uM β-mercaptoethanol, 0.1% Chaps, and 150 mM NaCl using a Centri-Sep gel filtration column per manufacturer's instructions (Princeton Separations, Inc., Adelphia, NJ). A final dye (Fluorescein-5-EX, succinimidyl ester dye; Molecular Probes, Eugene, OR) to protein ratio of 5:1 was used, and the target was labeled on ice for 2 h. The reaction was terminated with the addition of β-mercaptoethanol. Excess free dye was removed by exchange of the reaction into storage buffer (20 mM Tris/AcOH pH 7.5, 150 mM NaCl, 0.1% Chaps, 5 mM DTT). Glycerol was added to a final concentration of 50% and the labeled protein stored at −20 °C.

Affinity Capillary Electrophoresis (ACE) Instrumentation and Assay. The CE assay was developed on a Beckman PACE 5000 capillary electrophoresis system. High-throughput screening of extracts was performed on the Gemini capillary electrophoresis system developed at Cetek Corporation (Marlborough, MA). The Gemini is a CE electrophoresis system combining a robotic high-throughput pipetting station with multiple capillaries and laser-induced fluorescence (LIF, 490 nm ex/520 nm em) detectors. The CE assay was run at 600 V/cm with 15 °C capillary temperature over fused silica

capillaries treated with a polymeric coating. Software to analyze CE electropherograms was designed and written specifically for the Gemini instrumentation at Cetek Corporation. Any extract with constituents that bind to a target resulting in the generation of a qualitative change in CE profile or migration rate of greater than (approximately) 10% when compared to controls was scored as a "hit".[25]

Fungal Isolation, Identification, and Cultivation. The fungal strain F01895 was from Cetek's internal natural extract collection. The strain was identified as belonging to the genus *Oidiodendron* by analysis of the ITS regions of the ribosomal DNA as previously described.[26] Stock culture of strain F01895 was maintained as frozen whole broth at −80 °C in a final concentration of 10% glycerol. The seed medium contained the following ingredients (g/L): glucose, 10; soluble starch, 20; yeast extract (Difco, Plymouth, MN), 5; N-Z amine (Sigma, St. Louis, MO), 5; $CaCO_3$, 1.0; deionized H_2O, 1.0 L; the pH was not adjusted prior to autoclaving. A 250 mL Erlenmeyer flask containing 40 mL of this medium was inoculated with 1.0 mL of the stock culture. The flask was incubated at 28 °C on a rotary shaker (2 in. throw) at 220 rpm for 72 h. This seed culture (30 mL) was used to inoculate a Fernbach flask containing 300 mL of production medium. The production medium contained the following (g/L): sucrose, 30; ammonium tartrate, 5; yeast extract, 0.5; soytone peptone (Difco, Plymouth, MN), 1.0; $Ca(NO_3)_2$, 0.28; KNO_3, 0.08; KCl, 0.06; $MgSO_4 \cdot 7H_2O$, 0.36; $NaH_2PO_4 \cdot H_2O$, 0.1; deionized H_2O, 1.0 L; and trace metals, 1.0 mL, pH adjusted to 5.5 prior to autoclaving. Trace metals mix (mg/L): $FeSO_4 \cdot 7H_2O$, 4; $MnSO_4 \cdot H_2O$, 5; $ZnSO_4 \cdot 7H_2O$, 2.5; Borax, 1.4; $CoCl_2 \cdot 6H_2O$, 0.2; $CuSO_4 \cdot 5H_2O$, 0.5; $Na_2MoO_4 \cdot H_2O$, 0.2; deionized H_2O, 1.0 L. The Fernbach flasks (× 30) were incubated statically at 28 °C for 21 days.

Extraction and Isolation. A 300 mL static liquid culture was extracted with 1/2 volume of MeOH. The flask was then allowed to incubate at 4 °C in the refrigerator overnight. The MeOH was then filtered through a Chemglass extracourse glass filter under vacuum, and the cell mass was washed with an additional 1.0 L of MeOH. To the MeOH extract was added 200 mL of dry HP20 resin. The MeOH was removed by rotary evaporation to leave behind the dry HP20 resin with the adsorbed extract. The dried HP20 was then washed with 2.0 L of H_2O and eluted with 2.0 L of a 9:1 acetone/H_2O mixture. The 9:1 acetone/H_2O was removed by rotary evaporation to yield 3.5 g of crude extract. The crude extract was then dissolved in 800 mL of H_2O and extracted twice with 800 mL of methyl ethyl ketone (MEK). The MEK was removed by rotary evaporation, yielding 900 mg of extract. The 900 mg was chromatographed on Sephadex LH20 column (Amersham Pharmacia Biotech), collecting 40 15 mL fractions. Fractions showing Akt1 activity were combined to yield 65 mg of semipure compound. The 65 mg was subjected to further purification by HPLC (Agilent 1100 series) using a linear gradient starting with 5% solvent B (acetonitrile with 0.1% formic acid) and 95% solvent A (H_2O with 0.1% formic acid) to 30% B in 30 min with a flow rate of 3.5 mL/min on a 150 × 10 mm Synergi 4u hydro-RP-18 column (Phenomenex). The active fractions were analyzed by LC/MS and combined to yield 4.0 mg of 95% pure active compound **1**.

Compound 1: powder; UV λ_{max} 274 nm; ^{1}H NMR δ 9.89 (1H, s, 4-OH), 9.39 (1H, s, 5-OH), 9.31 (1H, s, 6-OH), 6.71 (1H, s, H-7), 5.49 (1H, q, J = 7.0 Hz, H-3), 1.52 (3H, d, J = 7.0 Hz, H-8); ^{13}C NMR δ 170.2 (C, C-1), 147.4 (C, C-6), 139.9 (C, C-4), 139.6 (C, C-5), 130.6 (C, C-7a), 115.1 (C, C-3a), 101.5 (CH, C-7), 75.5 (CH, C-3), 19.3 (CH_3, C-8); HRMS *m/z* 197.0448 $[M + H]^+$ (calcd for $C_9H_8O_5$, 197.0444).

Biochemical Assay and Western Blot Detection. Akt1 phosphorylation of recombinant soluble Bad protein was tested in the presence and absence of crude extracts or purified compound. Briefly, 5 μL of active Akt1 (50 nM final conc) (Upstate, Lake Placid, NY), 10 μL of assay dilution buffer I (Upstate), and test agent in DMSO (1.4% final conc) were incubated for 5 min at room temperature prior to addition of 15 μL of soluble Bad (final 1.2 μM) (Upstate) and 50 μL of the magnesium ATP solution (Upstate). The reaction then incubated for 30 min with agitation at 30 °C. Following SDS-PAGE separation, protein transfer was onto Immun-Blot PVDF membrane (BioRad, Hercules, CA). Detection of phosphorylated Bad protein was via rabbit polyclonal anti-phospho-Bad (ser 112) Ab (Upstate), HRP-conjugated goat-anti-rabbit Ab (Upstate), and SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). Enzymatic inhibition was quantified by scanning densitometry of autoradiographs using software from Scion Image (Frederick, MD) and charted using GraphPad Prism software (San Diego, CA).

Human Cancer-Derived Cell Line Testing. Cell lines tested at Cetek Corporation were propagated in the media recommended for each specific line by American Type Culture Collection (ATCC, Manassas, VA). Cell Titer blue assay reagent (Promega, Madison, WI) was used to estimate the number of viable cells by quantifying metabolic activity levels through reduction of resazurin in the presence or absence of compound. Fluorescence was measured at an excitation/emission of 530/590 on a Bio-Tek Synergy HT 96/384 well plate reader (Winooski, VT). Cell lines tested at the National Cancer Institute (NCI) through the Developmental Therapeutics Program were propagated in RPMI 1640 with 5% fetal bovine serum (FBS) and 2 mM L-glutamine. TCA fixation and sulforhodamine B staining for total protein was used to quantify the number of viable cells.

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