15α-methoxypuupehenol Induces Antitumor Effects In Vitro and In Vivo against Human Glioblastoma and Breast Cancer Models

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Abstract

Studies with 15α-methoxypuupehenol (15α-MP), obtained from the extracts of Hyrtios species, identified putative targets that are associated with its antitumor effects against human glioblastoma and breast cancer. In the human glioblastoma (U251MG) or breast cancer (MDA-MB-231) cells, treatment with 15α-MP repressed pT705Stat3, pErk1/2, pS107/119CyclinB1, pY507Alk (anaplastic lymphoma kinase), and pY788ezrin levels and induced pS10merlin, without inhibiting pAKT (Janus kinase) or pAkt induction. 15α-MP treatment induced loss of viability of breast cancer (MDA-MB-231, MDA-MB-468) and glioblastoma (U251MG) lines and glioblastoma patient–derived xenograft cells (G22) that harbor aberrantly active Stat3, with only moderate or little effect on the human breast-derived xenograft cells (G22) that harbor aberrantly active Stat3, compared with moderate or little effects on human lung fibroblast, WI-38, or normal mouse embryonic fibroblast (MEF Stat3−/−) lines that do not harbor constitutively active Stat3 or the Stat3-null (Stat3−/−) mouse astrocytes. 15α-MP–treated U251MG cells have severely impaired F-actin organization and altered morphology, including the cells rounding up, and undergo apoptosis, compared with a moderate, reversible morphology change observed for similarly treated mouse astrocytes. Treatment further inhibited U251MG or MDA-MB-231 cell proliferation, anchorage-independent growth, colony formation, and migration in vitro while only moderately or weakly affecting MCF7 cells or normal mouse astrocytes. Oral gavage delivery of 15α-MP inhibited the growth of U251MG subcutaneous tumor xenografts in mice, associated with apoptosis in the treated tumor tissues. Results together suggest that the modulation of Stat3, CyclinB1, Alk, ezrin, merlin, and Erk1/2 functions contributes to the antitumor effects of 15α-MP against glioblastoma and breast cancer progression.


Introduction

Glioblastoma is the most common primary malignant brain tumor in adults. Patients with glioblastoma have a poor prognosis and a median survival of approximately 15 months (1, 2). The tumors are aggressive, high-grade glial neoplasms, typically resistant to current chemotherapy and radiotherapy. They frequently infiltrate into the normal brain tissue, which complicates surgery (3). Despite the aggressive nature, up to 30% of breast cancer mortality remains high among women. While the estrogen receptor (ER), progesterone receptor (PR), and the HER2-positive breast cancer subtypes are managed with targeted therapies (4). In particular, HER2-positive tumors and TNBC generally show worse prognosis (5).

Stat3 has emerged as a validated target for the development of anticancer therapeutics. Stat3 is activated by tyrosine (Tyr) phosphorylation mediated by kinases, such as Janus kinases (JAKs) or Src. Normal Stat3 signaling is transient and receptive to growth factors to mediate cell growth and differentiation, inflammation, and other responses (6, 7). However, in many human cancers, including glioblastoma (8), breast (9), ovarian (10), lung (11), and pancreatic cancers (12), Stat3 is constitutively active, whereby it upregulates cell growth and proliferation, cell survival, migration and invasion, and tumor angiogenesis and promotes tumor immune tolerance (13, 14). It dysregulates gene expression, including CyclinD1, c-Myc, Bcl-2, Bcl-xL, VEGF receptor, and matrix metalloproteinases (13). In proof-of-concept studies, the inhibition of persistently active Stat3 by genetic or pharmacologic approaches blocks tumor cell growth, promotes tumor cell death, and inhibits tumor growth in vivo (15, 16).

As notable sources of bioactive molecules, natural products represent potential sources of chemical probes of potential anticancer value. 15α-methoxypuupehenol (15α-MP) was isolated from the Hyrtios species as a bioactive metabolite. Specifically, 15α-MP suppressed the viability, anchorage-independent growth, colony formation, and migration and altered the morphology in vitro of glioblastoma or breast cancer cells that harbor constitutively active Stat3, compared with moderate or little effects on...
the human breast cancer, MCF7, colorectal adenocarcinoma, Caco-2, or normal human lung fibroblast, WI-38 cells. In contrast, the natural product only had reversible effect on the morphology of normal mouse astrocytes or was weakly active against the viability, proliferation, or migration of the nontransformed cells.

The oral gavage delivery of 15e-MP inhibited the growth of subcutaneous U251MG tumor xenografts in vivo, with evidence of apoptosis in treated tumor tissues. Data together suggest that the antitumor effects of 15e-MP are, in part, due to the modulation of constitutively active Stat3, pErk1/2, pY478ezrin, merlin, pS147cyclinB1, and pY507Alk (anaplastic lymphoma kinase) induction.

Materials and Methods

Cell culture and reagents

The human glioblastoma, U251MG line, the patient-derived xenograft cells, G22, and the breast cancer, MDA-MB-231, MDA-MB-468, and MCF7 cells, have all been previously reported (17). The normal Stat3 floxed mouse embryonic fibroblast line (MEF Stat3fl/fl) and the mouse astrocytes (normal Stat3k0oxP/k0oxP and their knockout counterparts, Stat3fl/fl) were kind gifts of Drs. Valeria Poli, University of Turin (Turin, Italy; ref. 18) and Azad Bonni, Washington University (St Louis, MO; ref. 19), respectively. The human epithelial colorectal adenocarcinoma, Caco-2, and normal human lung fibroblast, WI-38 cells, were obtained from the ATCC. U251MG cells were grown in RPMI (Roswell Park Memorial Institute)-1640 (Cellgro), with 5% FBS (Gibco), and containing 1% nonessential amino acids (Cellgro) and 1% penicillin/streptomycin (Pen/Strep; Gibco). MDA-MB-231, MDA-MB-468, and MCF7 cells, normal MEFs (MEF Stat3fl/fl), the normal mouse astrocytes, Stat3k0oxP/k0oxP and their Stat3fl/fl counterparts were grown in DMEM, containing 10% FBS and 1% Pen/Strep. The G22 cells were cultured in DMEM containing 10% heat-inactivated FBS, whereas the Caco-2 and WI-38 cells were grown in Eagle’s Minimum Essential Medium, supplemented with 20% FBS and 1% Pen/Strep. The MDA-MB-231, MDA-MB-468, and MCF7 lines were obtained in 1996; U251MG and G22 cells were obtained in 2011 and 2013, respectively; Caco-2 and WI-38 cells in 2016, whereas the mouse astrocytes and fibroblast lines were all obtained in 2015. Except for MDA-MB-231 and MDA-MB-468, which were authenticated using American Type Tissue Culture Cell Line Authentication Service on December 16, 2015, all other cells have not been recently authenticated. Antibodies against Stat3, pStat3, c-Myc, CyclinD1, pErk1/2, Erk1/2, pAkt, Akt, pJAK2, JAK2, β-tubulin, PARP, and cleaved PARP (Asp214), ERα (Cell Signaling Technology), β-actin, β-tubulin, GAPDH (Santa Cruz Biotechnology, Inc.), pS10merlin, FLAG (Sigma-Aldrich), p-ezrin, pCyclinB1 (Abcam), ezrin, merlin, CyclinB1, pAlk, pS518merlin (Novus Biologicals), and Alk (SDIX) were purchased from the indicated sources.

Isolation and purification of 15e-MP

Details of the isolation of 15e-MP from the Hyrtios species and its structural characterization have been described in the Methods of Supplementary Materials and as previously reported (20).

Cell viability assay

CyQuant cell proliferation assay (Invitrogen/Life Technologies Corp) was performed as previously reported (17) and following the manufacturer’s instructions. Details are described in the Methods of Supplementary Materials.

Colony survival assay

This assay was performed as previously reported (17). More details are provided in the Methods of Supplementary Materials.

Soft-agar colony formation assay

Studies were conducted as previously reported (21) with some modifications. Details are provided in the Methods of Supplementary Materials.

Scratch and Boyden chamber assays

Studies were performed as previously reported (22) with some modifications. Details are provided in the Methods of Supplementary Materials.

Bromodeoxyuridine incorporation assay

Cells were seeded on glass coverslips in 24-well plates and synchronized by serum starvation for 18 hours. Following a 9-hour recovery period, cells were untreated (DMSO) or treated with 5 μM 15e-MP for 17 hours and subjected to fixation. Two hours prior to fixation, bromodeoxyuridine (BrdUrd; 10 μmol/L) was added to the culture media. Cells were fixed in 4% paraformaldehyde for 10 minutes, permeabilized by adding 1.5 mol/L HCl, blocked with 5% goat serum in 0.3% Triton X-100 in PBS, and stained with anti-BrdUrd antibody (Cell Signaling Technology) overnight at 4°C according to the manufacturer’s protocol. The coverslips were mounted on microscope slides using DAPI Fluoromount-G mounting medium (Southern Bio-tech). Images were visualized with an Olympus IX71 inverted fluorescence microscope (Olympus) and taken using a Retiga 2000R (Q Imaging) mounted camera. Cells were counted using the ImageJ software (NIH, Bethesda, MD).

Immunoprecipitation and immunoblotting analyses

Immunoprecipitation and immunoblotting analyses were performed as previously reported (17, 23). Details are provided in the Methods of Supplementary Materials. Whole-cell lysate preparation and immunoblotting analysis were performed as previously reported (17, 23).

Cloning and site-directed mutagenesis

Merlin sequence was amplified from the pCMV-XL4-merlin construct (Origene) and inserted between the KpnI and BamHI restriction sites in the pX40 plasmid (24), downstream from the FLAG coding sequence. Details of the primers and the mutagenesis are described in the Methods of Supplementary Materials.

Transient transfection

Details of the transfection using Lipofectamine 3000 and following the manufacturer’s instructions are provided in the Methods of Supplementary Materials.

Immunofluorescent imaging

Immunofluorescent studies were performed as previously reported (17). Details are provided in the Methods of Supplementary Materials.
15α-Methoxypropupehenol Induces Antitumor Effects

Phospho-protein profiling explorer array

The antibody array assay was performed according to the manufacturer's protocol (Full Moon Biosystems, Inc.) and described in details in the Methods of Supplementary Materials.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay

Staining was performed with Promega’s DeadEnd Colorimetric TUNEL System kit according to the manufacturer’s instructions. Briefly, the paraffin-embedded tissue sections were deparaffinized and rehydrated, fixed with 4% paraformaldehyde solution, and permeabilized with Proteinase K. Biotinylated nucleotides were incorporated into the 3′-OH DNA ends using the recombinant terminal deoxynucleotidyl transferase and detected by the horse-radish peroxidase–labeled streptavidin and 3,3′-diaminobenzidine (DAB) colorimetric staining method. The endogenous peroxidases were blocked by 0.3% hydrogen peroxide. Tissue sections processed without the TdT enzyme were used as negative controls, and positive controls were section-treated with DNase I. Under this procedure, the apoptotic nuclei in tissue sections were stained dark brown. A total of 14 to 15 tissue sections for each group were stained and analyzed. A scoring system was set for assessing the staining of all of the tissue sections. On the basis of the staining of the negative and positive controls, the number of positive or negative staining of tissue sections in each group was counted. The χ² test was used to assess whether there is a significant difference in staining between the 2 groups.

Antitumor activity studies in vivo

All mice studies were performed under an Institutional Animal Care and Use Committee (IACUC)-approved protocol. Four-week-old female athymic nude mice were purchased from the Harlan Laboratories and maintained in institutional animal facilities. Mice were injected subcutaneously in the right flank with the human glioblastoma U251MG (7.3 × 10⁶ in 200 μl of equal mixture of PBS and Matrigel) cells. Once tumors were palpable, treatment was initiated at 3 mg/kg, 3× per week, via oral gavage (100 μl). Animals were monitored daily and the body weights were taken, and tumor sizes were measured using calipers. Tumor volumes were calculated as V = 0.52 × a² × b, where a is the smallest diameter and b is the largest diameter. Tumor volumes of the treatment groups were compared with the control group using a paired t test.

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 < 0.05. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining quantification was analyzed using the χ² test comparing the treated tumor samples with the control, with significance of difference (*) between groups at P < 0.001.

Results

Isolation and characterization of 15α-MP

15α-MP was isolated from Hypo species sponge and identified as 15α-MP with a molecular weight of 360.49 (Fig. 1A). The compound was identified by comparison of mass spectrometric and NMR spectroscopic (1D and 2D) data with that previously reported (20).

15α-MP suppresses viability, colony survival, malignant transformation, cellular motility, and migration and induces morphology changes and apoptosis of human glioblastoma or breast cancer cells

Human glioblastoma (U251MG and G22), breast (MDA-MB-231, MDA-MB-468, and MCF7), colorectal (Caco-2) cancer cells, the normal human lung fibroblasts, WI-38, the normal MEF (MEF Stat3-fi/−), and Stat3 knockout mouse astrocytes (Stat3−−) were treated once with 0 to 15 μmol/L 15α-MP for 72 hours and analyzed by CyQuant viability assay for viable cells. Treatment with 15α-MP variably and preferentially inhibited the viability of U251MG, G22, MDA-MB-468, and MDA-MB-231 cells that harbor aberrantly active Stat3, with IC50 values of 3.5 ± 0.4, 8.5 ± 0.6, 3.0 ± 0.5, and 9.6 ± 0.1 μmol/L, respectively [Fig. 1B (i) and (ii)]. In contrast, the compound only moderately inhibited the viability of MCF7 cells, with IC50 of 12.0 ± 0.3 μmol/L [Fig. 1B (ii)] or was weak against the normal human lung fibroblasts, WI-38, the normal MEF Stat3-fi/−, the Stat3−− astrocytes, or the colorectal cancer Caco-2 cells, with IC50 values > 15 μmol/L [Fig. 1B (iii)]. Immunoblotting analysis shows cleaved PARP, indicating evidence of apoptosis of U251MG cells treated with 5 μmol/L 15α-MP for 24 or 48 hours or with 10 nmol/L paclitaxel for 48 hours (Fig. 1C). Colony survival assay shows that one-time treatment with 1- to 5 μmol/L 15α-MP dose dependently suppressed the colony numbers of glioma U251MG and G22 and breast cancer MDA-MB-231 and MDA-MB-468 cells while only weakly affecting that of MCF7 cells [Fig. 2A (i) and (ii)]. In soft-agar assay, one-time treatment with 5 μmol/L 15α-MP preferentially suppressed the anchorage-independent growth of both U251MG and MDA-MB-231 cells [Fig. 2B (i) and (ii)], with statistically insignificant effects on MCF7 cells [Fig. 2B (ii)]. Scratch assay shows that treatment with 10 μmol/L 15α-MP of glioma, U251MG or G22 cells for 5, 18 or 22 hours or of breast cancer MDA-MB-231 cells for 5 or 7 hours decreased their migration into the denuded areas (Fig. 2C). Similar results were obtained in the Boyden chamber assay where 2.5 or 5 μmol/L 15α-MP inhibited the migration of both the glioma U251MG and breast cancer MDA-MB-231 cells, with only a weak effect on that of MCF7 cells [Fig. 2D]. Moreover, U251MG cells treated for 17 hours with 5 μmol/L 15α-MP showed impaired BrdUrd incorporation, in contrast to no observable effect on that of normal mouse astrocytes Stat3fi/−, indicative of the preferential inhibition of glioblastoma cell proliferation (Fig. 2E).

One of the most striking effects of 15α-MP is observed on the morphology of tumor cells. Phase-contrast microscopy analysis shows that U251MG cells treated with 5 μmol/L 15α-MP become rounded up at 24 hours, in contrast to minimal morphology change observed for similarly treated normal mouse astrocytes Stat3fi/− (Fig. 3A, top, 24 hours). Upon the treatment with higher concentration (10 μmol/L), there were early dramatic morphology changes at 3 hours on U251MG cells, compared with minimal effect on the normal mouse astrocytes Stat3fi/− (Fig. 3A, bottom, 3 hours). Surprisingly, a similar treatment of the human breast cancer, MDA-MB-231 and MCF7 cells, had minimum effects on cell morphology up to 72 hours (Supplementary Fig. S1), despite that the viability of these cells are variably inhibited by 15α-MP (Fig. 1B).

We wanted to determine whether the effect on tumor cell morphology is reversible. U251MG cells or the normal mouse astrocytes Stat3fi/− were treated with 10 μmol/L
Figure 1. 15α-MP suppresses cell viability and induces apoptosis of human glioblastoma or breast cancer cells. A, Structure of 15α-MP. B, Human glioblastoma line (U251MG) and patient-derived xenograft cells (G22) (i), human breast cancer (MDA-MB-231, MDA-MB-468, MCF7) (ii), and human colorectal adenocarcinoma (Caco-2) cells, normal human lung fibroblasts, WI-38, mouse astrocytes (Stat3^+/−/−), and mouse embryonic fibroblasts (MEF Stat3^{fl/fl}) (iii) were untreated or treated once with 1 to 15 μmol/L 15α-MP for 72 hours. Cells were assayed for viability using CyQuant cell proliferation kit. IC50 values were derived from the dose–response curves. C, Immunoblots of cleaved PARP, PARP, and tubulin in whole-cell lysates of U251MG cells treated with the designated compounds for 24 or 48 hours. Control samples (0, con) represent cells treated with 0.05% DMSO. Values, mean ± SD of 3–4 independent experiments. Data are representative of 2 independent experiments.
15α-MP for 4 hours, replaced the culture media with fresh media free of 15α-MP, and incubated the cells further, while visualizing under the microscope after 4.5 hours (8.5 hours total) and 19 hours (23 hours total). As expected, the prior 4-hour treatment with 15α-MP induced a striking morphology change in U251MG cells, which persisted and was...
unrecoverable when the culture media was replaced with fresh media [Fig. 3B (ii), bottom, 4, 4.5, and 19 hours]. In the case of the normal astrocytes Stat3loxP/loxP, there was no substantial morphology change during the 4-hour treatment prior [Fig. 3B (i), bottom, 4, 4.5, 19 hours]. Consistent with the morphology changes, U251MG cells also displayed substantial disruption of the F-actin and the cytoskeletal structural organization that started at 30 minutes following treatment with 10 μmol/L 15α-MP, and which culminated in the rounding up of the cells by 3 hours (Fig. 4A). In comparison, similar treatment only had relatively marginal effects on F-actin organization in MDA-MB-231 cells (Fig. 4B) and no observable effect on that of the normal astrocytes Stat3loxP/loxP up to 3 hours (Fig. 4C). Therefore, 15α-MP more strongly promotes early irreversible changes in the F-actin and cytoskeletal structural organization and the morphology of glioblastoma cells.

Phospho-proteomics analysis reveals alterations in key proteins in 15α-MP–treated glioblastoma cells

To better understand the underlying mechanisms for the observed biologic responses, phospho-proteomic analysis (25) was performed. The U251MG cells were treated or untreated with 10 μmol/L 15α-MP for 30 minutes and 1 hour, and the cells for the 2 time points were pooled together and whole-cell lysates prepared and exposed to site-specific antibodies in the phospho explorer antibody microarray kit, as described in the Materials and Methods. Although the array contained more than 1,300 proteins, the notable phosphoproteins that were altered dramatically in response to 15α-MP are...
Figure 4.
15α-MP induces F-actin disorganization in human glioblastoma cells. A–C, Immunofluorescent microscopic images of U251MG cells (A), MDA-MB-231 cells (B), or normal mouse astrocytes Stat3loxP/loxP (C), untreated or treated with 10 μmol/L 15α-MP for 0.5 to 3 hours and stained with phalloidin for F-actin and DAPI for the nucleus. Control samples represent cells treated with 0.05% DMSO. Data are representative of 2 to 3 independent experiments.
shown in Supplementary Fig. S2. These include targets whose functions impact cellular structurally directed processes, such as pY731VE-cadherin, a glycoprotein of the cadherin superfamily involved in endothelial cell-cell adhesion (26), pY125WAVE1 (Wiskott-Aldrich syndrome protein), WASP family member involved in regulating the actin cytoskeletal organization (27), pS339CXCR4, a member of the chemokine receptors that regulates morphogenesis, angiogenesis, and immune responses (28), and pS10merlin levels, which all increased in response to the treatment with 15α-MP, compared with control (Supplementary Fig. S2). Other targets include pY478ezrin, pS147cyclinB1, and pY1507Alk levels, which decreased (Supplementary Fig. S2).

To further investigate the altered phospho-proteins in the phospho explorer antibody microarray studies, immunoblotting analysis was performed. Pooled whole-cell lysates of equal total protein from similarly treated U251MG or G22 cells were immuno-probed focusing on select proteins. Results confirmed decreased pY478ezrin, pS147cyclinB1, and pY1507Alk expressions and increased pS10merlin levels in U251MG cells (Fig. 5A, left), which are more sensitive to 15α-MP (Fig. 1B, U251MG).

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Modulatory effect of 15α-MP on Stat3, ezrin, merlin, CyclinB1, and Alk induction. Immunoblotting analysis of whole-cell lysates of equal total protein prepared from (A–C, F) U251MG or G22 or (D) MDA-MB-231 cells untreated or treated with 10 μmol/L 15α-MP for (A) 0.5 or 1 hour and pooled together or for (B) 0.5 to 5 hours or (C, D, F) 0.0167 to 24 hours or (E) U251MG cells untreated or treated with 10 μmol/L 15α-MP for 0.5 or 1 hour and pooled together (i) or untreated and transiently transfected with the pcDNA3 empty vector (mock) or the designated FLAG-tagged merlin constructs (WT, or mutants, merlinS10A or merlinS10E). The cropped S10E lane is part of the same gel (ii) and probing for pY705Stat3, Stat3, pY478ezrin, ezrin, pS10merlin, pS147cyclinB1, cyclinB1, pY1507Alk, Alk, pErk, Erk, pJAK2, JAK2, pAkt, Akt, c-Myc, CyclinD1, FLAG, actin, and α,β-tubulin. Positions of proteins in gel are labeled. Control lane (c) represents whole-cell lysates prepared from cells treated with 0.025% DMSO. Data are representative of 2 to 4 independent experiments.
Also, pY478ezrin, pS147CyclinB1, and pY1507Alk were all suppressed (Fig. 5A, right) in the moderately sensitive G22 cells (Fig. 1B, G22), without changes in merlin. Focusing on ezrin and merlin due to their counter roles in promoting or suppressing tumor phenotype (29), we note that the constitutive pS10merlin levels are unaffected by the 15α-MP treatment (Fig. 5B).

15α-MP also inhibits Stat3 phosphorylation, Erk1/2 induction, and the target gene, c-Myc, expression in glioblastoma or breast cancer cells

Given that U251MG, G22, and MDA-MB-231 cells all harbor constitutively active Stat3, we were interested in determining the effect of 15α-MP on Stat3 activation in the tumor cells. 15α-MP treatment inhibited the pY705Stat3 in a time-dependent manner, starting as early as 30 minutes, with a restoration of pY705Stat3 levels at 6 hours in U251MG or at 24 hours in MDA-MB-231 cells (Fig. 5C and D). The restoration of pStat3 levels after the initial inhibition has been similarly observed by us and others (30, 31) in different contexts and suggested to be, in part, because of the re-employment of redundant Tyr kinases as a compensatory mechanism to re-activate Stat3, despite that the treated tumor cells ultimately undergo cell death (32). 15α-MP treatment also decreased pErk1/2 levels after 3 to 24 hours in both U251MG and MDA-MB-231 cells (Fig. 5C and D). In contrast, the compound minimally affected pAkt2 or pAkt levels, except for the brief pAkt induction at 0.5 to 1 hour in MDA-MB-231 cells (Fig. 5D).

It is reported that the overexpression of merlin led to decreased pY705Stat3 (33). Given the dual modulation of pY705Stat3 and pS10merlin by 15α-MP, we sought to determine whether the 2 events are related. Similar to the alterations in pY478ezrin, pS147cyclinB1, pY1507Alk, and pS10merlin expression (Fig. 5A), immunoblotting analysis of the pooled whole-cell lysates from U251MG cells treated with 10 μmol/L 15α-MP for 30 minutes and 1 hour showed strongly suppressed pY705Stat3 levels [Fig. 5E (i)]. Moreover, U251MG cells were transiently transfected with Flag-tagged merlin constructs [wild-type (WT), merlinS10A (mimicking unphosphorylated merlin; ref. 34), and merlinS10E (mimicking phosphorylated merlin; ref. 35), and whole-cell lysates were prepared for immunoblotting analysis. The overexpression of each of the merlin constructs (WT, merlinS10A, or merlinS10E) decreased the levels of pY705Stat3, compared with mock-transfected cells [Fig. 5E (ii)]. Consistent with the inverse relationship in the protein expressions between merlin and ezrin, as observed in Fig. 5A and B and in the phospho-protein array (Supplementary Fig. S2), the overexpression of each of the exogenous merlin constructs led to decreased levels of pY478ezrin [Fig. 5E (iii)].

The overexpression of merlin, however, had no effect on the level of pYAK2 [Fig. 5E (iiii)]. Our results together suggest, however, that the inhibition of pY705Stat3 is not necessarily related to the phosphorylation status of merlin. Moreover, while the dephosphorylation of S518merlin site has been documented to support function (29), there is little information about the phosphorylation on the S10merlin site. Treatment with 15α-MP also induced a time-dependent suppression of the Stat3 target gene, c-Myc expression, in U251MG cells that occurred at 3 hours and persisted up to 24 hours, whereas cyclinD1 levels were not significantly altered (Fig. 5F).

Reports suggest a crosstalk between the ERα and the JAK2/Stat3 signaling pathway (36, 37). Using the ER-positive MCF7 cells, where ER is of critical functional and clinical significance, we were interested to determine whether there is any effect of 15α-MP treatment on the ERα/Stat3 crosstalk by performing co-immunoprecipitation and Western blotting analyses. Results show that Stat3 co-immunoprecipitates with the ERα, and this complex was not suppressed when the MCF7 cells were treated with 15α-MT (Supplementary Fig. S3).

Small-molecule ezrin inhibitor induces nearly similar responses in glioblastoma cells

Results together suggest a role for ezrin in the cellular responses to 15α-MP. We were interested to determine whether the inhibition of ezrin functions has similar biochemical and biologic effects. Immunoblotting analysis confirms that the treatment of U251MG cells with the ezrin inhibitor, NSC668394 (38), decreased pY478ezrin levels at 10 minutes up to 3 hours, and a further suppression at 24 hours, a trend comparable to that of 15α-MP (Fig. 6A, left and right). Moreover, the suppression of pY478ezrin level was accompanied by the elevation of pS10merlin level and a decreased pY705Stat3 level (Fig. 6B), as similarly observed for 15α-MP [Fig. 5D (i)], suggesting that NSC668394 inhibited pY705Stat3 induction in addition to its inhibition of pY478ezrin levels.

Treatment of U251MG cells with NSC668394 further suppressed cell viability, with an IC50 of 2.7 ± 0.7 μmol/L (Fig. 6C), and it also inhibited the migration of U251MG cells following a 22-hour treatment (Fig. 6D). These findings together suggest that the inhibition of ezrin and Stat3 functions contributes to the overall effect of NSC668394 on the growth and migration of glioblastoma cells in vitro. Interestingly, the effects of NSC668394 on U251MG cell growth and migration parallel that observed for 15α-MP. Therefore, the modulation of pY705Stat3, pY478ezrin, and pS10merlin functions constitutes a common pathway that is part of the mechanism to inhibit cell viability and migration of glioblastoma by both 15α-MP and NSC668394. Surprisingly, however, NSC668394 induced minimal morphology changes of glioblastoma cells up to 24 hours (Fig. 6E), suggesting that the underlying mechanisms for the observed 15α-MP-induced glioblastoma cell morphology changes likely extend beyond the effects on ezrin, Stat3, and merlin.

15α-MP inhibits growth of human glioblastoma xenograft in vivo

We were interested to determine the antitumor effects of 15α-MP against human tumors that harbor aberrantly active Stat3 and used the subcutaneous glioblastoma xenografts for proof-of-concept for in vivo effects. The oral gavage delivery of 15α-MP, 3 mg/kg, 3× a week, to mice bearing subcutaneous human glioblastoma U251MG tumor xenografts inhibited tumor growth (Fig. 7A). No significant changes in body weight or other signs of toxicity, including loss of appetite or decreased activity, were noticed throughout the study. TUNEL staining showed evidence...
of apoptotic cells in tumor tissues of 15α-MP–treated mice, compared with control (Fig. 7C, bottom vs. top), whereas tissue sections treated with DNase I were used as positive control (Fig. 7B, right). Samples were blindly scored by a board-certified anatomic pathologist and clinical pathologist on the basis of staining intensity (0, 1+, 2+, or 3+). For treated tumor tissues, of the 14 samples stained, 11 scored 2 (moderate, positive) whereas 3 scored 1 (weak, negative), similar to the negative control, which scored 1. In contrast, of the 15 stained samples of the control tissues, 14 samples scored 1 (weak, negative) whereas 1 scored 2 (moderate, positive). The $\chi^2$ shows statistically significant difference between the treated and control samples, with $P < 0.001$ (Supplementary Table S1).

**Discussion**

We present evidence that 15α-MP has inherent antitumor activity against human tumor cells, including breast cancer and glioblastoma. The antitumor activity is due, in part, to the inhibition of constitutively active Stat3, a common molecular abnormality observed in both glioblastoma and breast cancers and that promotes tumor progression (6, 39). Constitutively active Stat3 likely represents one of the putative targets of 15α-MP, which contributes to the preferential responses of tumor types, as the MCF7 breast cancer and Caco-2 colon adenocarcinoma cells that do not harbor aberrantly active Stat3 are relatively less sensitive. Among the responsive breast and glioblastoma tumor cells, there are differences in the sensitivities to 15α-MP. Notably, it is striking that 15α-MP promotes F-actin disorganization and irreversible morphology change leading to the rounding up of glioblastoma cells. Loss of tumor cell viability and tumor growth inhibition is due, in part, to the induction of apoptosis.

Studies using the glioblastoma cell model also reveal the modulation of other putative targets, including VE-cadherin (26), WAVE1 (27), pS339CXCR4 (28), merlin, ezrin, CyclinB1, Erk1/2, and Alk, which would constitute part of the mechanism leading to the antitumor responses to 15α-MP. Functionally, CyclinB1 is a known regulator of the cell cycle (40), Alk promotes growth and antiapoptotic pathways (41), whereas ezrin, radixin, moesin (ERM), and merlin comprise the ERM family of proteins.

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**Figure 6.**
Ezrin inhibitor suppresses pY705Stat3, upregulates pS10Merlin, downregulates pY478Ezrin, and inhibits cell viability and migration of human glioblastoma cells. **A** and **B**, Western blotting analysis of whole-cell lysates of equal total protein prepared from U251MG cells untreated or treated with 10 μmol/L 15α-MP or the ezrin inhibitor, NSC668394, for (A) 0.167 to 24 hours or (B) 0.5 and 1 hour and pooled together and probing for pY705Stat3, Stat3, pY478Ezrin, ezrin, pS10Merlin, and merlin. **C**, U251MG cells were untreated or treated once with 0.1 to 10 μmol/L NSC668394 for 72 hours and assayed for viability using CyQuant cell proliferation kit. IC50 values were derived from the dose-response curves. **D** and **E**, Phase-contrast microscopy images of cultured U251MG cells (**D**) in a scratch assay and the effects of treatment with 5 μmol/L NSC668394 for 5 and 22 hours on the migration into the denuded area and (**E**) untreated or treated with 10 μmol/L 15α-MP or NSC668394 for the designated times. Positions of proteins in gel are labeled. Control lane (C, 0) represents whole-cell lysates prepared from cells treated with 0.025% DMSO. Values, mean ± SEM. Data are representative of 2 to 4 experiments.
which link membrane proteins to the actin cytoskeleton (34). Notably, Alk is reportedly overexpressed in glioblastoma tumors (41), ezrin and its functions are associated with tumor cell migration and tumor invasion in glioblastoma and other cancers (42), and with increasing malignancy of astrocytic tumors (43), whereas merlin causes contact-dependent growth inhibition, decreased proliferation, increased apoptosis, and tumor growth inhibition (44, 45). We particularly note the pattern of a seemingly inverse correlation between the expression levels of pS10merlin and the pro-oncogenic pY478ezrin (46) in response to 15α-MP treatment, highlighting the importance of the 2 genes in the antitumor mechanisms of the natural product.

Merlin is phosphorylated on several sites (47), including the well-characterized S518merlin, which is reported to inactivate its tumor suppressor function, and the S10merlin that promotes changes in cellular morphology (48). While the resting elevated pS518merlin level is expected to promote glioblastoma progression as reported for other cancers (49, 50), the induction of pS10merlin by 15α-MP may alter merlin functions in favor of inhibiting tumor growth (44, 45). Accordingly, the inhibition

![Figure 7](https://example.com/figure7.png)

**Figure 7.**
15α-MP inhibits growth of subcutaneous human glioblastoma xenograft in mice. Mice bearing U251MG subcutaneous tumor xenograft were administered 15α-MP at 3 mg/kg (in 100 μL) or vehicle (1% DMSO) via oral gavage, 3 times per week for 24 days. **A,** Tumor sizes, measured every 3 days, were converted to tumor volumes and plotted against days of treatment. **B** and **C,** Tumor tissue sections were subjected to TUNEL. n = 6. *, p < 0.05. Tumor progression in U251MG xenograft treated with DMSO or 15α-MP.

**TUNEL staining**

- **A:** U251MG Xenograft
  - Control
  - 15α-MP (3 mg/kg)

- **B:** Negative
- **C:** Positive

- **Vehicle**
- **15α-MP (3 mg/kg)**
of ezrin by NSC668394 (38), which is associated with pS10merlin induction and pT705Stat3 suppression, blocked glioblastoma cell viability and migration. However, as observed for NSC668394, the modulation of ezrin, merlin, and Stat3 functions alone appears insufficient to induce changes in glioblastoma cell morphology. Altogether, present studies identify 150ψ-M as a chemical probe that suppresses human glioblastoma and breast cancer progression, in part, by modulating the functions of constitutively active Stat3, ezrin, merlin, Alk, Erk1/2, and CyclinB1.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: T.S. Hilliard, P. Yue, P. Williams, J. Turkson
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References

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Molecular Cancer Therapeutics

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