A resveratrol analogue promotes ERK\textsuperscript{MAPK}-dependent Stat3 serine and tyrosine phosphorylation alterations and antitumor effects \textit{in vitro} against human tumor cells

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MOL Manuscript #99093

kinase; mTOR, Mammalian target of rapamycin; PARP, Poly ADP ribose polymerase; PBS, Phosphate buffered saline; PI3-K, Phosphoinositide 3-kinase; PMSF, Phenylmethylsulfonyl fluoride; PVDF, Polyvinylidene fluoride; RIPA, Radioimmunoprecipitation assay; RPMI, Roswell Park Memorial Institute; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Stat1, Signal transducer and activator of transcription 1; Stat3, Signal transducer and activator of transcription 3; TBST, Tris Buffered Saline with Tween-20; TLA, aprotinin, leupeptin and antipain.
Abstract

(E)-4-(3,5-Dimethoxystyryl)phenyl acetate (Cmpd1) is a resveratrol analog that preferentially inhibits glioma, breast and pancreatic cancer cell growth, with IC₅₀ values of 6-19 μM. Notably, the human U251MG glioblastoma tumor line is most sensitive, with an IC₅₀ of 6.7 μM, compared to normal fibroblasts, with IC₅₀ >20 μM. Treatment of U251MG cells that harbor aberrantly-active Signal Transducer and Activator of Transcription (Stat)3 with Cmpd1 suppresses Stat3 tyrosine705 phosphorylation in a dose-dependent manner, in parallel with the induction of pserine727 Stat3 and pErk1/2MAPK (extracellular signal-regulated kinase/mitogen-activated protein kinase 1/2). Inhibition of pErk1/2MAPK induction by the MEK (mitogen-activated protein/extracellular signal-regulated kinase kinase) inhibitor, PD98059, blocked both the pserine727 Stat3 induction and ptyrosine705 Stat3 suppression by Cmpd1, indicating the dependency on the MEK-Erk1/2MAPK pathway for Cmpd1-induced modulation of Stat3 signaling. Cmpd1 also blocked epidermal growth factor (EGF)-stimulated pStat1 induction, while upregulating pSrc, pAkt, p-p38, pHsp (heat shock protein)27, and pmTOR (mammalian target of rapamycin) levels. However, pJanus kinase (Jak)2 and pEGFR (EGF receptor) levels were not significantly altered. Treatment of U251MG cells with Cmpd1 reduced in vitro colony formation, induced cell cycle arrest in G2/M phase and the cleavage of caspases 3, 8 and 9 and poly ADP ribose polymerase (PARP), and suppressed Survivin, Mcl-1, Bcl-xL, Cyclin D1, and Cyclin B1 expression. Taken together, these data identify a novel mechanism for the inhibition of Stat3 signaling by a resveratrol analog and suggest that the preferential growth inhibitory effects of Cmp1 occur in part by Erk1/2MAPK-dependent modulation of constitutively-active Stat3.
Introduction

Resveratrol is a stilbenoid phytoalexin present in red grapes, red wine, peanuts and other plant sources. Numerous studies in cardiovascular, neurological, immune regulation, and cancer chemoprevention models, and anticancer efficacy evaluations have all shown significant biological activity in vitro and in vivo for resveratrol and highlighted the potential benefits of this agent in diverse human diseases (Baur and Sinclair, 2006; Jang et al., 1997; Pervaiz and Holme, 2009; Rimando et al., 2002). In both chemo-preventative and anticancer efficacy studies using models of solid and hematological malignancies, resveratrol inhibited tumor cell proliferation, survival, and invasion, as well as tumor angiogenesis and tumor formation (Baur and Sinclair, 2006; Jang et al., 1997; Pervaiz and Holme, 2009; Shankar et al., 2007). It has been well recognized that the multitude of effects and the notable health benefits of resveratrol are due to its activities on multiple targets and processes. Among others, resveratrol affects COX-1 (cyclo-oxygenase-1) and COX-2, NAD⁺-dependent histone deacetylase SIRT1 (sirtuin 1), QR2 (quinone reductase 2), and ribonucleotide reductase enzymes, and Erk1/2MAPK, Src, protein kinase C (PKC), nuclear factor (NF)B, and phosphoinositide 3-kinase (PI3-K) signaling pathways, and alters DNA synthesis (Calamini et al., 2010; Fröjdö et al., 2007; Jeong et al., 2004; Pervaiz and Holme, 2009; Pirola and Fröjdö, 2008; Yu et al., 2001). Despite the clinical and anticancer potential of resveratrol, its development for clinical application has been hampered by its low potency and limited bioavailability (Baur and Sinclair, 2006; Jang et al., 1997; Shankar et al., 2007). Efforts have therefore been placed on discovering analogs that have enhanced potency and appreciable bioavailability suitable for clinical application.
Previous reports also showed that resveratrol suppresses Stat3 signaling via the inhibition of Src or Jak2 induction, thereby inducing growth inhibitory and apoptotic effects against cultured human breast, prostate and pancreatic cancer cells and the v-Src transformed mouse fibroblasts, and malignant natural killer cells (Kotha et al., 2006; Quoc Trung et al., 2013). The Stat family of cytoplasmic transcription factors are normally activated transiently via tyrosine (Tyr) phosphorylation by Jaks, Src, and growth factor receptor Tyr kinases. Phosphorylation leads to Stat:Stat dimerization, nuclear translocation and gene transcription that promote cell growth and proliferation, differentiation, inflammation and other physiological responses to growth factors and cytokines (Bromberg, 2000; Darnell, 1997). In contrast, constitutively-active Stat3 is prevalent in many human cancers, including glioblastoma, breast, and pancreatic cancers and represents a critical mediator of malignant transformation and tumor progression (Bowman et al., 2000; Miklossy et al., 2013). Aberrantly-active Stat3 promotes tumorigenesis in part via dysregulation of gene expression, leading to uncontrolled growth and survival of cells, enhanced tumor angiogenesis and metastasis (Bowman et al., 2000; Bromberg and Darnell, 2000; Miklossy et al., 2013; Turkson, 2004; Turkson and Jove, 2000; Yu and Jove, 2004), and the repression of tumor immune surveillance (Wang et al., 2004; Yu and Jove, 2004).

While the evidence suggests the inhibition of Stat3 signaling represents one of the mechanisms for the antitumor cell response to resveratrol (Kotha et al., 2006; Quoc Trung et al., 2013), the mechanism of resveratrol-induced Stat3 inhibition is less understood. The initial reports suggested the inhibition of Stat3 activity might occur indirectly through inhibition of Src and Jaks induction (Kotha et al., 2006; Quoc Trung et al., 2013). Several resveratrol analogs were
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screened for improved activity against human tumor cells that harbor aberrantly-active Stat3, leading to the identification of one potentially more active agent. The analogue, (E)-4-(3,5-Dimethoxystyryl)phenyl acetate (Cmpd1) more potently inhibited constitutive Stat3 tyrosine705 phosphorylation, while enhancing Stat3 serine727 phosphorylation in malignant cells. These events were associated with the induction of pErk1/2^{MAPK}. While the inhibition of MEK-Erk1/2^{MAPK} induction suppressed Cmpd1-induced pserine727 Stat3, it reversed the inhibitory effects of Cmpd1 on ptyrosine705 Stat3, suggesting that Erk1/2^{MAPK} induction is required for Cmpd1-dependent modulation of both Stat3 serine727 and tyrosine705 phosphorylation events. Cmpd1 further induced pSrc, pmTOR, pAkt, pHsp27 and p-p38, suppressed pStat1, and had no significant effects on pJak2 and pEGFR. The activities of Cmpd1 resulted in the loss of cell viability, growth, and survival of human glioma, breast, or pancreatic cancer cells, and induced cell cycle block at the G2/M phase and apoptosis with human glioma cells in culture.

Materials and Methods

Cell Lines and Reagents

The human glioma lines, U251MG and SF-295, were obtained from the Division of Cancer Treatment and Diagnosis, Tumor Repository of the National Cancer Institute (Frederick, MD) and cultured in Roswell Park Memorial Institute (RPMI) medium containing 10% FBS, or Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and supplemented with 1% nonessential amino acids (Corning Inc., Corning, NY). The human breast (MDA-MB-231 and MCF-7) and pancreatic (Panc-1) cancer cells, and the normal mouse fibroblasts (NIH3T3) and
their v-Src-transformed (NIH3T3/v-Src) or v-Ras-transformed (NIH3T3/v-Ras) counterparts have all been previously reported (Kotha et al., 2006; Zhang et al., 2012; Zhao et al., 2010). These cells were grown in DMEM containing 10% heat-inactivated fetal bovine serum (FBS). Resveratrol was purchased from Sigma Aldrich (St. Louis, MO). Pterostilbene and PD98059 were purchased from Cayman Chemical (Ann Arbor, MI). Cmpd1, Cmpd2, and Cmpd3 have all been previously described (Kondratyuk et al., 2011; Sun et al., 2010). Except for anti-β-actin, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (Santa Cruz Biotechnology, Inc., Dallas, Texas), and Cyclin D1 (SPM587) (Novus Biologicals, Littleton, CO), antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Recombinant human EGF was purchased from Invitrogen Corp/Life Technologies Corp (Carlsbad, CA). Where appropriate, cells were stimulated with recombinant human EGF (10 ng/ml) for 12 min. PD98059 (Cayman Chemical Company), SB202190 and LY294002 (Calbiochem, EMD Millipore, Billerica, MA), wortmannin and staurosporine (Sigma), dasatinib (ChemiTEK, Indianapolis, IN) were obtained from the designated sources.

Cyquant Cell Proliferation Assay

The CyQuAnt cell proliferation assay (Invitrogen Corp/Life Technologies Corp) was used following the manufacturer’s instructions to evaluate the biological activities of compounds, as previously reported (Zhang et al., 2012). Relative cell viability of the treated cells was normalized to the DMSO-treated control cells.

Trypan Blue Cell Counting
Cells in culture were treated once with compounds at increasing concentrations for 0-72 h. Treated and untreated cells were harvested every 24 h and viable cells were stained with Trypan blue dye and counted under microscope with a hemocytometer.

Colony Survival Assay

Five-hundred single cells were seeded on a 6 cm dish, cultured for 24 h, and treated once with Cmpd1. Cells were allowed to grow for 14-21 days until colonies were visible, which were stained with crystal violet for 1-3 hours, imaged and counted.

ESI-MS/MS Analysis of Cmpd1 and pterostilbene

Studies were performed by the Analytical Biochemistry Shared Resource (ABSR) at the University of Hawaii Cancer Center. The pterostilbene standard (10 µM in methanol, MeOH) was serially diluted in MeOH/H2O (1/1 v/v) to calibrate the system. Cell lysates were extracted with ethyl acetate. The organic phase was dried under nitrogen and reconstituted in MeOH/H2O for liquid chromatography–mass spectrometry (LCMS) analysis. LCMS analysis was carried out on a Q-Exactive mass spectrometer coupled to HTC autosampler and Accela pump (all from Thermo Scientific, Waltham, MA). Twenty-five microliters (25 µL) of standard or extracts were injected onto an Ascentis Express C18 column (15 x 3 mm; 2.7µm; Supelco); chromatographic separation was achieved with a linear gradient consisting of (A) 0.1% formic acid in water, (B) 0.1% formic acid in MeOH, and (C) 0.1% formic acid in acetonitrile at 0.3 mL/min: A/B/C= 80/10/10 to 20/40/40 in 10 min and keeping at 20/40/40 for another 10 min. Mass spectrometric measurements were performed in full scan ESI mode with positive and negative switching. Quantitation of the pterostilbene ([M+H]+ 257.11722, [M-H]− 255.10212) and acetyl-pterostilbene ([M+H]+ 299.12779, [M-H]− 297.11268) was performed with Xcalibur software using a 5 ppm window from the exact masses.
Western Blot Analysis

Immunoblotting analysis was performed as previously reported (Kotha et al., 2006; Zhang et al., 2012; Zhao et al., 2010). Briefly, whole-cell lysates were prepared using ice-cold radioimmunoprecipitation assay (RIPA) buffer containing phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), sodium orthovanadate (Na$_3$VO$_4$) and TLA (aprotinin, leupeptin and antipain). Lysates were spun down at 13,300 rpm for 20 min at 4 °C using a microcentrifuge and the supernatants were collected. Protein concentrations were determined by the Bradford Assay (Pierce Biotechnology, Rockford, IL). Equivalent amounts of total protein were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to a nitrocellulose or PVDF (polyvinylidene fluoride) membrane (BioRad, Hercules, CA). The membranes were blocked for 1 h with 5% milk in TBST (Tris Buffered Saline with Tween-20), incubated with primary antibody overnight, washed three-times and then incubated with secondary antibody at room temperature for 1 h. Membranes were then treated with enhanced chemiluminescence reagent (BioRad, Hercules, CA) for imaging analysis.

Immunoprecipitation Assay

Immunoprecipitation/SDS-PAGE analyses were performed as previously reported (Zhang et al., 2012). Briefly, following treatment, cells were washed with 1x PBS (phosphate-buffered saline) followed by 1x NTN buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05%NP-40) and 1x low-salt HEPES buffer (10 mM HEPES [pH7.8], 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT). Cells were lysed with 1x low-salt buffer containing 10% Nonidet P-40 (NP-40), and cell lysates were spun down at 13,300 rpm for 20 min at 4 °C to collect the
supernatants. Lysates were incubated with protein A/G beads (Santa Cruz Biotechnology, Inc, Dallas, TX) in the cold room on an orbital rocker for 30 min, and subsequently centrifuged at 3,000 rpm at 4 °C for 3 min. The supernatants were collected and incubated with the primary antibody for 12 h overnight in a cold room, and then with protein A/G beads for 1 h. Subsequently, samples were spun down at 3,000 rpm at 4 °C for 3 min to collect the bead pellets, which were washed four-times with NTN buffer, added to 30 µL of 1x SDS loading buffer, and boiled for 5 min. Samples were subjected to SDS-PAGE and immunoblotting analysis.

Cell Cycle Analysis by Flow Cytometry

Following treatment with 1 for 24 or 72 h, cells in culture in 6 cm plates were trypsinized, centrifuged at 1,000 rpm at 4 °C for 10 min, and washed with 1x PBS. Cells were fixed with fixation buffer (70% ethanol and 3% FBS in PBS) overnight at -20 °C. Subsequently, cells were washed two-times with 1x PBS and once with propidium iodide (PI)/RNase staining buffer (BD Biosciences, San Jose, CA), and stained by incubating with PI/RNase buffer at room temperature for 15 min. Samples were placed on ice and analyzed using a FACS Calibur (BD Biosciences, San Jose, CA). Data were collected with CellQuest Pro (BD Biosciences, San Jose, CA) and with FlowJo (Ashland, OR).

Statistical analysis
Statistical analysis was performed on mean values using Prism GraphPad Software, Inc. (La Jolla, CA). The significance of differences between groups was determined by the paired t-test at $p < 0.01^*$. 

Results

Resveratrol analogs, Cmpd1, Cmpd2, and Cmpd3 more potently inhibit human tumor cell growth

Initial screening of 98 resveratrol analogs (data not shown) identified three analogs, Cmpd1, Cmpd2 and Cmpd3 (Fig. 1A) that showed stronger activity against the viability of the human glioma U251MG and breast MDA-MB-231 cancer cells than resveratrol. Of these, Cmpd1 inhibited tumor cell viability the greatest (Fig. 1B).

Human glioma (U251MG and SF295), breast (MDA-MB-231 and MCF7), and pancreatic (Panc-1) cancer cells, and normal mouse fibroblast (NIH3T3) in culture were treated once with 0-20 µM of Cmpd1, Cmpd2 or Cmpd3 for 72 h and harvested for the CyQuant cell proliferation assay to determine viable cell numbers. Cmpd1 showed a dose-dependent inhibition of cell viability that is strongest against U251MG, moderate against MCF7, Panc-1 and MDA-MB-231, and weak against SF295 cells, with IC$_{50}$ values of 6.7, 10.8, 18.1, 18.5 and >20 µM, respectively [Fig. 1B(i); Table 1]. By contrast, the inhibitory effect of Cmpd1 against normal NIH3T3 was insignificant, except at 20 µM, with an IC$_{50}$ >20 µM [Fig. 1B(i); Table 1]. Cmpd2 showed moderate activity against MDA-MB-231 and no effect on U251MG up to 20 µM [Fig. 1B(ii)], while Cmpd3 was weak against both cell lines at concentrations up to 20 µM [Fig. 1B(iii)]. Similar Cyquant cell
proliferation assay results obtained with U251MG cells showed only a moderate effect with resveratrol [Fig. 1C(i)], with an IC$_{50}$ $>$50 µM, while its structural analog, pterostilbene (Pan et al., 2008; Rimando et al., 2002), induced a strong and dose-dependent response [Fig. 1C(ii)], with an IC$_{50}$ of 6.3 µM. From literature reports and our studies, the activities observed for Cmpd1 are significantly superior to that of resveratrol against the same cell lines [Table 1; Fig. 1B(i) and C(i)], while Cmpd1 and pterostilbene are equally active against U251MG cell growth.

Trypan blue exclusion/phase-contrast microscopy for viable cells treated once with Cmpd1 (0-20 µM) for 0-72 h confirmed the dose-dependent suppression of human glioma U251MG cell growth (Fig. 1D), compared to a marginal decline in cell growth induced against normal NIH3T3 (Fig. 1E). In the clonogenic assay, one time treatment with Cmpd1 (0-20 µM) of single-cell cultures for 14-21 days significantly suppressed colony numbers for U251MG, SF-295 and MCF7, while only moderately affecting that of Panc-1 cells, except at a concentration of 20 µM (Fig. 1F). Unlike the limited effects on cell proliferation and cell growth (Fig. 1B and E), treatment of single-cell cultures of NIH3T3 with Cmpd1 in the clonogenic assay reduced the colony numbers (Fig. 1F). However, we note the larger relative effects against U251MG, SF295 and MCF7 colonies at 15 and 20 µM, compared to that against NIH3T3, which suggest higher tumor-cell sensitivity. We infer that the reduced colony numbers of normal NIH3T3 cells could be due to small colony sizes that were the result of apparent slowness in growth rate in response to Cmpd1 (Fig. 1E). Small colonies may be excluded from staining and quantification (Supplementary Fig. S1, NIH3T3).
Cmpd1 inhibits Stat3 tyrosine705 phosphorylation, while up-regulating Stat3 serine727 phosphorylation

A previous report had shown that resveratrol treatment suppresses Stat3 tyrosine705 phosphorylation in v-Src-transformed mouse fibroblasts (NIH3T3/v-Src) (Kotha et al., 2006). The most sensitive U251MG cell line and NIH3T3/v-Src fibroblasts, both of which harbor constitutively activated Stat3, were used to investigate the effects of Cmpd1 on Stat3 signaling. Cells (U251MG and NIH3T3/v-Src) in culture were treated with 0-20 µM Cmpd1 for 3 h or 15 µM for 0-24 h, and whole-cell lysates were prepared for immunoblotting analysis. Cmpd1 inhibited constitutive Stat3 tyrosine705 phosphorylation at 5 µM and higher (Fig. 2A and Supplementary Fig. S2A), and as early as 1 h (Fig. 2B) in U251MG cells, or at a later time, in 10-24 h, in NIH3T3/v-Src fibroblasts (Fig. S2A). Notable differences in the effectiveness and the kinetics of inhibition of ptyrosine705 Stat3 were observed between the two cell lines. For U251MG cells, ptyrosine705 Stat3 inhibition by Cmpd1 was complete at 1-3 h, and was followed by a gradual recovery at 10-24 h (Fig. 2B). By contrast, ptyrosine705 Stat3 inhibition by Cmpd1 in NIH3T3/v-Src fibroblasts was only moderate and occurred at a later time of 10-24 h (Supplementary Fig. S2A).

For comparison, 20 µM resveratrol mediated a time-dependent, progressive suppression of ptyrosine705 Stat3 that occurred at 1-10 h, followed by a recovery at 24 h in both U251MG and NIH3T3/v-Src lines (Fig. 2C and Supplementary Fig. S2B). These data indicate that unlike Cmpd1, resveratrol inhibits Stat3 tyrosine705 phosphorylation with nearly similar kinetics and potencies in both U251MG and NIH3T3/v-Src lines. However, Cmpd1 shows improved inhibitory activity
against ptyrosine705 Stat3 that reflects its structural modifications. Surprisingly, pterostilbene (20 µM) only moderately inhibited Stat3 tyrosine705 phosphorylation in U251MG cells, albeit with similar kinetics as Cmpd1 (Fig. 2D). Therefore, the subtle, acetoxy group change at the 4’ position between Cmpd1 and pterostilbene (Fig. 1A) is sufficient to alter the inhibitory activity against Stat3 tyrosine705 phosphorylation, despite the fact that both agents are equally active against U251MG cell growth [Fig. 1B(i), C(ii)]. These results suggest that ptyrosine705 Stat3 inhibition may only moderately contribute to the effects of pterostilbene against U251MG cell growth. On the other hand, electrospray ionization tandem mass spectrometry (ESI-MS/MS) employed to qualitatively analyze the levels of Cmpd1 (acetyl pterostilbene) in triplicate cellular samples of Cmpd1 (15 µM, 1h)-treated cells, relative to DMSO-treated (control) cells, showed detectable levels of pterostilbene (retention time of 12.5 min) and minimal detection of acetyl pterostilbene (data not shown), suggesting that by 1 h, Cmpd1 has converted to and exists predominantly as pterostilbene in tumor cells. We hypothesize that the early, more potent effect of Cmpd1 on pYStat3 is likely due to the combined activities of both the acetylated and de-acetylated forms.

Stat3 is phosphorylated on serine727, and this modification is associated with increased transcriptional function (Wen et al., 1995). Unexpectedly, Stat3 serine727 phosphorylation was induced in U251MG cells in response to treatment with Cmpd1 as early as 1-3 hours, and returned to near baseline levels by 10-24 h (Fig. 2E). We note that the kinetics of the pserine727 Stat3 induction parallel the inhibition of ptyrosine705 Stat3 (Fig. 2B vs. 2E). Studies have also shown that resveratrol directly or indirectly activates the cellular metabolism master
regulator, 5' adenosine monophosphate-activated protein kinase (AMPK) and the protein deacetylase SIRT1 (Calamini et al., 2010; Fullerton and Steinberg, 2010; Pervaiz and Holme, 2009), and that the activities of SIRT1 leads to the suppression of Stat3 lysine685 acetylation (Jain et al., 1998). Immunoblotting analysis however showed no significant change in acetylated Stat3 (aStat3) in U251MG cells at 3 and 10 h post treatment with Cmpd1 (Fig. 2F) and no significant alteration in the expression of SIRT1 levels (data not shown). To determine whether Cmpd1 could inhibit other Stat family members, mouse fibroblasts over-expressing the human EGFR (NIH3T3/hEGFR) were treated for 1-24 h, stimulated with EGF, and whole-cell lysate prepared and subjected to immunoblotting analysis. Pre-treatment with Cmpd1 diminished both EGF-induced pY705Stat3 and pY701Stat1 (Supplementary Fig. S1C), with similar kinetics. Despite the inhibition of pY701Stat1, Cmpd1 did not show evidence of general cytotoxicity against NIH3T3 cells at 15-20 µM [Fig. 1B(i), 1E].

Cmpd1 induces Src, Erk1/2MAPK, Akt, Hsp27, p38 and mTOR phosphorylation, and has no effects on pJak2 or pEGFR induction

Resveratrol was previously reported to inhibit Stat3 tyrosine705 phosphorylation via inhibition of upstream kinases, including Src and Jak2 (Kotha et al., 2006; Quoc Trung et al., 2013). We asked whether Cmpd1 could similarly inhibit tyrosine kinases, such as pSrc, pJak2, or pEGFR. Surprisingly, pY416Src was strongly induced in U251MG cells in response to Cmpd1 (15 µM) at 30 min to 10 h, and down-regulated by 24 h, with no significant change in total protein levels (Fig. 3A). The enhanced pY416Src, which is a reflection of its autophosphorylation, suggest Src could not be the responsible target site for the inhibition of pY705Stat3 that is observed at 1-10
h in Cmpd1-treated U251MG cells (Fig. 2B). However, the pattern of effect of Cmpd1 on Src was different in v-Src-transformed NIH3T3/v-Src fibroblasts, where pSrc levels were marginally induced at 30 min-3 h post treatment with the compound, and then suppressed or returned to baseline level at 10-24 h (Supplementary Fig. S3A). The decrease in pSrc levels in NIH3T3/v-Src at 10-24 h occurred with similar kinetics as the suppression of Stat3 tyrosine705 phosphorylation in the same cell line (Supplementary Fig. S2A), suggesting the two events may be causally related in the Cmpd1-treated NIH3T3/v-Src, as was similarly reported for the effects of resveratrol in the same cell line (Kotha et al., 2006). By comparison, treatment with 20 µM resveratrol induced an early (30 min) and sustained inhibition of pSrc in both U251MG (Fig. 3B) and NIH3T3/v-Src cell lines (Supplementary Fig. S3B). The early and sustained decline in pSrc levels correlates with the inhibition of ptyrosine705 Stat3 at 1 h and later in both cell lines (Fig. 2C), as was previously reported (Kotha et al., 2006). Data herein therefore reveal differences between Cmpd1 and resveratrol in regards to their respective effects on pSrc induction in U251MG and NIH3T3/v-Src lines and of the possible mechanisms for their inhibitory effects on Stat3 tyrosine705 phosphorylation.

In light of the observation that Src may not be responsible for Cmpd1-mediated inhibition of ptyrosine705 Stat3, we sought to investigate if Src activity could contribute to promoting Stat3 tyrosine705 phosphorylation in U251MG cells by treating cells with the small-molecule Src inhibitor, dasatinib (100 nM). Immunoblotting analysis showed complete suppression of both pSrc and ptyrosine705 Stat3 levels (Fig. 3C) suggesting Src activity promotes pYStat3 induction in the glioma U251MG cell line. However, ptyrosine705 Stat3 levels rebounded at 18-24 h,
despite the fact that pSrc levels remained attenuated, suggesting that other tyrosine kinases contribute to Stat3 tyrosine705 phosphorylation and act as compensatory mechanisms. Tyrosine kinases responsible for Stat3 tyrosine705 phosphorylation include Jaks and EGFR. Immunoblotting analysis showed no change in pJak2 and an apparent moderate induction of pEGFR at 30 min in U251MG cells treated with Cmpd1 (15 µM) (Fig. 3D, 3E). These results together suggest that Jaks, EGFR, and Src are not involved in the mechanisms leading to the reduction of ptyrosine705 Stat3 levels in Cmpd1-treated U251MG cells. By contrast, resveratrol inhibits Stat3 tyrosine705 phosphorylation in part through its suppressive effects on Src activity (Kotha et al., 2006). Treatment with Cmpd1 (15 µM) also promoted early (30 min – 3 h) induction of pErk1/2^{MAPK}, p-p38, pmTOR, and pAkt, while pHsp27 was moderately induced at a later (24 h) time (Fig. 3F-J), indicating that Cmpd1 also triggers the induction of multiple cellular signaling pathways.

*Erk1/2^{MAPK} activation by Cmpd1 leads to pserine727 Stat3 induction and the inhibition of Stat3 tyrosine705 phosphorylation*

It was previously reported that Erk1/2^{MAPK} induction led to suppression of Stat3 tyrosine705 phosphorylation (Jain et al., 1998). Of significance, Cmpd1-mediated induction of pErk1/2^{MAPK} at 30 min (Fig. 3F) occurred prior to Stat3 serine727 phosphorylation at 1 h (Fig. 2E), raising the possibility that pErk1/2^{MAPK} could be responsible for inducing Stat3 serine727 phosphorylation in response to Cmpd1. To assess whether pErk1/2^{MAPK} induction has a causal role in the parallel ptyrosine705 Stat3 inhibition and pserine727 Stat3 induction, U251MG cells were treated with the MEK inhibitor, PD98059, for 30 min, prior to treating the cells with Cmpd1 for 15 or 30 min
and preparing whole-cell lysates for immunoblotting analysis. The results showed parallel suppression of ptyrosine705 Stat3 by Cmpd1 (Fig. 4A, lane 2, pY705Stat3), together with the induction of both pErk1/2^{MAPK} and pserine727 Stat3 in the absence of PD98059 (Fig. 4A, lane 2, and 4B, lanes 2 and 3, pErk1/2^{MAPK}, pS727Stat3), which were all prevented when cells were pretreated with the MEK inhibitor, PD98059 (Fig. 4A, lane 4, and 4B, lanes 6 and 7, pY705Stat3, pS727Stat3, pErk1/2^{MAPK}). The MEK inhibitor alone also strongly suppressed the background pErk1/2^{MAPK} levels and induced ptyrosine705 Stat3 (Fig. 4A, lane 3, and 4B, lanes 4 and 5, pErk1/2^{MAPK}, pY705Stat3). Taken together, these results indicate that Cmpd1 promotes pErk1/2^{MAPK} induction that is sensitive to MEK inhibitor, which in turn phosphorylates serine727 Stat3, and these events are associated with the suppression of ptyrosine705 Stat3.

While treatment with the MEK inhibitor eliminated the background pErk1/2 levels (Fig. 4A, lane 3, 4B, lanes 4 and 5), it failed to completely abrogate pErk1/2 induction in the presence of Cmpd1 (Fig. 4A, lane 4, 4B, lanes 6 and 7), leaving a residual, pErk1/2 activation that may be MEK-independent. To determine whether other pathways are involved in Cmpd1-mediated pErk1/2 induction, we pre-treated U251MG cells with the inhibitors of p38 (SB202190), Src (Dasatinib), mTOR (rapamycin), phosphoinositide 3-kinase (PI 3-kinase)/Akt (LY294002), or the general protein kinase inhibitor, Staurosporine (Staur) prior to treatment with Cmpd1 and whole-cell lysates were prepared for immunoblotting analysis. As noted, pretreatment with PD98059 substantially, albeit not completely, suppressed Cmpd1-induced pErk1/2 levels (Supplementary Fig. S4A). Pre-treatment with the other inhibitors on the other hand had no suppressive effects on Cmpd1-induced pErk1/2 levels (Supplementary Fig. S4A). Pterostilbene
similarly promoted pErk1/2 induction, which was sensitive to the MEK inhibitor, PD98059, but not the inhibitors of p38, PI 3-kinase, Src, mTOR or the general protein kinase inhibitors (Supplementary Fig. S4B). We sought to investigate further the induction of pErk1/2 by Cmpd1 in the context of v-Ras-transformation (NIH3T3/v-Ras) in which Erk1/2 signaling is under the regulation of oncogenic Ras, compared to normal cells (NIH3T3). Cells were pre-treated for 1 h with PD98059 (20 µM) prior to treatment with Cmpd1 (15 µM, 30 min). Immunoblotting analysis of whole-cell lysates showed minimal to moderate modulation by Cmpd1 of pY705Stat3, pS727Stat3 or pErk1/2 in both cell lines (Supplementary Fig. S5). Results further showed the suppression by PD98059 of the resting levels of pErk1/2 in both lines and of the background levels of pS727Stat3 in the transformed NIH3T3/v-Ras line (Supplementary Fig. S5). These results together indicate that Cmpd1 is unable to substantially promote pErk1/2 and pS727Stat3 induction in normal fibroblasts and in transformed cells where pErk1/2 is constitutively induced by the upstream oncogenic Ras.

*Cmpd1 treatment suppresses cell cycle regulatory and apoptotic genes, arrests the cell cycle and induces apoptosis of glioma cells*

With respect to the importance of constitutively-active Stat3 in tumor cell growth and survival (Miklossy et al., 2013), inhibition of Stat3 activity in tumor cells leads to induction of cell cycle arrest and apoptosis by down-regulating Stat3-regulated genes (Zhang et al., 2010; Zhang et al., 2012). Resveratrol was previously shown to induce cell cycle arrest at the G0/G1 or S-phase (Kotha et al., 2006). Western blotting analysis shows 24-h-treatment with Cmpd1 leads to suppression of survivin, mcl-1, bcl-xl, cyclin D1 and cyclin B1 expression in U251MG cells (Fig
5A). Analysis by flow cytometry revealed that similar treatment with Cmpd1 induced cell cycle arrest at the G2/M phase as early as 24 h and at 72 h post treatment, which is associated with decreased populations at GO/G1 and/or S phases (Fig. 5B, upper panel). By contrast, treatment with Cmpd1 only moderately induced a temporary G2/M block in normal NIH3T3 fibroblasts at 24 h, which was reversed by 72 h (Fig 5B, lower panel). Moreover, Cmpd1 treatment induced the cleavage of caspases 3, 8 and 9, and PARP at 48 h post treatment (Fig 5C).

Discussion

First reported as compound 55 (Sun et al., 2010), Cmpd1 [(E)-4-(3,5-dimethoxystyrlyl)phenyl acetate] was subsequently evaluated as compound 86 and found to exhibit no significant activity in quinone reductase (QR) 1 activation, QR2 inhibition, nitric oxide production, aromatase, nuclear factor (NF)κB, COX-1/2, antiproliferative activity, and other in vitro assays (Kondratyuk et al., 2011). In the present study, however, it is notable that this analog is more potent than resveratrol in suppressing the viability and growth of the human glioma, breast, and pancreatic cancer cells, with a preferential effect against U251MG glioma cells, while only weakly affecting normal NIH3T3 at the highest dose tested. The cell growth inhibition, cell cycle block at G2/M phase, and apoptosis induced by Cmpd1 in U251MG cells all parallel the reported inhibitory effects of resveratrol in human breast, pancreatic, prostate, rhabdomyosarcoma, and other tumor cells (Chow et al., 2005; Hadi et al., 2000; Kotha et al., 2006; Meeran and Katiyar, 2008; Quoc Trung et al., 2013). Cmpd1-induced biological responses are preceded by decreases in the expression of cell cycle regulatory and apoptotic genes. Interestingly, Cmpd1 and pterostilbene showed similar inhibitory potency against glioma cell
growth, suggesting the acetoxy group change at the 4’ position has a minimal impact on in vitro activity. Given the susceptibility of ester groups to hydrolysis by intracellular esterases (Fukami and Yokoi, 2012), the 4’ acetoxy group on Cmpd1 appears to be hydrolyzed in tumor cells, thereby generating pterostilbene from Cmpd1, as ESI-MS/MS studies show. It is proposed that the rapid modulation of Stat3 in response to Cmpd1 is likely the combined activities of the acetylated and de-acetylated forms.

The resveratrol analog, Cmpd1 promotes early inhibition of aberrantly-active Stat3 in tumor cells, which would be expected to contribute to the growth inhibitory effects against U251MG, MDA-MB-231, and Panc-1 cells (Kotha et al., 2006; Miklossy et al., 2013). However, the present studies reveal differences between Cmpd1 and resveratrol in the mechanisms leading to the inhibition of Stat3 signaling in tumor cells. In contrast to inhibition of pSrc or Jak2 induction by resveratrol (Kotha et al., 2006; Quoc Trung et al., 2013), Cmpd1 promotes pSrc induction and has no substantial effects on Jaks or EGFR Tyr kinases. Worthy of note is the induction of pErk1/2MAPK, which has been previously reported to suppress Stat3 activity (Jain et al., 1998). Data provided herein show that Cmpd1 mediated pErk1/2MAPK induction that in turn promotes Stat3 serine727 phosphorylation, and the two events have a causal relationship with the inhibition of Stat3 tyrosine705 phosphorylation (Fig. 6). Accordingly, the inhibition of MEK by PD98059 blocked Cmpd1-mediated pErk1/2MAPK and pserine727 Stat3 induction, and prevented the ptyrosine705 Stat3 suppression. The induction of pErk1/2MAPK may promote the formation of an Erk1/2MAPK/Stat3 complex, which would interrupt Stat3 recruitment to activating kinases,
thereby suppressing its tyrosine705 phosphorylation (Fig. 6), as has been previously reported (Tian and An, 2004).

Despite the fact that tumor cells harbor constitutively-active Stat3, they exhibit differential responses that could not all be explained by the inhibition of aberrant Stat3 signaling. Furthermore, human breast cancer MCF7 cells that are also responsive to Cmpd1 do not harbor constitutive Stat3 activity, suggesting other factors contribute to the responsiveness of tumor cells to Cmpd1. Notably, in addition to Erk1/2MAPK activation, Akt, mTOR, p38 and Hsp27 are all induced in response to Cmpd1 (Fig. 6), indicative of pleiotropic effects, as has been observed for resveratrol and its metabolites (Calamini et al., 2010; Fröjdö et al., 2007; Pervaiz and Holme, 2009; Pirola and Fröjdö, 2008; Yu et al., 2001). To what extent the combination of these changes facilitates the growth inhibitory effects of Cmpd1 is presently unclear.

The increased potency of Cmpd1 as compared to resveratrol is likely due to the 3,5-dimethoxy groups (Fig. 1). This is supported by the similar antitumor potency of pterostilbene, which shares the 3,5-dimethoxy groups, and the published reports that methoxylated resveratrol analogues have increased activity against cell viability compared to the parent compound (Mazué et al., 2010). However, pterostilbene had a moderate effect on Stat3 tyrosine705 phosphorylation, compared to Cmpd1, underscoring the differences in the underlying molecular mechanisms between the two compounds. It is notable that Cmpd1 lacks the side chain hydroxyl groups that are known to drive the antioxidant properties and the oxidative stress/redox mechanisms of resveratrol and pterostilbene, mechanisms known to promote...
biological responses (Bhat et al., 2001; Farghali et al., 2013; McCormack and McFadden, 2013; Pan et al., 2008; Rimando et al., 2002), although it remains feasible that the 4’ acetoxy group may become hydrolyzed inside tumor cells. While Stat3 signaling has been reported to be influenced by redox changes (Li et al., 2010; Liu et al., 2012), whether Cmpd1 induces oxidative stress events that contribute to inhibition of Stat3 activation and the induction of antitumor responses in vitro is unknown. The inhibitory activity of Cmpd1 against the Stat3 pathway suggests the potential that this agent could be useful as part of treatment strategies tailored to tumors harboring aberrantly-active Stat3 following in vivo validation of antitumor efficacy in Stat3 relevant tumor models. The present study suggests that gliomas and to a lesser extent breast cancer harboring constitutive Stat3 activity may be responsive to Cmpd1.
Acknowledgements

We thank the members of our laboratory for the stimulating discussions. Flow cytometry services were provided by the Molecular and Cellular Immunology Core of the John A. Burns School of Medicine, University of Hawaii. ESI-MS/MS studies to qualitatively determine the levels of Cmpd1 and pterostilbene were performed by the Analytical Biochemistry Shared Resource (ABSR) at the University of Hawaii Cancer Center.
Authorship Contributions

Participated in research design: Chelsky, Yue and Turkson.

Conducted experiments: Chelsky and Yue.

Contributed new reagents or analytic tools: Kondratyuk, Pezzuto, Cushman and Turkson.

Performed data analysis: Chelsky, Yue, Paladino and Turkson.

Wrote or contributed to the writing of the manuscript: Chelsky, Paladino and Turkson.
References


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Footnotes

This work was supported by the National Institutes of Health/National Cancer Institute rant [CA161931] and the University of Hawaii start-up funds. The Molecular and Cellular Immunology Core is supported in part the National Institute of General Medical Sciences Centers of Biomedical Research Excellence grant [P20GM103516]. The Analytical Biochemistry Shared Resource is supported by the National Institute of Health/National Cancer Institute P30 Cancer Center Support Grant [P30 CA71789].
Figure Legends

Fig. 1. **Resveratrol and analogs, Cmpd1, Cmpd2, Cmpd3 and pterostilbene differentially suppress tumor cell growth and survival.** (A) Structures of Cmpd1, Cmpd2, Cmpd3, resveratrol and pterostilbene; (B, C) CyQuant cell proliferation assay for the effects of 72-h treatment with (B) 0-20 µM [i] Cmpd1, [ii] Cmpd2 and [iii] Cmpd3, or (C) [i] 0-80 µM resveratrol (Res) or [ii] 0-50 µM pterostilbene (PTE) on human glioma (U251MG and SF295), breast (MDA-MB-231) or pancreatic (Panc-1) cancer cells harboring activated Stat3, or breast cancer (MCF7) cells and normal mouse fibroblasts (NIH3T3) that do not. Viable cell numbers as percent of control are plotted; (D and E) U251MG cells (D) or normal NIH3T3 fibroblasts (E) growing in culture were treated once with 0-20 µM Cmpd1 for 0-72 h and viable cells counted at the indicated times by Trypan blue exclusion/phase-contrast microscopy. Viable cell numbers are plotted; and (F) cultured human glioma (U251MG and SF295), breast (MDA-MB-231 and MCF7), and pancreatic (Panc-1) cancer cells, and normal NIH3T3 mouse fibroblast were seeded as single-cells and treated once with 0-20 µM Cmpd1 and allowed to culture until large colonies were visible, which were stained with crystal violet, counted and plotted. Values are the mean ± S.D., n=3-4. *p <0.01*.

Fig. 2. **Effects of Cmpd1, resveratrol and pterostilbene on Stat3 tyrosine and serine phosphorylation and Stat3 acetylation in human glioma cells.** Immunoblots of pYStat3, pSer727Stat3, Stat3, acetylated Stat3 (aStat3), and β-actin from whole-cell lysate preparation from the human glioma U251MG cells harboring aberrantly-active Stat3 untreated or treated for (A) 3 h with 0-20 µM Cmpd1, or (B) 0-24 h with 15 µM Cmpd1, (C) 0-24 h with 20 µM resveratrol (Res), (D) 0-
24 h with 20 µM pterostilbene (PTE), or (E and F) 0-24 h with 15 µM Cmpd1. The positions of proteins in the gel are labeled; bands corresponding to the phospho-Stat3 or acetylated Stat3 protein levels in the gel were quantified by ImageQuant and calculated as a percent of control (DMSO) relative to the total proteins and the β-actin levels; control lane (0) represents whole-cell lysates prepared from 0.025% DMSO-treated cells. Data are representative of three independent determinations.

Fig. 3. **Cmpd1 promotes phospho- Src, Erk1/2MAPK, Akt, mTOR, p38 and Hsp27 induction and has no significant effects on Jak2 and EGFR in glioma U251MG cells.** Immunoblotting analysis of whole-cell lysate preparation from the human glioma U251MG cells untreated or treated with (A, D-J) 15 µM Cmpd1, (B) 20 µM resveratrol (Res), or (C) 100 nM dasatinib (Das) for 0-24 h and probing for pY416Src, Src, pYStat3, Stat3, pJak2, Jak2, pY1173EGFR, EGFR, pErk1/2MAPK, Erk1/2MAPK, p-p38, p38, pmTOR, mTOR, pAkt, Akt, pHsp27, Hsp27, β-actin or GAPDH. The positions of proteins in the gel are labeled; bands corresponding to the phospho-protein levels in the gel were quantified by ImageQuant and calculated as percent of control (DMSO) relative to the total proteins and β-actin or GAPDH levels; control lane (0) represents whole-cell lysates prepared from 0.025% DMSO-treated cells. Data are representative of three independent determinations.

Fig. 4. **Cmpd1-mediated inhibition of pYStat3 is associated with Erk1/2MAPK-induction that promotes pS727Stat3 and is reversed by MEK inhibitor, PD98059.** Immunoblotting analysis of whole-cell lysate preparation from U251MG cells untreated (-) or treated with 15 µM Cmpd1
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for 15 or 30 min following pre-treatment with or without 50 µM PD98059 for the indicated times and probing for pYStat3, pS727Stat3, Stat3, pErk1/2MAPK, Erk1/2MAPK and GAPDH. The positions of proteins in the gel are labeled; bands corresponding to the phospho-protein levels in the gel were quantified by ImageQuant and calculated as percent of control (DMSO) relative to the total proteins and GAPDH levels; control lane (-) represents whole-cell lysates prepared from 0.025% DMSO-treated cells. Data are representative of three independent determinations.

Fig. 5. Cmpd1 suppresses the expression of cell cycle and apoptotic regulatory genes, inhibits cell cycle, and induces caspases and PARP cleavage. (A, C) Immunoblotting analysis of whole-cell lysate preparation from U251MG cells untreated (0) or treated with 15 µM compound 1 for 24 or 48 h and probing for (A) Survivin, Mcl-1, Bcl-xL, Cyclin D1, Cyclin B1 and β-actin or (C) caspase 8, 9, or 3, PARP and β-actin; and (B) cell cycle distribution analysis of U251MG or NIH3T3 cells treated or untreated (DMSO) with 15 µM Cmpd1 for 24 or 72 h, processed by propidium iodide (PI) staining, and analyzed by flow cytometry for DNA content, which is plotted. The positions of proteins in the gel are labeled; bands corresponding to the phospho-protein levels in the gel were quantified by ImageQuant and calculated as percent of control (DMSO) relative to the total proteins and β-actin or GAPDH levels; control lane (0) represents whole-cell lysates prepared from 0.025% DMSO-treated cells. Data are representative of three independent determinations. Values are the mean ± S.D., n=3-4. p <0.01*. 

Molecular Pharmacology Fast Forward. Published on July 2, 2015 as DOI: 10.1124/mol.115.099093
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Fig. 6. Model of Cmpd1-mediated modulation of Stat3 signaling and the role of MEK-Erk1/2 pathway, and Cmpd1-dependent induction of mTOR, p38, Akt, Hsp27 and Src signaling in human glioma cells. Cmpd1-mediates inhibition of Stat3 tyrosine phosphorylation (pY705Stat3) and Stat3-dependent gene transcription, in parallel with the upregulation of pS727Stat3, which is dependent on MEK-Erk1/2 induction, and Cmpd1 further upregulates mTOR(pS2448), p38(pT180/Y182), Akt (pS473), Hsp107 (p…) and Src(pY416) induction in glioma U251MG cells. Question mark (?), not definitively determined; up arrow (↑), induction; down arrow (↓), suppression.
**Table 1.** Inhibitory constants on the effects of Cmpd1 or resveratrol (Res) on growth of human tumor and normal cells.

<table>
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<th>Cell Line</th>
<th>Cmpd1 (IC50, µM)</th>
<th>Res (IC50, µM)</th>
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<td>6.7</td>
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<tr>
<td>MDA-MB-231</td>
<td>18.5</td>
<td>40 ± 5 (Baur and Sinclair, 2006)</td>
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<tr>
<td>MCF-7</td>
<td>10.8</td>
<td>68.3 (Burns et al., 2002)</td>
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<tr>
<td>NIH3T3</td>
<td>≥20</td>
<td>&gt;20 (Jang et al., 1997)</td>
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<tr>
<td>Panc-1</td>
<td>18.1</td>
<td>70 ± 10 (Baur and Sinclair, 2006)</td>
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<tr>
<td>SF-295</td>
<td>&gt;20</td>
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</table>

nd, not determined
Fig. 2.

A Dose-response effects of Cmpd1 on pYStat3
Cmpd1 (µM, 3 h) 0 5 15 20

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<tr>
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<td>100%</td>
<td>100%</td>
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</table>

B Time-course effects of Cmpd1 on pYStat3
15 µM Cmpd1 (h) 0 1 3 10 24

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<td>82%</td>
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<tr>
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<td>100%</td>
<td>100%</td>
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</table>

C Time-course effects of resveratrol on pYStat3
20 µM Res (h) 0 1 3 10 24

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D Time-course effects of pterostilbene on pYStat3
20 µM PTE (h) 0 1 3 10 24

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<tr>
<td>β-actin</td>
<td>100%</td>
<td>100%</td>
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</table>

E Time-course effects of Cmpd1 pSer727Stat3
15 µM Cmpd1 (h) 0 1 3 10 24

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<td>100%</td>
<td>100%</td>
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F Time-course effects of Cmpd1 of Stat3 acetylation
15 µM Cmpd1 (h) 0 1 3 10 24

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<td>91%</td>
<td>82%</td>
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</tr>
<tr>
<td>β-actin</td>
<td>100%</td>
<td>100%</td>
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</tbody>
</table>
Fig. 3.

A Time-course effects of Cmpd1 on pY416Src
15 µM Cmpd1 (h) 0 0.5 3 10 24
pSrc 100% 166% 144% 172% 40%
Src
β-actin

B Time-course effects resveratrol on pY416Src
20 µM Res (h) 0 0.5 3 10 24
pSrc 100% 32% 85% 46% 78%
Src
GAPDH

C Time-course effects of dasatinib on pYStat3
100 nM Das (h) 0 0.5 1 3 6 18 24
pYStat3 100% 6% 3% 5% 5% 89% 119%
Stat3
pSrc
Src
β-actin

D Time-course effects of Cmpd1 on pJak2
15 µM Cmpd1 (h) 0 0.5 3 10 24
pJak2 100% 76% 91% 73% 110%
Jak2
β-actin

E Time-course effects of Cmpd1 on pY1173EGFR
15 µM Cmpd1 (h) 0 0.5 1 3
pY1173EGFR 100% 134% 98% 93%
EGFR
β-actin

F Time-course effects of Cmpd1 on pErk1/2
15 µM Cmpd1 (h) 0 0.5 3 10 24
pErk1/2 100% 382% 168% 87% 187%
Erk1/2
GAPDH

G Time-course effects of Cmpd1 on p-p38
15 µM Cmpd1 (h) 0 0.5 1 3
p-p38 100% 136% 125% 187% 169%
p38
p-p38
β-actin

H Time-course effects of Cmpd1 on pmTOR
15 µM Cmpd1 (h) 0 0.5 3 10 24
pmTOR 100% 204% 314% 212% 186%
mTOR
GAPDH

I Time-course effects of Cmpd1 on pAkt
15µM Cmpd1 (h) 0 0.5 3 10 24
pAkt 100% 187% 167% 262% 245%
Akt
β-actin

J Time-course effects of Cmpd1 on pHsp27
15 µM Cmpd1 (h) 0 0.5 3 10 24
pHsp 27 100% 127% 75% 106% 157%
Hsp 27
GAPDH
**Fig. 4.**

(A) Protein expression levels of phosphor-Stat3 (pS727Stat3), Stat3, phosphor-Stat3 (pYStat3), Erk1/2, and GAPDH. Assays were conducted with 15 μM Cmpd1 (min) and 50 μM PD98059 (min) in Lane 1-4.

(B) Protein expression levels of phosphor-Stat3 (pS727Stat3), Stat3, phosphor-Erk1/2, and GAPDH. Assays were conducted with 15 μM Cmpd1 (min) and 50 μM PD98059 (min) in Lane 1-7.
Fig. 5.

A

15 μM Cmpd1 (h)

Survivin

β-actin

Mcl-1

β-actin

Bcl-xL

β-actin

Cyclin D1

β-actin

Cyclin B1

β-actin

B

U251MG

24 h

72 h

% Distribution

Sub G1 G0/G1 S G2/M

% Distribution

Sub G1 G0/G1 S G2/M

C

15 μM Cmpd1 (h)

Caspase 8

Caspase 9

Caspase 3

PARP

β-actin

NIH3T3

DMSO

15 μM Cmpd1

DMSO

15 μM Cmpd1

DMSO

15 μM Cmpd1

DMSO

15 μM Cmpd1

DMSO

15 μM Cmpd1

DMSO

15 μM Cmpd1

DMSO

15 μM Cmpd1

DMSO

15 μM Cmpd1

DMSO

15 μM Cmpd1

DMSO

15 μM Cmpd1

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15 μM Cmpd1

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15 μM Cmpd1

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15 μM Cmpd1

DMSO

15 μM Cmpd1

DMSO

15 μM Cmpd1

DMSO

15 μM Cmpd1

DMSO

15 μM Cmpd1

DMSO
Fig. 6.

Glioma Cells

MEK

pErk1/2

pS727Stat3

Stat3-mediated gene transcription

pS473Akt

pY416Src

pS78/82Hsp27

pT180/Y182p38

pS2448mTOR

pS78/82Hsp27
A resveratrol analogue promotes ERK\textsuperscript{MAPK}-dependent Stat3 serine and tyrosine phosphorylation alterations and antitumor effects \textit{in vitro} against human tumor cells

Zachary L. Chelsky, Peibin Yue, Tamara P. Kondratyuk, David Paladino, John M. Pezzuto, Mark Cushman, and James Turkson
Figure Legends

Fig. S1. **Cmpd1 preferentially suppress colony formation of malignant cells.** Cultured human glioma (U251MG and SF295), breast (MDA-MB-231 and MCF7), and pancreatic (Panc-1) cancer cells, and normal NIH3T3 mouse fibroblast were seeded as single-cells and treated once with 0-20 μM Cmpd1 and allowed to culture until large colonies were visible, which were stained with crystal violet and imaged. Data are representative of three independent determinations.

Fig. S2. **Effects of Cmpd1 and resveratrol on constitutive or ligand-induced Stat3 and Stat1 activation in normal or mouse transformed fibroblasts.** Immunoprobes of pYStat3, pSer727Stat3, Stat3, pY701Stat1, Stat1 and β-actin from whole-cell lysate preparation from (A and B) v-Src-transformed mouse fibroblasts (NIH3T3/v-Src) harboring aberrantly-active Stat3 untreated or treated for 0-24 h with (A) 15 μM Cmpd1 or (B) 20 μM resveratrol (Res); or (C) mouse fibroblasts over-expressing the human EGF receptor (NIH3T3/hEGFR) pre-treated with or without 15 μM Cmpd1 for 0-24 h and stimulated with EGF for 15 min. The positions of proteins in the gel are labeled; bands corresponding to the phospho-protein levels in the gel were quantified by ImageQuant and calculated as a percent of control (DMSO) relative to the total proteins and the β-actin levels; control lane (-, 0) represents whole-cell lysates prepared from 0.025% DMSO-treated or un-stimulated cells. Data are representative of three independent determinations.

Fig. S3. **Differential effects of Cmpd1 and resveratrol on Src induction in v-Src-transformed mouse fibroblasts.** Immunoblots of pY416Src, Src and β-actin from whole-cell lysate preparation from
v-Src-transformed mouse fibroblasts (NIH3T3/v-Src) harboring aberrantly-active Stat3 untreated or treated for 0-24 h with (A) 15 µM Cmpd1 or (B) 20 µM resveratrol (Res). The positions of proteins in the gel are labeled; bands corresponding to the phospho-protein levels in the gel were quantified by ImageQuant and calculated as a percent of control (DMSO) relative to the total proteins and the β-actin; control lane (0) represents whole-cell lysates prepared from 0.025% DMSO-treated cells. Data are representative of three independent determinations.

Fig. S4. Phospho-Erk1/2MAPK induction mediated by Cmpd1 and pterostilbene is reversed by MEK inhibitor, PD98059, but not the general protein kinase inhibitor, staurosporine or the inhibitors of p38, mTOR, PI 3-kinase/Akt, or Src. Immunoblotting analysis of whole-cell lysate preparation from U251MG cells pre-treated with (+) or without (-) the designated concentrations of PD98059, SB202190, LY294002 (LY), dasatinib (Das), rapamycin (Rap) or staurosporine (Staur) for 1 h prior to treatment with (A) 15 µM Cmpd1 or (B) 20 µM pterostilbene (PTE) and probing for pErk1/2MAPK and Erk1/2MAPK. The positions of proteins in the gel are labeled; control lane (-) represents whole-cell lysates prepared from 0.025% DMSO-treated cells. Data are representative of two independent determinations.

Fig. S5. Lack of modulation by Cmpd1 of Stat3 and Erk1/2MAPK phosphorylation in normal and v-Ras-transformed mouse fibroblasts. Immunoblotting analysis of whole-cell lysate preparation from normal (NIH3T3) and v-Ras-transformed (NIH3T3/v-Ras) mouse fibroblasts pre-treated with (+) or without (-) 20 µM PD98059 for 1 h prior to treatment with 15 µM Cmpd1 and
probing for pY705Stat3, pS727Stat3, Stat3, pErk1/2MAPK, and Erk1/2MAPK. The positions of proteins in the gel are labeled; control lane (-) represents whole-cell lysates prepared from 0.025% DMSO-treated cells. Data are representative of two independent determinations.
Supplementary Figures

Fig. S1

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Fig. S2

A

15 µM Cmpd1 (h) 0 1 3 10 24
pYStat3
Stat3
β-actin
NIH3T3/v-Src

B

20 µM Res (h) 0 1 3 10 24
pYStat3
Stat3
β-actin
NIH3T3/v-Src

C

Time (h) - 1 1 3 10 24 24
EGF - + + + + +
15 µM Cmpd1 - - + + + + -
DMSO - + - - - - +
pYStat3 100% 89% 80% 82% 82% 85%
Stat3 100% 69% 59% 69% 59% 88%
pY701Stat1 100% 69% 59% 69% 59% 88%
Stat1 100% 69% 59% 69% 59% 88%
β-actin 100% 69% 59% 69% 59% 88%

NIH3T3/hEGFR
Fig. S3

A 15 µM Cmpd1 (h) 0 0.5 3 10 24

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B 20 µM Res (h) 0 0.5 3 10 24

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Fig. S4

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B

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Fig. S5

20 µM PD98059 (1 h) - - + +
15 µM Cmpd1 (30 min) + - - +

pY705Stat3
pS727Stat3
Stat3
pErk1/2
ERK1/2

NIH3T3
NIH3T3/v-Ras