Galiellalactone Is a Direct Inhibitor of the Transcription Factor STAT3 in Prostate Cancer Cells**

The transcription factor STAT3 is constitutively active in several malignancies including castration-resistant prostate cancer and has been identified as a promising therapeutic target. The fungal metabolite galiellalactone, a STAT3 signaling inhibitor, inhibits the growth, both in vitro and in vivo, of prostate cancer cells expressing active STAT3 and induces apoptosis of prostate cancer stem cell-like cells expressing phosphorylated STAT3 (pSTAT3). However, the molecular mechanism of this STAT3-inhibiting effect by galiellalactone has not been clarified. A biotinylated analogue of galiellalactone (GL-biot) was synthesized to be used for identification of galiellalactone target proteins. By adding streptavidin-Sepharose beads to GL-biot-treated DU145 cell lysates, STAT3 was isolated and identified as a target protein. Confocal microscopy revealed GL-biot in both the cytoplasm and the nucleus of DU145 cells treated with GL-biot, appearing to co-localize with STAT3 in the nucleus. Galiellalactone inhibited STAT3 binding to DNA in DU145 cell lysates without affecting phosphorylation status of STAT3. Mass spectrometry analysis of recombinant STAT3 protein pretreated with galiellalactone revealed three modified cysteines (Cys-367, Cys-468, and Cys-542). Here we demonstrate with chemical and molecular pharmacological methods that galiellalactone is a cysteine reactive inhibitor that covalently binds to one or more cysteines in STAT3 and prevents STAT3 binding to DNA. This further validates galiellalactone as a promising direct STAT3 inhibitor for treatment of castration-resistant prostate cancer.

The transcription factor signal transducer and activator of transcription 3 (STAT3)2 is involved in many cellular processes including proliferation, survival, and immune response, and the transient activation of STAT3 is tightly regulated under normal conditions (1). Furthermore, the STAT3 signaling pathway is constitutively activated in several malignancies, including multiple forms of solid and hematological cancers, and is shown to be involved in anti-apoptotic effects and drug resistance (1–3).

The mode of constitutive activation of STAT3 through post-translational modifications, e.g. tyrosine and serine phosphorylation and lysine acetylation, may vary between different types of cancer (4). Aberrant signaling of upstream tyrosine kinases may be due to mutations or gene amplifications as well as increased expression of growth factors, cytokines, and ligand receptors, which all may lead to constitutive activation of STAT3 and malignant transformation (5, 6). Somatic mutations of the STAT3 gene are uncommon, although such are described in leukemia and hepatocellular adenomas (7, 8).

Prostate cancer is the second most common cancer in men world wide and the fifth most common cancer overall (2008) (9). Initially, prostate cancer cells respond to androgen deprivation therapy, but within 12–18 months many patients develop castration-resistant prostate cancer with a need for second-line therapy (10). Although cytotoxic drugs and recently approved drugs for more efficient blockade of androgen signaling, including abiraterone and enzalutamide, are available, there is an obvious need for new and efficient treatment strategies in metastatic castration-resistant prostate cancer (11).

Activated STAT3 has been correlated to the malignant potential of prostate cancer cells, disease progression, and increased Gleason score (12–14) and shown to promote metastatic progression of prostate cancer (15). Furthermore, the JAK/STAT signaling pathway is associated with a cancer stem
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cell-like phenotype in prostate cancer, and blocking of this pathway may inhibit the initiation of tumors (16). Targeting the transcription factor STAT3 appears to be a promising treatment strategy for patients with advanced prostate cancer, and STAT3 has been identified as a relevant target protein for the development of new therapies in this group of patients (17, 18).

The fungal metabolite galiellalactone is a small non-toxic and non-mutagenic molecule that has been shown to prevent STAT3 signaling by blocking the binding of STAT3 to STAT3-specific transcriptional DNA elements (19). We have previously demonstrated that galiellalactone inhibits the growth, both in vitro and in vivo, of prostate cancer cells expressing activated STAT3 and inhibits the expression of STAT3-regulated genes and proteins (20). Furthermore, galiellalactone inhibits growth and induces apoptosis of prostate cancer stem cell-like cells expressing phosphorylated STAT3 (pSTAT3) (21).

Galiellalactone contains a reactive α,β-unsaturated lactone functionality, and galiellalactone has been demonstrated to react with nitrogen- and sulfur-nucleophiles to produce inactive adducts (22). With the reactive potential of galiellalactone toward biological nucleophiles in consideration, we were interested in investigating whether galiellalactone can alkylate STAT3 and thereby inhibit the DNA binding as there is precedence that direct covalent modification of STAT3 with small molecules (23, 24) or through cysteine oxidation (25) can block the transcriptional activity of STAT3. The aim of the present study is to elucidate in more detail the mechanism of action of galiellalactone using human prostate cancer cells as a model.

**EXPERIMENTAL PROCEDURES**

**Biotinylated Galiellalactone Analogue**—Biotinylated galiellalactone analogues were synthesized to be used as a tool for the transcriptional activity of STAT3. The aim of the present study was to elucidate in more detail the mechanism of action of galiellalactone using human prostate cancer cells as a model.

**EXPERIMENTAL PROCEDURES**

**Biotinylated Galiellalactone Analogue**—Biotinylated galiellalactone analogues were synthesized to be used as a tool for identification of target proteins bound to galiellalactone. The complete synthetic procedure will be published elsewhere.\(^3\) Galiellalactone was prepared by synthesis as described previously (26).

**Synthesis of Galiellalactone-Cysteine Adduct**—3.5 mg (0.02 mmol) of galiellalactone was dissolved in 1.0 ml of MeOH with 2.4 mg (0.018 mmol) of L-cysteine. The solution was stirred at room temperature for 1 h. The solvent was removed under reduced pressure, and the residue was washed with chloroform to afford 5.6 mg (89%) of the pure adduct. \(^1\)H NMR (DMSO-\(d_6\)) d 0.50 (1H, ddd, \(J = 25.4\) Hz, \(J_2 = 12.2\) Hz, \(J_3 = 12.2\) Hz), 1.05 (3H, \(d, J = 6.55\) Hz), 1.84 (1H, m), 1.86 (1H, m), 2.01 (1H, m), 2.06 (1H, m), 2.70 (1H, dd, \(J_1 = 25.4\) Hz, \(J_2 = 12.2\) Hz), 2.97 (1H, dd, \(J_1 = 13.8\) Hz, \(J_2 = 8.0\) Hz), 3.14 (1H, dd, \(J_1 = 13.7\) Hz, \(J_2 = 3.5\) Hz), 3.42 (1H, dd, \(J_1 = 8.0\) Hz, \(J_2 = 3.7\) Hz), 3.53 (1H, s), 4.47 (1H, s); \(^13\)C NMR (DMSO-\(d_6\)) d 20.5, 28.8, 31.3, 31.9, 32.8, 37.0, 45.2, 47.3, 52.0, 54.0, 83.1, 88.7, 168.7, 175.6; HRMS (FAB-reduced pressure, and the residue was washed with chloroform

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\(^3\) Z. Escobar, M. Johansson, A. Bjartell, R. Hellsten, and O. Sterner, manuscript in preparation.
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care), the membrane was treated with enhanced chemiluminescent reagent (Santa Cruz Biotechnology or Millipore) followed by exposure to x-ray film or visualized using a ChemiDoc XRS system (Bio-Rad).

Isolation of Galiellalactone-interacting Proteins Using Biotinylated Galiellalactone Analogue—To isolate target proteins, intact cells or whole cell lysates of DU145 or LNCaP cells were treated with GL-biot for 1 h. Cell lysates were incubated with magnetic streptavidin-Sepharose beads (GE Healthcare), and bound proteins were eluted in 2% SDS and identified using Western blot analysis. For competition assays, DU145 cells or DU145 cell lysates were pretreated with galiellalactone (0–100 μM) for 1 h followed by the addition of 25 μM GL-biot for 1 h. The reverse was also investigated where DU145 cell lysates were pretreated with GL-biot for 1 h before the cells were fixed with paraformaldehyde with subsequent methanol fixation and permeabilized and blocked with 3% BSA, 0.3% Triton-X in PBS for 1 h. Slides were incubated with STAT3 primary antibody (Cell Signaling Technology) overnight, washed in PBS, and subsequently incubated with Alexa Fluor 594-conjugated streptavidin and secondary Alexa Fluor 488-conjugated anti-mouse antibody (Invitrogen) for 1 h. Slides were washed in PBS and mounted using DAPI-containing VECTASHIELD mounting medium (Vector Laboratories). The cells on the slides were analyzed by confocal laser scanning microscopy using the Zeiss LSM 710 system (Carl Zeiss AG).

Confocal Microscopy—Localization of GL-biot and possible co-localization of GL-biot with STAT3 were assessed using confocal microscopy. DU145 cells were grown on chamber slides. After 24 h, the cells were treated with 50 μM GL-biot for 2 h before the cells were fixed with paraformaldehyde with subsequent methanol fixation and permeabilized and blocked with 3% BSA, 0.3% Triton-X in PBS for 1 h. Slides were incubated with STAT3 primary antibody (Cell Signaling Technology) overnight, washed in PBS, and subsequently incubated with Alexa Fluor 594-conjugated streptavidin and secondary Alexa Fluor 488-conjugated anti-mouse antibody (Invitrogen) for 1 h. Slides were washed in PBS and mounted using DAPI-containing VECTASHIELD mounting medium (Vector Laboratories). The cells on the slides were analyzed by confocal laser scanning microscopy using the Zeiss LSM 710 system (Carl Zeiss AG).

Electric Mobility Shift Assay—STAT3 consensus sequence (Santa Cruz Biotechnology) was labeled with digoxigenin-ddUTP (Roche digoxigenin gel shift kit, Roche Applied Science) according to the manufacturer’s protocol. Lysates from DU145 cells treated with galiellalactone for 1 h or DU145 cell lysates treated with galiellalactone for 15 min on ice were incubated with labeled probes for 15 min at room temperature. Supershift assays were performed by pretreating the cell lysate with STAT3 antibody (Cell Signaling Technology). The complexes were resolved on a 6% DNA retardation gel (Invitrogen) in 0.5× Tris-borate-EDTA buffer. The DNA protein complex was transferred onto a positively charged membrane and cross-linked. Visualization of bands was performed according to the manufacturer’s protocol.

Recombinant pSTAT3 Protein—A synthetic version of the STAT3 gene, codon-optimized for Escherichia coli (DNA 2.0) encoding amino acids 127–722, was amplified by PCR and ligated into pETm11-SUMO3 (European Molecular Biology Laboratory (EMBL)). The resulting plasmid, pETm11-SUMO3-STAT3, encodes STAT3 with a cleavable N-terminal His6-SUMO tag. The plasmid was verified by sequencing and transformed into E. coli BL21(DE3) TKB1 (Stratagene). The resulting protein is a homobioconjugate of tyrosine kinase with a monomeric catalytic activity.

Mass Spectrometry Analysis—Samples obtained after galiellalactone derivatization were partly denatured with 2 M UREA prior to in-solution digestion. The samples were diluted with 50 mM ammonium bicarbonate to raise the pH, and an aliquot of trypsin solution (Promega) was added in a 1:50 trypsin:protein ratio (w/w). Enzymatic digest was carried out at 37 °C overnight and was stopped by the addition of 10% trifluoroacetic acid. The experiments were performed with an EasyLC nanoflow HPLC interfaced with a nanoEasy spray ion source (Proxeon Biosystems, Odense, Denmark) connected to an LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific). The chromatographic separation was performed at 40 °C on a 15-cm (75-μm inner diameter) EASY-Spray column packed with 3 μm of resin (Proxeon Biosystems). The nano-HPLC was operating at 300 nl min⁻¹ with a gradient of 5–20% solvent B (0.1% (v/v) formic acid, 100% (v/v) acetonitrile in water) in solvent A (0.1% (v/v) formic acid in water) during 60 min and then 20–40% during 30 min followed with an increase to 90% during 5 min. An MS scan (400–1400 m/z) was recorded in the Orbitrap mass analyzer set at a resolution of 60,000 at 400 m/z, 1 × 10⁶ automatic gain control target, and 500–ms maximum ion injection time. The MS was followed by data-dependent collision-induced dissociation MS/MS scans on the eight most intense multiply charged ions. The general mass spectrometric conditions were as follows: spray voltage, 2.0 kV; no sheath or auxiliary gas flow; S-lens 60%; ion transfer tube temperature, 275 °C. Raw data were processed by Mascot distiller searching the Swiss-Prot database with an in-house Mascot database. The search parameters for the Mascot searches were: Taxonomy: Homo sapiens; Enzyme, No enzyme; Variable Modifications, Galiellalactone (Cys); Precursor Tolerance, 20 ppm; MS/MS Fragment Tolerance, 0.1 Da.
**RESULTS**

**Activity of Biotinylated Galiellalactone Analogues and Cysteine Reactivity of Galiellalactone**—Two biotinylated analogues of galiellalactone (ZE139 and ZE140) were synthesized (Fig. 1A), and ZE139 was chosen for further experiments based on the inhibitory effect on cell proliferation and STAT3 activity (Fig. 1, B and C). From previous studies, we knew that an added phenyl group in position 7 of the tricyclic structure of galiellalactone gave analogues with increased potency.3 Galiellalactone and the biotinylated analogues ZE139 and ZE140 inhibit proliferation of DU145 cells with growth IC50 values of 3.6, 6.6, and 14 μM, respectively, after 72 h. C, the inhibitory effect of galiellalactone (GL) and ZE139 on STAT3 activity was investigated in a luciferase reporter gene assay using IL-6-stimulated LNCaP cells. The STAT3-dependent luciferase activity is expressed as the percentage of activation of relative control and presented as mean ± S.E. from two separate experiments. D, galiellalactone undergoes a Michael addition with L-cysteine in PBS or MeOH to form a stable adduct.

**Biotinylated Galiellalactone Analogue Binds Directly to STAT3**—To investigate the suggested covalent protein interactions of galiellalactone in an unbiased way, DU145 cell lysates and intact DU145 cells were treated with GL-biot, and the proteins bound were precipitated using streptavidin-Sepharose beads. STAT3 was successfully isolated and identified as a target protein by immunoblotting (Fig. 2A). GL-biot was able to

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**FIGURE 1. Chemical structure and activity of biotinylated galiellalactone analogues.** A, chemical structures of galiellalactone and the two biotinylated derivatives utilized in this study. B, galiellalactone and biotinylated galiellalactone analogues ZE139 and ZE140 inhibit proliferation of DU145 cells with growth IC50 values of 3.6, 6.6, and 14 μM, respectively, after 72 h. C, the inhibitory effect of galiellalactone (GL) and ZE139 on STAT3 activity was investigated in a luciferase reporter gene assay using IL-6-stimulated LNCaP cells. The STAT3-dependent luciferase activity is expressed as the percentage of activation of relative control and presented as mean ± S.E. from two separate experiments. D, galiellalactone undergoes a Michael addition with L-cysteine in PBS or MeOH to form a stable adduct.
bind to STAT3 both in intact tumor cells and in cell lysates. In addition, GL-biot was able to bind to recombinant STAT3 protein (Fig. 2A). Furthermore, pretreatment of DU145 cell lysates with galiellalactone prior to the addition of GL-biot prevented the binding of the GL-biot to STAT3 in a dose-dependent manner (Fig. 2B) demonstrating competitive binding. In whole cell lysates of GL-biot-treated DU145 cells subjected to Western blot analysis, several bands were detected using anti-biotin antibody, clearly demonstrating that GL-biot had bound to several protein targets (Fig. 2C).

DU145 cells treated with GL-biot were subjected to confocal microscopy to demonstrate the localization of the bound compound in cellular compartments (Fig. 2D). GL-biot was observed in both the cytoplasm and the nucleus of DU145 cells, appearing to co-localize with STAT3 in the nucleus. STAT3 was mainly present in the nucleus. The endogenous levels of biotin were below the detection limit.

Galiellalactone Inhibits STAT3 Binding to DNA without Affecting Phosphorylation Status—When extracts from DU145 cells pretreated with galiellalactone and DU145 cell extracts incubated with galiellalactone were subjected to EMSA with STAT3 probes, we demonstrated a dose-dependent decrease in STAT3 DNA binding (Fig. 3A). Galiellalactone did not affect phosphorylation of STAT3 Tyr-705 and Ser-727 or the expression of total STAT3 in DU145 cells (Fig. 3B). GL-biot was found to bind to both pSTAT3 Tyr-705 and pSTAT3 Ser-727 in DU145 cells (Fig. 3C). In LNCaP cells, which do not express constitutively active STAT3, GL-biot was able to bind to unphosphorylated STAT3 (Fig. 3D).

Galiellalactone Modifies Cysteine Residues in STAT3—When phosphorylated recombinant STAT3 protein pretreated with various concentrations of galiellalactone was digested by trypsin and subjected to mass spectrometric analysis, we found three modified cysteine residues in most of the experiments. The results show that Cys-367, Cys-468, and Cys-542 in recombinant STAT3 were the predominant cysteines modified by galiellalactone, and at higher concentrations of galiellalactone, we could also identify modification of Cys-251, Cys-259, and Cys-687 (Table 1). Kinetic studies showed that cysteines Cys-367, Cys-468, and Cys-542 all were alkylated after 1 min at molar ratios 1:25 (protein:galiellalactone) (Table 1). A list of galiellalactone-modified STAT3 peptides identified by MS/MS...
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FIGURE 3. Galiellalactone interferes with STAT3 DNA binding without inhibiting phosphorylation. A, the STAT3 DNA binding activity was investigated by EMSA in DU145 cell lysates treated with GL for 15 min and in DU145 cells treated with GL in culture for 1 h. GL inhibited the STAT3 DNA binding in a dose-dependent manner. Supershift assay was performed with STAT3 antibody (STAT3 ab) showing a loss of band. B, DU145 cells were treated with 5 μM GL for 24 h and immunoblotted for STAT3, pSTAT3 Tyr-705, and pSTAT3 Ser-727. GL did not affect the phosphorylation status of STAT3. C, DU145 cell lysates were treated with GL-biot for 1 h and precipitated using streptavidin-Sepharose beads. The elution was immunoblotted for pSTAT3 Tyr-705 and pSTAT3 Ser-727. D, LNCaP cell lysates were treated with GL-biot (25 μM) for 1 h and precipitated using streptavidin-Sepharose beads. The elution was immunoblotted for STAT3. LNCaP cells treated with IL-6 (50 ng/ml for 1 h) were used as positive control for pSTAT3 expression.

TABLE 1
Galiellalactone-modified cysteine residues in STAT3

| Ratio, GL:STAT3 | Cys-251 | Cys-259 | Cys-328 | Cys-367 | Cys-418 | Cys-468 | Cys-542 | Cys-550 | Cys-687 |
|----------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| 250:1          | 2       | 1       | —       | 6       | 15      | 23      | —       | 1       |
| 50:1           | 1       | —       | —       | 4       | 12      | 16      | —       | 4       |
| 25:1           | —       | —       | —       | 3       | 10      | 7       | —       | —       |
| 6.25:1         | —       | —       | —       | 1       | 7       | 3       | —       | —       |
| 2.5:1          | —       | —       | —       | —       | 9       | 2       | —       | —       |

| Incubation time (min) | Cys-251 | Cys-259 | Cys-328 | Cys-367 | Cys-418 | Cys-468 | Cys-542 | Cys-550 | Cys-687 |
|-----------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| 5                     | —       | —       | —       | 2       | —       | 10      | 2       | —       |
| 10                    | —       | —       | —       | 2       | —       | 5       | 2       | —       |
| 15                    | —       | —       | —       | 1       | —       | 7       | 2       | —       |
| 30                    | —       | —       | —       | 2       | —       | 13      | 2       | —       |
| 60                    | —       | —       | —       | 2       | —       | 9       | 3       | —       |

The number of observations for galiellalactone-modified STAT3 peptides is shown. To regard a peptide as identified, a mascot score above 20 was required and manual validation of MS/MS was performed. Five different molar ratios, from 2.5 to 250 times excess of galiellalactone to protein, were used. The experiments were performed in triplicate. Incubation was made during 30 min. For kinetic studies, the experiments were conducted with a 25:1 galiellalactone to STAT3 ratio. Five different incubation times between 1 and 60 min were used. Experiments were performed in duplicate. Dash indicates no observed modification.

and database search is given in Table 2. Annotation of galiellalactone-modified and non-modified 452–428 STAT3 peptides using tandem mass spectrometry is shown in Fig. 4, A and B. The observed sequence coverage was above 90% in all experiments (Fig. 4C). Fig. 4D shows a representation of the STAT3 monomer bound to DNA. The galiellalactone alkylated cysteine residues in STAT3 are located in the linker domain (Cys-542) and DNA binding domain (Cys-367 and Cys-468) where Cys-468 is in direct contact with bound DNA.

Irreversible Binding of Galiellalactone to STAT3—To investigate the binding characteristics of galiellalactone to STAT3, the inhibition of STAT3 activity was studied with wash-out experiments where galiellalactone was removed from the cell culture solution by washing the cells with PBS after 1 h of treat-
FIGURE 4. Modification of STAT3 cysteines by galiellalactone. Annotation of galiellalactone-modified and non-modified 452–488 STAT3 peptides using tandem mass spectrometry and database search is shown. A, MS/MS of galiellalactone-modified 452–488 peptide. B, MS/MS of peptide 452–488 where Cys-468 is found without modification. Fragments of the 452–488 peptides are indicated with the following legends: /c141 denotes fragments that are common for both galiellalactone-modified and non-modified peptide, F denotes fragments that contain galiellalactone-modified Cys-468, and f denotes fragments that contain non-modified Cys-468. C, sequence coverage of the recombinant expressed STAT3 protein from a typical mass spectrometry experiment. 94% of the sequence was identified in this example. The C-terminal peptide, including Cys-712 and Cys-718, was never observed in the experiments.

D, surface (gray) and ribbon (turquoise) representation of STAT3 monomer bound to DNA (orange) with the three cysteine residues alkylated by galiellalactone labeled (green with sulfur in yellow). Cys-542 is located in the linker domain, and both Cys-367 and Cys-468 are located in the binding domain. Cys-468 is in direct contact with bound DNA.

TABLE 2
Galiellalactone-modified STAT3 peptides identified by mass spectrometry

| Amino acid | Sequence | Mascot Score | Charge | Experimental weight | Theoretical weight |
|------------|----------|--------------|--------|---------------------|-------------------|
| 247–262    | QQIACIGPPNCILDLR | 23 | 2+  | 1890.9429 | 1890.9332 |
| 247–262    | QQIACIGPPNCILDLR | 46 | 3+  | 2085.0223 | 2085.0275 |
| 364–379    | IVCIGACISADPL   | 35 | 4+  | 1895.9943 | 1896.0026 |
| 366–379    | QTCIGGDSDVLAAR  | 58 | 3+  | 1654.8102 | 1654.8236 |
| 452–488    | IDLESLWVSNICQMNAPISILWYNMLTNNFK | 90 | 4+  | 4418.1933 | 4418.2153 |
| 455–488    | ETSLVPVVSNTICQMNAPILWYNMLTNNFK | 54 | 3+  | 4076.9493 | 4077.0202 |
| 519–548    | GLSIEQLTTLAELLGPGNVSNQCCITWAK | 45 | 3+  | 3383.7763 | 3383.7687 |
| 532–546    | LGLPGVSNQCCITWAK | 72 | 2+  | 1800.8644 | 1800.8757 |
| 532–548    | LGLPGVSNQCCITWAK | 109 | 2+  | 1999.9970 | 2000.0077 |
| 539–548    | YSGQCCITWAK   | 40 | 2+  | 1349.6276 | 1349.6326 |
| 686–707    | YCRPESQEHFEADPQSAAPYLK | 34 | 3+  | 2638.1902 | 2638.2009 |
| 686–707    | YCRPESQEHFEADPQSAAP(p)YLK | 50 | 3+  | 2718.1540 | 2718.1673 |

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The results indicate that the STAT3 signaling inhibitor galiellalactone binds directly and covalently to STAT3 and that this interaction prevents DNA binding without interfering with upstream activation by phosphorylation. This further validates galiellalactone as a promising STAT3 inhibitor for the treatment of castration-resistant prostate cancer and other malignancies with constitutive activation of STAT3.

Galiellalactone is a reactive Michael acceptor that can react with both N-nucleophiles and S-nucleophiles including cysteine. Nucleophiles add diastereoselectively to the double bond of the α,β-unsaturated lactone moiety giving inactive compounds, thus demonstrating the importance of the reactive functionality for the biological activity of galiellalactone. Here we demonstrate that galiellalactone does form a stable adduct with cysteine. Galiellalactone is thought to covalently modify cysteine residues in the DNA binding region of STAT3, thus preventing DNA binding. Here we saw that galiellalactone modifies the cysteine residues Cys-367, Cys-468, and Cys-542 in recombinant STAT3. Cys-542 is located in the linker domain, and Cys-367 and Cys-468 are located within the DNA binding region of STAT3 (Fig. 4D). The linker domain may be involved in DNA binding as well as transcriptional activity (27).

The binding of galiellalactone to one or more of these cysteines, which are located in domains involved with DNA binding, may prevent the binding of STAT3 to DNA. The corresponding cysteine residues to the cysteines in STAT3, which were observed to be modified by galiellalactone, are not present in STAT1 and STAT5 (28), possibly making this mode of action by galiellalactone selective for STAT3. Nevertheless, the effect of galiellalactone on STAT1 and STAT5 remains to be investigated.

Buettner et al. (23) demonstrated that the small molecule STAT3 inhibitor C48 alkylates Cys-468 in the STAT3 DNA binding domain, although at a high compound concentration. Stattic, a STAT3 inhibitor that has been shown to prevent STAT3 dimerization and phosphorylation (29), was shown to alkylate four cysteine residues in unphosphorylated STAT3 (Cys-251, Cys-259, Cys-367, and Cys-426) (24). Furthermore, it has been shown that the transcriptional activity of STAT3 is sensitive to changes in cellular redox conditions as treatment of STAT3 with hydrogen peroxide leads to covalent modifications (oxidation) of specific cysteine residues (25). Taken together, these studies, including this one, demonstrate that STAT3 transcriptional activity can be modulated by covalent modifications.

Strategies for the direct targeting of STAT3 include inhibition of the STAT3 dimerization process by binding of small molecules or peptide mimetics to the SH2 domain and inhibiting STAT3 DNA binding with oligonucleotides (30). These strategies have been thoroughly investigated and have produced compounds with in vivo activity. Recently, it has been shown that alkylation of reactive cysteine residues in STAT3 is a potential novel strategy for direct inhibiting of STAT3 function. Alkylation of Cys-468 in the DNA binding domain by the compound C48 is shown to prevent DNA binding, and methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate (CDDO-Me) prevented STAT3 dimerization by alkylation of Cys-259 (23, 31). In addition, the reactive compound Stattic inhibits STAT3 by binding to multiple cysteine residues in unphosphorylated STAT3 as determined by mass spectrometry analysis. These findings are in line with STAT3 signaling being sensitive to redox-dependent cysteine modifications (25, 32).

Galiellalactone shows a strong irreversible binding to STAT3 and sustains inhibitory activity after the compound is removed, which could prolong the effect of the compound even after the compound exposure is below the level of detection thus potentially disconnecting pharmacokinetics from pharmacodynamics. The long half-life of STAT3 (>8 h) indicates that STAT3 activity is not regulated by protein degradation (33). In addition to binding to STAT3, other binding proteins were detected using GL-biot, which is not unexpected considering the reactive nature of the inhibitor. The surprisingly small number of additional target proteins, considering the reactivity of galiellalactone toward cysteine, was not identified in this study. However, galiellalactone has been shown to inhibit NF-κB signaling by direct interaction with the NF-κB subunit p65 (34). Furthermore, galiellalactone has been observed to inhibit TGF-β signaling by preventing binding of SMAD2/3 to DNA (35). This demonstrates that galiellalactone is not specific for STAT3, although the identification of STAT3 as a direct binding target correlates well with the inhibitory effects seen in

FIGURE 5. Irreversible binding of galiellalactone to STAT3. A, STAT3 luciferase reporter gene transfected LNCaP cells were treated with GL for 1 h and stimulated with IL-6 (50 ng/ml) for 4 h to induce STAT3 activity. For wash-out (wo) experiments, the cells were treated with GL for 1 h and washed with PBS before stimulation with IL-6. The STAT3-dependent luciferase activity is expressed as the percentage of activation of relative control and presented as mean ± S.E. from three separate experiments. B, DU145 cell lysates were treated with 25 μM GL-biot for 1 h before the addition of increasing concentrations (10–100 μM) of GL for 1 h. Cell lysates were incubated with streptavidin-Sepharose beads, and elutions were immunoblotted for STAT3. GL-biot was not displaced by GL, indicating a strong binding to STAT3.

DISCUSSION

The inhibitory effect on STAT3 activity was then measured after an additional 4 h (Fig. 5A). The results show that inhibition of STAT3 activity by galiellalactone persisted 4 h after removal of galiellalactone from the cell medium. Furthermore, the binding of GL-biot to STAT3 was not disrupted by the addition of galiellalactone, indicating a strong and irreversible binding to STAT3 (Fig. 5B).

The binding of galiellalactone to one or more of these cysteines, which are located in domains involved with DNA binding, may prevent the binding of STAT3 to DNA. The corresponding cysteine residues to the cysteines in STAT3, which were observed to be modified by galiellalactone, are not present in STAT1 and STAT5 (28), possibly making this mode of action by galiellalactone selective for STAT3. Nevertheless, the effect of galiellalactone on STAT1 and STAT5 remains to be investigated.

Buettner et al. (23) demonstrated that the small molecule STAT3 inhibitor C48 alkylates Cys-468 in the STAT3 DNA binding domain, although at a high compound concentration. Stattic, a STAT3 inhibitor that has been shown to prevent STAT3 dimerization and phosphorylation (29), was shown to alkylate four cysteine residues in unphosphorylated STAT3 (Cys-251, Cys-259, Cys-367, and Cys-426) (24). Furthermore, it has been shown that the transcriptional activity of STAT3 is sensitive to changes in cellular redox conditions as treatment of STAT3 with hydrogen peroxide leads to covalent modifications (oxidation) of specific cysteine residues (25). Taken together, these studies, including this one, demonstrate that STAT3 transcriptional activity can be modulated by covalent modifications.

Strategies for the direct targeting of STAT3 include inhibition of the STAT3 dimerization process by binding of small molecules or peptide mimetics to the SH2 domain and inhibiting STAT3 DNA binding with oligonucleotides (30). These strategies have been thoroughly investigated and have produced compounds with in vivo activity. Recently, it has been shown that alkylation of reactive cysteine residues in STAT3 is a potential novel strategy for direct inhibiting of STAT3 function. Alkylation of Cys-468 in the DNA binding domain by the compound C48 is shown to prevent DNA binding, and methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate (CDDO-Me) prevented STAT3 dimerization by alkylation of Cys-259 (23, 31). In addition, the reactive compound Stattic inhibits STAT3 by binding to multiple cysteine residues in unphosphorylated STAT3 as determined by mass spectrometry analysis. These findings are in line with STAT3 signaling being sensitive to redox-dependent cysteine modifications (25, 32).

Galiellalactone shows a strong irreversible binding to STAT3 and sustains inhibitory activity after the compound is removed, which could prolong the effect of the compound even after the compound exposure is below the level of detection thus potentially disconnecting pharmacokinetics from pharmacodynamics. The long half-life of STAT3 (>8 h) indicates that STAT3 activity is not regulated by protein degradation (33). In addition to binding to STAT3, other binding proteins were detected using GL-biot, which is not unexpected considering the reactive nature of the inhibitor. The surprisingly small number of additional target proteins, considering the reactivity of galiellalactone toward cysteine, was not identified in this study. However, galiellalactone has been shown to inhibit NF-κB signaling by direct interaction with the NF-κB subunit p65 (34). Furthermore, galiellalactone has been observed to inhibit TGF-β signaling by preventing binding of SMAD2/3 to DNA (35). This demonstrates that galiellalactone is not specific for STAT3, although the identification of STAT3 as a direct binding target correlates well with the inhibitory effects seen in
galiellalactone-treated DU145 cells expressing constitutively active STAT3. In addition, galiellalactone has been shown to be well tolerated in animal studies with repeat dosing and displays minimal cytotoxic effects in non-STAT3-dependent cells, which would not be expected with a non-discriminate and highly reactive compound (20).

Considering the multitude of STAT3 activation mechanisms in malignancies and the risk of developing therapy resistance to kinase inhibitors, it is in theory desirable to develop inhibitors acting directly on STAT3. Direct targeting of STAT3 instead of upstream activators of STAT3 such as tyrosine kinases may lead to a decrease in side effects and increased efficacy. Furthermore, direct targeting of STAT3 circumvents the nature of upstream activation pathway. Here we show that galiellalactone exerts its inhibitory actions by direct interaction with STAT3.

STAT3 may have different roles in the various stages of cancer and cancer development with different activation modes of STAT3 (phosphorylation of pSTAT3 Tyr-705 or Ser-727) (4). The activation of STAT3 by phosphorylation of Tyr-705 or Ser-727 induces the transcription of different subsets of genes. The binding of galiellalactone to STAT3 was independent of phosphorylation status and bound to both phosphorylated and unphosphorylated STAT3. Unphosphorylated STAT3 has been shown to regulate the expression of genes involved in oncogenesis (36). Binding of galiellalactone to unphosphorylated STAT3 in prostate cancer cells may be an additional way to prevent tumor progression; however, the role of unphosphorylated STAT3 in prostate cancer has not yet been evaluated.

Here we demonstrate with chemical and molecular pharmacological methods that the reactive fungal metabolite galiellalactone is a cysteine reactive inhibitor that covalently binds to one or more cysteines in STAT3 and that this leads to inhibition of STAT3 binding to DNA and thus blocks STAT3 signaling without affecting tyrosine phosphorylation. Considering the difficulty in targeting transcription factors with small, drug-like molecules and the rekindled interest in covalent inhibitors, this study further validates galiellalactone as a promising chemical starting point for designing highly specific STAT3 inhibitors and as a promising STAT3 inhibitor in itself for the treatment of castration-resistant prostate cancer and other malignancies with constitutive activation of STAT3.

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