Orally bioavailable small-molecule inhibitor of transcription factor Stat3 regresses human breast and lung cancer xenografts

Xiaolei Zhang¹,², Peibin Yue¹,², Brent D. G. Page³, Tianshu Li³, Wei Zhao³, Andrew T. Namanja³, David Paladino⁵, Jihe Zhao⁶, Yuan Chen⁷, Patrick T. Gunning⁷, and James Turksona,b,2

¹Burnett School of Biomedical Sciences, University of Central Florida College of Medicine, Orlando, FL 32827; ²Cancer Biology and Experimental Therapeutics Programs, University of Hawaii Cancer Center, Honolulu, HI 96813; ³Department of Chemistry, University of Toronto at Mississauga, Mississauga, ON, Canada, L5L 1C6; and ⁴Department of Molecular Medicine, Beckman Research Institute of the City of Hope, Duarte, CA 91010

Edited by James E. Darnell, The Rockefeller University, New York, NY, and approved April 13, 2012 (received for review January 4, 2012)

Computer-aided lead optimization derives a unique, orally bioavailable inhibitor of the signal transducer and activator of transcription (Stat3) Src homology 2 domain. BP-1-102 binds Stat3 with an affinity (K_d) of 504 nM, blocks Stat3–phospho-tyrosine (pTyr) peptide interactions and Stat3 activation at 4–6.8 μM, and selectively inhibits its growth, survival, migration, and invasion of Stat3-dependent tumor cells. BP-1-102–mediated inhibition of aberrantly active Stat3 in tumor cells suppresses the expression of c-Myc, Cyclin D1, Bcl-xl, Survivin, VEGF, and Krüppel-like factor 8, which is identified as a Stat3 target gene that promotes Stat3-mediated breast tumor cell migration and invasion. Treatment of breast cancer cells with BP-1-102 further blocks Stat3–NF-κB cross-talk, the release of granulocyte colony-stimulating factor, soluble intercellular adhesion molecule 1, macrophage migration-inhibitory factor/glycosylation-inhibiting factor, interleukin 1 receptor antagonist, and serine protease inhibitor protein 1, and the phosphorylation of focal adhesion kinase and paxillin, while enhancing E-cadherin expression. Intravenous or oral gavage delivery of BP-1-102 furnishes micromolar or microgram levels in tumor tissues and inhibits growth of human breast and lung tumor xenografts.

Signal transducer and activator of transcription (STAT) proteins mediate responses to cytokines and growth factors (1). Recruitment via the Src homology 2 (SH2) domain to receptor phospho-tyrosine (pTyr) peptide motifs facilitates STAT phosphorylation on a key tyrosyl residue by growth factor receptors and the Janus kinase (Jak) and Src kinase families. Phosphorylation induces STAT–STAT dimerization through a reciprocal pTyr–SH2 domain interaction. The active dimers in the nucleus induce gene transcription by binding to specific DNA-response elements in the promoters of target genes.

The aberrant activation of Stat3 occurs in many human cancers (2) and promotes tumor progression. The mechanisms of Stat3-mediated tumorigenesis include dysregulation of gene expression that leads to uncontrolled growth and survival of tumor cells, enhanced tumor angiogenesis, and metastasis (3, 4). Tumor cell–associated constitutively active Stat3 also regulates proinflammatory cytokine expression, including RANTES (regulated upon activation normal T cell expressed and secreted) and CXC motif chemokine 10 (CXCL10) in T cells (5), Stat3 signaling that inhibits Stat3 activation and functions in vitro and in vivo, and thereby inhibits growth of mouse xenografts of human breast and non–small-cell lung cancers.

Results

Computer-Aided Design of BP-1-102 as an Analog of S3I-201.1066.

The structural composition and topology of the Stat3 SH2 domain–binding “hotspot” shows three solvent-accessible subpockets on the protein surface, including the key pTyr705-binding region, which are accessed by S3I-201.1066 and most of the reported Stat3 inhibitors. BP-1-102 (Fig. L4) has appendages that promote interactions with all three subpockets (Fig. 1B).

Inhibition of Stat3 Signaling and Function.

BP-1-102 binds Stat3 with a K_d of 504 nM, determined by surface plasmon resonance (SPR) analysis (Fig. S1A). It preferentially disrupts Stat3 binding to phosphorylated, native high-affinity, IL-6R/gp130 peptide (pTyr, pY904), with an IC50 of 4.1 μM (Fig. S1B), as determined by fluorescence polarization (FP) assay, compared with its weaker activity against Stat1 binding to the IFN-γ receptor peptide (GpYDKPHVL-NH2) or Stat5 binding to the erythropoietin receptor peptide (GpYLVLDKW-NH2), with an IC50 of 25–30 μM (Fig. S1C). Isothermal titration calorimetry (ITC) was further used to examine BP-1-102 effects on the interaction between pY904 and Stat3 (Fig. S1D). ITC experiments in the absence and presence of BP-1-102 were conducted using identical sample concentrations and conditions (SI Materials and Methods).

Pre-complexing Stat3 with BP-1-102 dramatically alters the pY904 profile, with significantly reduced enthalpic contribution by ~6 kcal/mol (Fig. S1D, Left vs. Right, respective p-axis intercepts). This result indicates a direct interference of pY904 binding to the SH2 domain. Similarly, the reverse ITC titration of BP-1-102 into free or pY904-bound Stat3 (Fig. S1E, Left and Right, respectively)...


The authors declare no conflict of interest. This article is a PNAS Direct Submission. Freely available online through the PNAS open access option.

X.Z. and P.Y. contributed equally to this work.

1To whom correspondence should be addressed. E-mail: jturkson@cc.hawaii.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1121606109/-/DCSupplemental.
confirms that these two ligands interfere with each other’s binding to the SH2 domain. In fact, titration of BP-1-102 to pY904-bound Stat3 shows an increasing endothermic profile (heat absorption), consistent with BP-1-102 displacing pY904 from Stat3. Taken together, these results suggest direct competition of the ligands to the same binding site on the SH2 domain.

The R2 relaxation filter NMR approach (18) was used to study pY904 binding to Stat3 and verify that BP-1-102 competes with pY904. The pY904 signal (Fig. S1F, black tracing) was attenuated upon addition of Stat3 (Fig. S1F, red tracing), indicating binding. Addition of BP-1-102 resulted in further attenuation (Fig. S1F, blue tracing), suggesting direct interference with pY904 binding to the SH2 domain. The interference could be more significant if BP-1-102 did not form soluble aggregates in the aqueous environment used for NMR studies (Fig. S1G and SI Materials and Methods). This result further supports the findings by SPR, FP, and ITC. BP-1-102 inhibits Stat3 DNA-binding activity in vitro, with an IC50 value of 6.8 ± 0.8 μM (Fig. 2A), and preferentially inhibits Stat3–Stat3, over Stat1–Stat1, or Stat5–Stat5 DNA-binding activity (Fig. 2B), as measured by EMSA analysis (9, 11, 17). BP-1-102 is substantially improved over the lead, S3I-201,1066 (SPR, KD of 2.7 μM; FP, IC50 of 23 μM; EMSA, IC50 of 36 μM) (17).

BP-1-102 inhibited constitutive Stat3 DNA-binding activity (Fig. 2C and Fig. S2A). Tyr705 phosphorylation (Fig. 2D and E, and Fig. S2B, pYStat3), and Stat3-dependent luciferase reporter (pLucTKS3) (19, 20) induction (Fig. S2C, TKS3). The inhibition occurs in a dose- and time-dependent manner and as early as 30 min (Fig. 2 C and D and Fig. S2B). Stat3 is distributed in the cytoplasm, nucleus, and mitochondria (Fig. 2D and Fig. S2 D and E). Levels of pY705Stat3 are higher in the nucleus than in the cytoplasm, except in DU145 cells (Fig. S2 D and E), and are undetectable in mitochondria (Fig. S2E). BP-1-102 treatment attenuated both nuclear and cytoplasmic pY705Stat3 (Fig. 2D and Fig. S2E). By contrast, BP-1-102 had little or no effect on phospho-Shc, Src, Jak-1/2, Erk1/2, or Akt levels (Fig. 2E), induction of the Stat3-independent luciferase reporter, pLucSRE, which is driven by the serum-response element (SRE)/c-fos promoter (19, 20) (Fig. S2C, SRE), or the phosphorylation of many cellular kinases (Table S1). BP-1-102 treatment further suppressed c-Myc, Cyclin D1, Bcl-xl, Survivin, and VEGF expression (Fig. 2F), which occurred subsequent to Stat3 inhibition (Fig. 2D). Thus, inhibition of aberrantly active Stat3 suppresses Stat3-dependent gene regulation.

**BP-1-102 Selectively Suppresses Growth, Survival, Malignant Transformation, Migration, and Invasion of Malignant Cells Harboring Constitutively Active Stat3.** Consistent with the dependency on abnormal Stat3 signaling of NIH 3T3/v-Src fibroblasts and malignant cells harboring aberrantly active Stat3 for the transformed phenotype (2, 4, 19, 20), BP-1-102 treatment suppressed cell proliferation, anchorage-dependent and -independent growth, and colony numbers of NIH 3T3/v-Src (v-Src), MDA-MB-231 (231), Panc-1, DU145, and A549 cells harboring aberrantly active Stat3 (Fig. 3 A and B). Overexpression of the artificially engineered, constitutively active Stat3C mutant (21) rendered MDA-MB-231 cells refractory to BP-1-102 (Fig. 3 A, 231/St3C), Stat3C expressing in cells is insensitive to BP-1-102 (Fig. S2F).

Moreover, BP-1-102 induced apoptosis in MDA-MB-231 cells (Fig. 3B, Lower, bars 3 and 4), which was attenuated (Fig. 3B, Lower, bars 6 and 7 vs. bars 3 and 4) by overexpressing Flag-tagged Stat3 SH2 domain (Fig. 3B, **Upper**, immunoblot), the target for BP-1-102. The data together show the specificity of BP-1-102 effects against Stat3-dependent tumor cells, which are dependent on disrupting Stat3 SH2 domain function. Treatment with BP-1-102 for 16 h, before observing the effect on proliferation (Fig. S3A, **Insets**), inhibited migration (Fig. 3C and Fig. S3C) and in-
Fig. 3. BP-1-102 induces antitumor cell effects in vitro and suppresses tumor-supporting factors. (A) Cultured MDA-MB-231, DU145, Panc-1, and NIH 3T3vSrc cells harboring aberrantly active Stat3 and NIH 3T3, NIH 3T3vRas, mouse thymus stromal epithelial cells, TE-71, Stat3-null mouse embryonic fibroblasts (Stat3−/−MEFs), cispalatin-sensitive ovarian cancer cells, A2780S cells that do not, were treated once with 0–30 μM BP-1-102 and subjected to CyQUANT cell proliferation assay. (B) Annexin V/flow cytometry analysis of MDA-MB-231 cells transfected with pcDNA-3 (mock) or Flag-tagged Stat3 (St3) SH2 domain and treated with 0–15 μM BP-1-102 (Lower); Flag immunoblot (Upper). (C) Cultured malignant melanoma cells were treated with BP-1-102, wounded, and allowed to migrate into a denuded area. (D) Number of invaded MDA-MB-231 cells in a BioCoat invasion chamber assay and the effects of BP-1-102. (E and F) Immunoblotting analysis of whole-cell lysates prepared from MDA-MB-231 cells (E and F, i) treated with 0–15 μM BP-1-102 or (F, ii) transfected with control (−) or Stat3 siRNA (+) and probing for FAK, pY576/577FAK, paxillin, pY118paxillin, E-cadherin, Snail, KLF8, EPST11, or β-actin. (G) Number of invaded MDA-MB-231 cells in a BioCoat invasion chamber assay and the impact of KLF8 overexpression on BP-1-102 effects. (H–J) Immunoblotting analysis of (H) whole-cell (WC), nuclear (Nuc), or cytosolic (Cyto) lysates, (I) immunocomplexes of Stat3 (Upper) or RelA (Lower) prepared from MDA-MB-231 cells treated with 0–15 μM BP-1-102, or (I) whole-cell lysates of MDA-MB-231 cells transfected with control (−) or Stat3 siRNA (+) and probing for pY705Stat3, Stat3, p5356RelA, RelA, β-actin, or HDAC1. (K) G-CSF, sICAM, and MIF/GIF levels assessed in conditioned medium from cultures of MDA-MB-231 cells treated for 48 h with BP-1-102. Positions of proteins in the gel are shown. Data are representative of three or four independent determinations. Values, mean ± SD, n = 4 or 9. *P < 0.05, **P < 0.01, ***P < 0.005.

BP-1-102 Reduces pRelA–pRelA Interaction in a Stat3-Independent Manner. BP-1-102 reduced pRelA–pRelA interaction (Fig. 3H, lane 2) compared to bar 2, which was validated by Stat3 knockdown by siRNA (Fig. 3I). Further, we compared bar 2 and 5, and in turn minimized the BP-1-102-induced effect that is otherwise observed in the wild-type, uninduced cells (Fig. 3J, compare bars 2 and 3 to 5), which is detected in the nucleus by coimmunoprecipitation analysis (Fig. 3J, lane 1) and as colocalization in immunofluorescence/confocal microscopy (Fig. S6A, control, merged). BP-1-102 treatment diminished the pStat3–pRelA interaction (Fig. 3J, lane 2 and Fig. S6A, compare 25 μM, 16 h to control), which was validated by Stat3 knockdown by siRNA (Fig. 3J). By contrast, BP-1-102 treatment had no effect on 1Kb–RelA interactions (Fig. S6B). Per the published report (26) that nuclear Stat3–NF-κB complex promotes nuclear NF-κB retention, BP-1-102-mediated suppressive events that promote tumor progression. BP-1-102-treatment breast cancer MDA-MB-231 cells showed decreased phosphorylation of paxillin and FAK and increased E-cadherin expression (Fig. 3E). To exclude nonspecific effects, ovarian cancer A2780S cells that do not harbor aberrantly active Stat3 were treated with BP-1-102. No changes in p-FAK or p-paxillin levels were observed (Fig. 3E). The data that Stat3 inhibition occurs as early as 30 min (Fig. 2D), when FAK and paxillin are little affected (Fig. S4), suggest that the decreased p-FAK and p-paxillin levels at 24 h (Fig. 3E) are secondary events to Stat3 inhibition. BP-1-102 further suppressed Snail expression (Fig. 3E), a Stat3-regulated gene that controls E-cadherin expression.

FAK promotes Krüppel-like factor (KLF8) induction (23). KLF8 and the tumor–stroma interaction factor, epithelial–stromal interaction 1 (EPST11) protein, promote tumor cell spread and invasiveness (24, 25). BP-1-102–treated breast cancer cells showed reduced KLF8 and EPST11 levels (Fig. 3F, i), which was validated by Stat3 knockdown by siRNA (Fig. 3F, ii). Furthermore, in normal NIH 3T3 fibroblasts, transient cotransfection with the KLF8 promoter-driven luciferase reporter (pLucKLF8) and v-Src vector, the activation of Stat3 by v-Src (20) induced pLucKLF8 expression by over twofold, which was repressed by BP-1-102 (Fig. S5A). The KLF8 promoter has three putative Stat3 binding sites (Fig. S5B, i). Site-specific mutation in the nucleotide sequence −253/−245 (site 1) severely impaired the mutual pLucKLF8/−253/G reporter induction (Fig. S5A, compare bars 2 and 5). In vitro DNA-binding/EMSA analysis further showed a strong Stat3 binding to an oligonucleotide probe incorporating site 1 (−253/−245), compared with the standard high-affinity sis-inducible element (hSIE) probe, which was diminished by blocking anti-Stat3 antibody in a supershift assay (Fig. S5B, ii). No Stat3 binding was observed to site 2 or 3 (Fig. S5B, ii), although we do not exclude the possibility they contribute to Stat3 responsiveness. Thus, Stat3 directly induces the KLF8 gene.

To further study KLF8 importance, we evaluated BP-1-102 effects on cell motility and invasiveness in a KLF8 overexpression or knockdown background. By contrast to inhibition of invasiveness of the wild-type cells (Fig. 3D and G, bar 2), KLF8 overexpression (24) in MDA-MB-231 cells abolished BP-1-102 effects (Fig. 3G, bars 3–5). Further, studies using tetracycline-inducible KLF8 shRNA (24) in MDA-MB-231-SiKld cells show that KLF8 knockdown, as expected, suppressed cell migration (Fig. S5C, compare bar 2 vs. 5) and invasiveness (Fig. S5D, compare bar 2 vs. 5) and in turn minimized the BP-1-102–induced effect that is otherwise observed in the wild-type, uninduced cells (Fig. S5C and D, compare the relative change between bars 2 and 3 to that of bars 5 and 6). Thus, KLF8 expression is one of the underlying mechanisms of Stat3-mediated tumor cell migration and invasiveness.

BP-1-102 Reduces pRelA–pRelA Interaction in a Stat3-Independent Manner. BP-1-102 reduces pRelA–pRelA interaction (Fig. 3H, lane 2) compared to bar 2, which was validated by Stat3 knockdown by siRNA (Fig. 3I). Further, we compared bar 2 and 5, and in turn minimized the BP-1-102–induced effect that is otherwise observed in the wild-type, uninduced cells (Fig. S5C and D, compare the relative change between bars 2 and 3 to that of bars 5 and 6). Thus, KLF8 expression is one of the underlying mechanisms of Stat3-mediated tumor cell migration and invasiveness.
inhibition of activated Stat3 diminishes nuclear Stat3 that in turn down-regulates nuclear pNF-κB.

To explore further the BP-1-102 effect on Stat3 cross-talks, we examined the production of soluble factors by tumor cells. Culture medium from BP-1-102-treated MDA-MB-231 cells showed lower granulocyte colony-stimulating factor (G-CSF), soluble intercellular adhesion molecule (sICAM) 1, and macrophage migration-inhibitory factor (MIF)/glycosylation-inhibiting factor (GIF) levels (Fig. 3K). Moreover, treatment of cells with exogenous G-CSF further induced Stat3 and RelA phosphorylation above constitutive levels (Fig. S6C, compare lanes 1 and 3) and consequently blocked the BP-1-102-repressive effect (Fig. S6C, compare lanes 2 and 4). Thus, BP-1-102 inhibits the production of soluble factors by tumor cells.

BP-1-102 Inhibits Growth of Human Breast and Non–Small-Cell Lung Tumor Xenografts and Modulates Stat3 Activity, Stat3 Target Genes, and Soluble Factors in Vivo. BP-1-102 inhibited growth of mouse xenografts of human breast (MDA-MB-231) and non–small-cell lung (A549) tumors that harbor aberrantly active Stat3 when administered i.v. (tail vein injection, 1 or 3 mg/kg, every 2 or 3 d for 15 d) (Fig. 4 A and C) or oral gavage (3 mg/kg, 100 μL, every day) (Fig. 4B). No significant changes in body weights (Fig. S7 A and B) or obvious signs of toxicity, such as loss of appetite, decreased activity, or lethargy, were observed during the efficacy study or in a separate toxicity study where animals were dosed 1 or 3 mg/kg i.v. every 2 or 3 d for 21 d and monitored over 42 d, as shown by body weight and gross anatomical examination of organs (Fig. S7 C and D). The apparent stronger antitumor response to oral gavage is likely due to the daily dosing.

Analysis of tumor tissue lysates shows decreased Stat3 DNA-binding activity in treated tumors (T1 and T3) compared with nontreated control (Fig. S8A, Upper). Immunoblotting analysis of lysates from residual tumor tissues also showed suppression of pY705Stat3, c-Myc, Cyclin D1, Bcl-xL, Survivin, and VEGF expression that occurred in a dose-dependent manner (Fig. S8A, Lower), decreased pFAK, phospho-paxillin, KLF8, and EPSTI1 levels, enhanced E-cadherin expression (Fig. S8B), and diminished pRelA (Fig. S8C), compared with control tumors. Analysis of residual tumor tissue lysates (T1 and T3) also showed suppression of sICAM-1, MIF/GIF, serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (Serpine 1), and interleukin 1 receptor antagonist (IL-1RA) production (Fig. S8D), whereas G-CSF was undetectable.

BP-1-102 Is Detectable at Micromolar Concentrations in Plasma and in Micrograms in Tumor Tissues. In vivo pharmacokinetic profiling of plasma samples from a cohort of three mice collected at 15, 30, 60, 90, 180, and 360 min post-i.v. treatment (3 mg/kg) with a single dose showed BP-1-102 levels upward of 35 μM at 15 min postdosing, which declined by 30 min to a steady 5–10 μM level for up to 6 h (Fig. 4D, i), whereas plasma samples post-oral dosing at 3 mg/kg showed peak BP-1-102 levels of about 30 μM at 30 min, which steadily declined to 5–10 μM over a 6-h period (Fig. 4D, ii). Thus, blood levels over a prolonged period can exceed the IC50 values against Stat3. Moreover, BP-1-102 was detectable at 55 or 32 μg/g tumor tissue, respectively, for i.v. or oral delivery of 3 mg/kg, 15 min after the last dosing, and at 25 or 15 μg/g tumor tissue, respectively, for i.v. or oral delivery of 3 mg/kg, 24 h after the last dosing (Fig. 4E). Data together confirm that BP-1-102 is orally bioavailable and that the agent accumulates in tumor tissues at levels sufficient to inhibit aberrantly active Stat3 functions and inhibit tumor growth.

Discussion

BP-1-102 is designed as a Stat3–Stat3 dimerization disruptor with optimized structural features to enhance inhibitory activity. Stat3 binding is supported by SPR, FP, ITC, and NMR data that also show disruption of Stat3 SH2 domain–pTyr peptide interaction. Modeling predicts it binds to the three solvent-accessible subpockets of the Stat3–Stat3 dimer interface (27) (Fig. 1B), making hydrogen bonds in the third subpocket, and additional interactions with the charged Lys side chain via the unique pentfluorobenzene, which contribute to increased activity. Binding at the pTyr peptide binding site of the SH2 domain would disrupt pre-existing Stat3–Stat3 dimers and block de novo Stat3 phosphorylation at the receptor and dimer formation. The reduced nuclear pYStat3 is the combination of diminished nuclear translocation, due to activation inhibition, and Stat3 nuclear exit upon dimer disruption (28).

The antitumor cell effects and the in vivo efficacy of BP-1-102 are consistent with thwarting Stat3’s key role in tumorigenesis, including dysregulation of gene expression leading to uncontrollable growth, survival, and angiogenesis (2, 4). The present study identifies KLF8 (24) as a Stat3 target gene that promotes motility, migration, and invasion. Further, the induction of FAK and paxillin phosphorylation, EPSTI1 (25) expression, and the down-regulation of E-cadherin likely contribute to Stat3-mediated malignant progression (Fig. S9). Both KLF8 and EPSTI1 promote epithelial–mesenchymal transition and tumor invasiveness and are up-regulated in invasive and metastatic tumors (24, 25), as is aberrantly active Stat3. The induction of these events would repress epithelial cell assembly and cadherin-based cell–cell adhesions and promote dynamic regulation of cell–matrix adhesions that would drive tumor migration and invasiveness.

In the strong interplay of tumor cells with the microenvironment, Stat3 modulates inflammatory cytokines/chemokines that in turn suppress immune and inflammatory cells’ functions and tumor-immune surveillance (6). Of the factors regulated by Stat3 (5), IL-6, VEGF, and NF-κB also in turn promote Stat3 activation or are in cross-talks that perpetuate protumorigenic processes (6, 7, 26). The present study raises the possibility that sICAM-1 (29), G-CSF, MIF/GIF, Serpine 1, and IL-1RA induction support Stat3-dependent tumor processes, including angiogenesis (30) (Fig. S9); MIF is overexpressed in breast cancer (31) and promotes disease progression (32), Serpine 1 expression correlated with advanced clear cell renal cell carcinoma and promoted tumor angiogenesis and aggressiveness (33). Further, IL-1RA blocked IL-1–induced antitumor cell effects in prostate cancer cells (34) and enhanced the proliferation (35) and growth
of hepatic and glioblastoma cells (36), and sIL-1RA mRNA expression correlated with lymph node and hepatic metastases in gastric carcinoma patients (37). BP-1-102-mediated repression of these tumor-supporting factors likely contributes to its anti-tumor effects.

We present a unique Stat3 inhibitor, BP-1-102, that sufficiently accumulates in tumor tissues and induces antitumor responses in human tumor xenografts that harbor aberrantly active Stat3. The oral bioavailability of BP-1-102 represents a substantial advancement in the discovery of small-molecule Stat3 inhibitors as unique anticancer agents.

**Materials and Methods**

**Cells and Reagents.** NIH 3T3, NIH 3T3xv-Src, and NIH 3T3xv-Ras cells, the human breast cancer line MDA-MB-231 and counterpart expressing inducible KLF8 shRNA (231-K8kd), and human prostate (DU145), non–small-cell lung (A549), and pancreatic (Panc-1) cancer cells have all been previously reported (11, 17, 20, 24). Stat3-dependent (pLucTKS3), Stat3-independent (pLucSRE), and pLucKLF8 luciferase reporters, and the v-Src, β-galactosidase, Stat3 SH2 domain, Stat3C, and pLVUT-TRAKR-KLF8 vectors have all been reported (9, 19–21, 23, 24). Other reagents are the Human Cytokine Array Kit (R&D Systems), G-CSF (Sigma-Aldrich) used at 100 ng/mL, and Stat1 and Stat5 (9, 19).

**Materials and Methods.**

**Cloning and Protein Expression.** Molecular cloning, expression, and purification of His-tagged Stat3 have previously been published (17). The cloning of the pXJ-FLAG-Stat3 SH2 domain and mutant KLF8 promoter is described in detail in *SI Materials and Methods.*

**Cell Viability, Proliferation, Colony Survival, and Wound-Healing Assays.** Studies were performed as described in detail (17). Details are provided in *SI Materials and Methods.*

**Immunostaining with Laser-Scanning Confocal Imaging.** Studies are described in detail in *SI Materials and Methods.*

**Fluorescence Polarization Assay.** Assays were conducted as previously reported (17).

**Surface Plasma Resonance Analysis.** Studies were performed as previously reported (17).

**Cell Migration/Invasion Assays.** Experiments were carried out and quantified as previously described (9, 24, 28). Details are provided in *SI Materials and Methods.*

**Cytokine Analysis.** The assay is described in detail in *SI Materials and Methods.*

**Tumor Xenografts, Efficacy, and Pharmacokinetic Studies.** Studies were performed as previously reported (9, 17, 28). BP-1-102 concentrations in mouse plasma and tumor tissue lysates were assayed using a validated analytical procedure via HPLC. Details are provided in *SI Materials and Methods.*

**Statistical Analysis.** Statistical analysis was performed on mean values using Prism (GraphPad Software). The significance of differences between groups was determined by the paired *t* test at *P < 0.05, **P < 0.01, and ***P < 0.005.

**ACKNOWLEDGMENTS.** We thank all colleagues and members of our labora-
tories for stimulating discussions, and the Sanford-Burnham Medical Research Institute Pharmacology Core for conducting the pharmacokinetic studies. This work was supported by National Cancer Institute Grants CA106439 (to J.T.), CA128865 (to J.T.), and CA132977 (to J.Z.); the University of Hawaii (J.T.); and the University of Toronto (P.T.G.).
SI Results

The lead agent, S3I-201.1066 (1), is a moderately potent signal transducer and activator of transcription (Stat)3 Src homology (SH)2 domain-binding ligand (2) and accesses the three solvent-accessible subpockets on the SH2 domain surface. We hypothesized that improved binding to the third pocket would enhance inhibitory activity. By extensive structure–activity relationship analysis of S3I-201.1066 and analogs structurally designed with appendages that promote interactions with all of the three subpockets on the Stat3 SH2 domain surface, BP-1-102 (Fig. 1A) was identified. BP-1-102 has appendages that promote interactions with all three subpockets (Fig. 1B). It retains the 4-aminosalicylic acid group as an effective phosphotyrosine (pTyr) mimetic (2), which binds to the pTyr-binding portion of the SH2 domain, making interactions with Lys591, Glu594, and Arg609 (Fig. 1B), and contains the hydrophobic cyclohexylbenzyl substituent, which forms van der Waals interactions with a series of predominantly hydrophobic residues, including Val637, Ile659, and Trp623 (Fig. 1B) that comprise the pY+1 (Leu)-binding pocket. The key modification is the pentafluorobenzene sulfonamide component of the molecule, linked via a glycine unit to the salicylic acid, which interacts with the previously unexplored third subpocket composed of Lys591, Glu594, Ile634, and Arg595 (Fig. 1B). Critically, the pentafluorobenzene may better interact with the Stat3 SH2 domain surface by participating in hydrogen bonds and also better interacting with the charged Lys side chain, as previously noted in a different context (3). The more polar pentafluorobenzene unit also confers enhanced solubility and oral bioavailability.

Scheme S1. (A) BnBr (2 equivalents), KOtBu, DMF, 0 °C, 16 h, 73%. (B) 4-Cyclohexylbenzaldehyde, AcOH, NaCNBH3, room temperature (rt), 16 h, 79%. (C) (CF3CO)2O, DIPEA (diisopropylethylamine), CH2Cl2, rt, 3 h, 96%. (D) TFA/CH2Cl2, 1:1, rt, 5 h, 100%. (E) 3, PPh3Cl2, CHCl3, 60 °C, 12 h, 97%. (F) LiOH·H2O, THF/H2O, 3:1, rt, 10 min, 98%. (G) PhF5SO2Cl, DIPEA, CH2Cl2, rt, 16 h, 93%. (H) H2, 10% Pd/C, MeOH/THF, 1:1, rt, 16 h, 95%.
SI Materials and Methods

Chemical Methods. Anhydrous solvents—methanol, DMSO, CH2Cl2, THF, and dimethylformamide (DMF)—were purchased from Sigma-Aldrich and used directly from Sure/Seal bottles. Molecular sieves were activated by heating to 300 °C under vacuum. All reactions were performed under an atmosphere of dry nitrogen in oven-dried glassware and were monitored for completeness by TLC (visualized by UV light, or developed by treatment with KMnO4 stain or phosphomolybdic acid stain).1 Hand13CN M R spectra were recorded on a Bruker 400 MHz spectrometer in either CDCl3, CD3OD, or d6-DMSO. Chemical shifts (δ) are reported in parts per million after calibration to residual isotopic solvent. Coupling constants (J) are reported in Hz. Before biological testing, inhibitor purity was evaluated by reversed-phase HPLC (rpHPLC). Analysis by rpHPLC was performed using a Microsorb-MV 300 A

(1) Benzyl 2-(benzyloxy)benzoate. To a stirred solution of primary aniline (3.00 g, 19.6 mmol) in DMF (0.1 M) at 0 °C was added KOtBu (2.42 g, 21.6 mmol). After 15 min, benzyl bromide (2.57 mL, 21.6 mmol) was added dropwise. The suspension was allowed to stir at room temperature (rt) for a further 4 h before the reaction vessel was again cooled to 0 °C. A further 1.1 equivalents of KOtBu (2.42 g, 21.6 mmol) were added before the dropwise addition of benzyl bromide (2.57 mL, 21.6 mmol). The reaction was then stirred overnight before quenching with H2O. The solution was then repeatedly extracted with ethyl acetate and the organics were combined. The organics were then washed with H2O and brine and dried over Na2SO4 and concentrated in vacuo (3.40 g, 74%): δH (400 MHz, d6-DMSO) 5.07 (s, 2H, CH2), 5.21 (s, 2H, CH2), 5.99 (br s, 2H, NH2), 6.17 (d, J = 8.6 and 2.0 Hz, 1H, CH); δC (100 MHz, d6-DMSO) 65.8, 70.2, 99.1, 106.7, 109.0, 126.3, 126.8, 127.5, 127.7, 127.9, 128.1, 128.3, 128.4, 134.3, 136.6, 136.7, 152.2, 160.7, 165.7; LRMS (low-resolution mass spectrometry) (ES+) calculated for [C21H19NO3 + H] 334.14, found 334.17.

(2) Benzyl 4-amino-2-(benzyloxy)benzoate. To a stirred solution of 4-aminosalicylic acid (I) (3.00 g, 19.6 mmol) in DMF (0.1 M) at 0 °C was added carboxylic acid (0.70 g, 1.4 mmol) and DIPEA [3.65 g (4.80 mL), 27.5 mmol] in CHCl3 (0.1 M) was added trifluoro anhydride (2.54 g, 12.1 mmol). The solution was allowed to stir at rt for 3 h before quenching with water and extraction into CH2Cl2. The combined organic layers were washed with water and brine and dried over Na2SO4 and the solution was concentrated under reduced pressure to give 5 (1.44 g, 88%): δH (400 MHz, d6-CDCl3) 1.46 (s, 9H, 3 CH3), 3.08 (s, 1H, CH3), 3.18 (d, J = 6.0 Hz, 1H, CH); δC (100 MHz, d6-CDCl3) 26.0, 26.7, 34.3, 39.8, 73.8, 90.9, 104.0, 110.6, 114.3, 129.8, 130.9, 132.6, 133.1, 147.2, 149.4, 152.1, 155.5, 163.0; LRMS (ES+) calculated for [C34H35NO3 + H] 556.27, found 556.22.

(3) Benzyl 2-(benzyloxy)-4-(4-cyclohexylbenzylamino)benzoate. To a solution of primary aniline (2) (1.7 g, 5.0 mmol) and acetic acid (0.36 g, 6.0 mmol) stirred in anhydrous MeOH (0.1 M) with 4-Å molecular sieves was added 4-cyclohexylbenzaldehyde (1.0 g, 5.5 mmol). The solution was then heated to 45 °C for 3 h and allowed to cool to rt. Next, NaCNBH3 (0.47 g, 7.5 mmol) was added and the reaction was allowed to stir at rt overnight. When TLC indicated the reaction was complete, the reaction was diluted with CH2Cl2, filtered, and concentrated in vacuo. The crude product was dissolved in CH2Cl2, washed with saturated NaHCO3, H2O, and brine, and then dried over Na2SO4. Compound 3 was isolated using flash chromatography in a mixture of CH2Cl2 and EtOAc (2.4 g, 83%): δH (400 MHz, d6-CDCl3) 1.25–1.48 (m, 5H, CH2), 1.74–1.95 (m, 5H, CH2), 2.48–2.52 (m, 1H, CH), 4.28 (s, 2H, CH2), 4.49 (br s, 1H, NH), 5.08 (s, 2H, CH2), 5.32 (s, 2H, CH2), 6.17 (J = 2.0 Hz, 1H, CH), 6.21 (dd, J = 8.6 and 2.0 Hz, 1H, CH), 7.19–7.27 (m, 4H, 4 CH), 7.28–7.37 (m, 6H, 6 CH), 7.40–7.49 (m, 4H, 4 CH), 7.85 (dd, J = 8.6 Hz, 1H, 1 CH); δC (100 MHz, d6-CDCl3) 26.0, 26.7, 34.3, 44.1, 47.3, 65.7, 70.3, 97.1, 104.8, 108.2, 126.8, 127.0, 127.4, 127.5, 127.6, 127.9, 128.2, 134.2, 135.4, 136.7, 136.8, 147.4, 152.9, 160.8, 165.8; LRMS (ES+) calculated for [C34H35NO3 + H]+ 556.27, found 556.22.

(4) Benzyl 2-(benzyloxy)-4-(4-cyclohexylbenzylamino)benzoate. To a solution of primary aniline (2) (1.7 g, 5.0 mmol) and acetic acid (0.36 g, 6.0 mmol) stirred in anhydrous MeOH (0.1 M) with 4-Å molecular sieves was added 4-cyclohexylbenzaldehyde (1.0 g, 5.5 mmol). The solution was then heated to 45 °C for 3 h and allowed to cool to rt. Next, NaCNBH3 (0.47 g, 7.5 mmol) was added and the reaction was allowed to stir at rt overnight. When TLC indicated the reaction was complete, the reaction was diluted with CH2Cl2, filtered, and concentrated in vacuo. The crude product was dissolved in CH2Cl2, washed with saturated NaHCO3, H2O, and brine, and then dried over Na2SO4. Compound 3 was isolated using flash chromatography in a mixture of CH2Cl2 and EtOAc (2.4 g, 83%): δH (400 MHz, d6-CDCl3) 1.25–1.48 (m, 5H, CH2), 1.74–1.95 (m, 5H, CH2), 2.48–2.52 (m, 1H, CH), 4.28 (s, 2H, CH2), 4.49 (br s, 1H, NH), 5.08 (s, 2H, CH2), 5.32 (s, 2H, CH2), 6.17 (J = 2.0 Hz, 1H, CH), 6.21 (dd, J = 8.6 and 2.0 Hz, 1H, CH), 7.19–7.27 (m, 4H, 4 CH), 7.28–7.37 (m, 6H, 6 CH), 7.40–7.49 (m, 4H, 4 CH), 7.85 (dd, J = 8.6 Hz, 1H, 1 CH); δC (100 MHz, d6-CDCl3) 26.0, 26.7, 34.3, 44.1, 47.3, 65.7, 70.3, 97.1, 104.8, 108.2, 126.8, 127.0, 127.4, 127.5, 127.6, 127.9, 128.2, 134.2, 135.4, 136.7, 136.8, 147.4, 152.9, 160.8, 165.8; LRMS (ES+) calculated for [C34H35NO3 + H]+ 556.27, found 556.22.
3.4 mmol). The reaction was then heated to 60 °C and stirred overnight. The reaction was allowed to cool and the solvents were removed under reduced pressure. The concentrate was absorbed directly onto silica for column chromatography (hexanes:EtOAc 2:1) purification yielding compound 7 (0.91 g, 97%): δH (400 MHz, CDCl3) 1.35–1.44 (m, 5H, CH2), 1.71–1.90 (m, 5H, CH2), 3.17 (m, 4H, CH2), 3.79 (s, 2H, CH2), 4.84 (s, 2H, CH2), 4.97 (s, 2H, CH2), 5.35 (s, 2H, CH2), 6.65 (s, 1H, CH), 6.78 (dd, J = 8.4 and 1.6 Hz, 1H, CH), 7.10–7.19 (m, 4H, CH2), 7.29–7.43 (m, 10H, CH), 7.86 (d, J = 8.4 Hz, 1H, CH); δC (100 MHz, CDCl3) 25.8, 26.6, 34.2, 36.7, 44.0, 51.2, 52.6, 66.7, 70.4, 77.2, 113.9, 115.0, 117.6, 119.8, 126.8, 126.9, 127.7, 128.0, 128.3, 128.3, 128.4, 132.9, 133.6, 135.5, 135.6, 144.6, 147.5, 157.0, 158.4, 156.1, 156.4; LRMS (ES+) calculated for [C37H40N2O4] [M+Na]+ m/z 695.27, found 695.36.

(8) Benzyl 2-(benzoyloxy)-4-(N-(4-cyclohexylbenzyl)-2-(methylamino)-acetamido)benzoate. Compound 7 (2.68 mmol) was dissolved in a THF:H2O (3:1) solution and treated with LiOH-H2O (337 mg, 8.04 mmol). After 10 min, the reaction was completed and diluted with H2O. The product was extracted into EtOAc and the combined extracts were washed with saturated NaHCO3, H2O, and brine and dried over Na2SO4 and concentrated under reduced pressure to yield 8 (1.57 g, 99%): δH (400 MHz, CDCl3) 1.35 (m, 5H, CH2), 1.66–1.84 (m, 5H, CH2), 2.28 (s, 2H, CH2), 2.44 (m, 1H, CH), 3.02 (s, 2H, CH2), 4.81 (s, 2H, CH2), 4.89 (s, 2H, CH2), 5.30 (s, 2H, CH2), 6.52 (s, 1H, CH), 6.54 (d, 1H, J = 8.0 Hz, CH), 7.05–7.13 (m, 4H, CH), 7.24–7.37 (m, 12H, CH), 7.80 (d, J = 8.0 Hz, 1H, CH); δC (100 MHz, CDCl3) 25.6, 26.3, 34.0, 35.4, 43.7, 52.0, 52.2, 66.4, 70.0, 77.2, 113.6, 119.6, 119.9, 126.4, 126.6, 127.5, 127.6, 127.8, 128.0, 128.1, 128.3, 132.5, 133.9, 135.3, 135.4, 145.0, 147.0, 158.1, 164.8, 169.6; LRMS (ES+) calculated for [C37H40N2O4] [M+Na]+ m/z 695.27, found 695.36.

(9) Benzyl 2-(benzoyloxy)-4-(N-(4-cyclohexylbenzyl)-2-(3,4,5,6-pentafluorophenyl)sulfonamido)acetamido)benzoate. To a stirred solution of amine (8) (55 mg, 0.1 mmol) dissolved in CH2Cl2 (0.1 M) was added DPEA (N,N-disopropylethylamine) (163 mg, 0.15 mmol) and pentafluorobenzensulfonyl chloride (28.2 mg, 0.11 mmol). After 1 h, the reaction was diluted with CH2Cl2, washed with water followed by a brine wash, and dried over Na2SO4. The organic layer was then concentrated under reduced pressure and purified by silica gel column chromatography to yield 9 (49 mg, 63%): δH (400 MHz, d-CDCl3) 1.34–1.42 (m, 5H, CH2), 1.70–1.86 (m, 5H, CH2), 2.43–2.52 (m, 1H, CH), 3.05 (s, 3H, CH3), 3.86 (s, 2H, CH2), 4.67 (s, 2H, CH2), 4.94 (s, 2H, CH2), 5.35 (s, 2H, CH2), 6.44 (s, 1H, CH), 6.66 (d, J = 8.0 Hz, 1H, CH), 6.96 (d, J = 7.2 Hz, 2H, CH), 7.12 (d, J = 7.2 Hz, 2H, CH), 7.30–7.41 (m, 10H, CH), 7.84 (dd, J = 8.0 and 1.2 Hz, 1H, CH); δC (100 MHz, d-CDCl3) 26.0, 26.7, 34.4, 35.4, 44.1, 51.9, 52.7, 67.0, 70.70, 111.8, 114.0, 115.8, 119.9, 121.1, 127.0, 127.1, 128.1, 128.2, 128.5, 128.6, 128.7, 133.3, 133.4, 135.6, 135.6, 137.8, 141.6, 142.9, 144.2, 147.9, 158.7, 165.2, 165.8; LRMS (ES+) calculated for [C39H39F3N2O5] [M+Na]+ m/z 807.84, found 807.79.

Identification of Putative Stat3 Binding Sites in the Human Krüppel-Like Factor 8 Promoter. The human Krüppel-like factor (KLF8) promoter sequence (−441 to +946; GenBank accession no. NM_007250.4) was searched, and three putative Stat3 binding sites [TTAGCTCTATGA (−253 to −245), TTGCGTGAAG (+217 to +225), and TTCTTACCA (+260 to +268)] were selected for further investigation into Stat3 DNA-binding activity by electrophoretic mobility shift assay (EMSA) analysis. The 32P-labeled oligonucleotide probes used in the assay, with putative Stat3 binding sites underlined, are 5′-AGCTGAGAATTAGCTCAGGAGT-3′, 5′-AGCTGCCATTTTGCTGGAACCCCTTG-3′, and 5′-AGCTCTATGTCTTACCATCTGGCCG-3′. Oligonucleotide sequences were purchased from Invitrogen.

Cloning of pXJ-Flag-Stat3 SH2 Domain and Site-Directed Mutagenesis of the KLF8 Promoter. The DNA fragment of Stat3 SH2 domain (residues 582–668) flanked by BamHI at the 5′ end and KpnI at the 3′ end was cloned into pXJ4-FLAG [a kind gift of X. Cao, Institute of Molecular and Cellular Biology, Singapore (4, 5)]. The new construct was confirmed by sequencing. The human KLF8 promoter-driven luciferase reporter construct pLucKLF8 was previously described (6). The putative Stat3 binding site TTAGCTCAGA (−253 to −245) in the human KLF8 promoter (GenBank accession no. NM_007250.4) was mutated to GTAGCTCAGA us-
ing Stratagene’s QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) and following the manufacturer’s instructions to generate pLucKLF8/–253T/G.

**Nuclear Extract Preparation, EMSA, and Densitometric Analysis.** Nuclear extract preparations and EMSA analysis were carried out as previously described (1, 7–9). The 32P-labeled oligonucleotide probes used were hSIE (high-affinity Six-inducible element from the c-fos gene, m67 variant, 5′-AGCTTTATTTCCGTATATCCTA-T-3′) that binds Stat1 and Stat3 (8, 10) and the mammary gland factor element from the bovine β-casein gene promoter (sense strand, 5′-AGATTTTCAGGAATTCAA-3′) that binds Stat5 (11, 12). For the direct effect of BP-1-102 on STAT DNA-binding activity, nuclear extracts containing activated STATs prepared from NIH 3T3/n-Src or EGF-stimulated NIH 3T3/hEGFR cells were preincubated with the agent for 30 min at room temperature before incubation with the radiolabeled probe for 30 min at 30 °C before subjection to EMSA analysis. Where appropriate, supershift analysis was performed in which nuclear extracts were preincubated with the anti-Stat3 antibody directed against Stat3 N-terminal amino acid residues 5–240 (F-2) (Santa Cruz Biotechnology) before incubation with the radiolabeled probe. Bands corresponding to DNA-binding activities for each concentration of BP-1-102 were scanned and quantified using ImageQuant (GE Healthcare) and plotted as a percentage of BP-1-102 IC50 values were derived from these plots, as previously reported (13, 14).

**Transient Transfection of Cells, Treatment with BP-1-102, and in Vitro Studies.** Transient transfection and luciferase assays were performed as previously reported (9, 15, 16). Transfection was performed 18 h following seeding for 3 h using Lipofectamine Plus (Invitrogen) and following the manufacturer’s protocol. For luciferase studies, cells in 12-well plates were transiently cotransfected with the appropriate plasmids, 100 ng β-galactosidase (internal control vector for normalizing), 900 ng pLucTKS3, pLucSRE, pLucKLF8, or pLucKLF8/–253T/G, and with or without 300 ng pMyv-Src. Where appropriate, cells in 96-well plates were transfected with Stat3C (0.2 μg) or in 6-well plates were transfected with 4 μg Stat3C, Stat3 SH2 domain, or pCDNA3 (mock). Twelve hours after transfection, cells were treated or untreated with BP-1-102 (0–30 μM) for 16–48 h and harvested. Subsequently, cytosolic extracts were performed for luciferase assay or nuclear extracts were prepared for EMSA analysis, as previously reported (9, 15), whole-cell lysates were prepared for immunoblotting analysis, or the cells were processed for CyQUANT proliferation or Annexin V/flow cytometry analysis.

The Stat3 siRNA smart pool Stat3 (M-003544) and the control, SiGENOME nontargeting siRNA pool (Dharmacon RNAi Technologies, Thermo Scientific), were transiently transfected into cells using Lipofectamine RNAiMAX (Invitrogen) in serum-free Opti-MEM culture medium (5 mL) (Invitrogen) according to the manufacturer’s instructions and using 200 pmol siRNA with 10 μL of Lipofectamine.

**Immunoprecipitation and Western Blotting Analyses.** Whole-cell lysates or tumor tissue lysates from pulverized tumor tissues were prepared in boiling SDS sample loading buffer to extract total proteins, as reported previously (9, 17, 18). Lysates of equal total protein were electrophoresed on an SDS/7.5% (g/vol) polyacrylamide gel and transferred to a nitrocellulose membrane. Nitrocellulose membranes were probed with primary antibodies, and the detection of horseradish peroxidase-conjugated secondary antibodies by enhanced chemiluminescence (Amer sham) was performed. Immunoprecipitation studies were performed as previously reported (7) using whole-cell lysates or nuclear extracts (250 μg total protein) and 2 μg of anti-Stat3, anti–NF-κB/p65RelA, or anti-IκB polyclonal antibody (Santa Cruz Bio-technology) or 5 μL of monoclonal anti-Stat3 antibody (Cell Signaling Technology).

**CyQUANT Cell Proliferation, Viability, Colony Survival, and Wound-Healing Assays.** These studies were performed as previously reported (1, 16). Briefly, proliferating cells in 6- or 96-well plates were treated once with 0–30 μM BP-1-102 for 24 h or with 10 μM BP-1-102 for up to 96 h. Viable cells were counted by trypan blue exclusion/phase-contrast microscopy or assessed by a CyQUANT Cell Proliferation Kit, according to the manufacturer’s (Invitrogen) instructions. For colony-survival studies, cells were seeded as a single-cell culture. On the next day following seeding, cells were treated once or not with BP-1-102 and allowed to culture until large colonies were visible, which were fixed with methanol and stained with crystal violet (Thermo Fisher Scientific) for 2 h. The number of colonies was counted or photomicrographs were taken under a phase-contrast microscope. For wound-healing assays, subconfluent cultures of cells in 6-well plates were wounded using pipette tips and treated with or without BP-1-102 and allowed to migrate into the denuded area over a 16-h period. The migration of cells was visualized at a 10x magnification using an Axiovert 200 inverted fluorescence microscope (Zeiss), with pictures taken using a mounted Canon Powershot A640 digital camera. Cells that migrated into the denuded area were quantified.

**Immunostaining with Laser-Scanning Confocal Imaging.** Studies were performed as previously reported (19). Briefly, cells were grown on glass coverslips in multiwell plates, fixed with ice-cold methanol for 15 min, washed three times with 1x PBS, permeabilized with 0.2% Triton X-100 for 10 min, and further washed three or four times with PBS. Specimens were then blocked in 1% BSA for 30 min and incubated with anti-pY705Stat3 (Cell Signaling) or anti–pS536NF-κB/p65 (Cell Signaling) antibody at 1:50 dilution (in 0.1% BSA) at 4 °C overnight. Subsequently, cells were rinsed three times with PBS and incubated with two Alexa Fluor secondary antibodies, Alexa Fluor 546 (goat anti-mouse) and Alexa Fluor 488 (donkey anti-rabbit) (Molecular Probes, Invitrogen) for pY705Stat3 and pS536NF-κB/p65 detection, respectively, for 1 h at room temperature in the dark. Specimens were then washed three times with PBS, mounted on slides with VECTASHIELD mounting medium containing DAPI (Vector Labs), and examined immediately under a Leica TCS SP5 confocal microscope. Images were captured and processed using Leica TCS SP 5 software.

**KLF8 Knockdown and Overexpression and Cell Migration/Invasion Assays.** Cell migration/invasion experiments were carried out and quantified as previously reported (7, 8, 20) using BioCoat migration/invasion chambers (BD Biosciences) of 24-well companion plates with cell-culture inserts containing 8-μm pore size filters and following the manufacturer’s protocol, with some modifications. Briefly, for doxycycline (Dox) induction, cells were maintained uninduced (U; in the absence of Dox) or induced (I; in the presence of Dox) for 3 d. Cells were then resuspended in serum-free medium with or without Dox, transferred to the top chambers of the 24-well transwell plates, and incubated for 16 h to allow the migration or invasion toward the serum-containing medium in the bottom chamber, and cells on the lower side were then counted. BP-1-102 (0–10 μM) was added to both the top and bottom chambers during the 16-h incubation. Where appropriate, the migration or invasion rates were normalized to the control, U cells in the absence of serum and in the bottom chambers.

For KLF8 overexpression, lentiviral particles were prepared and packaged following a published procedure (21) by transfecting cultures of HEK293FT cells with tetracycline-inducible KLF8 expression vector, pLVUT-tTR-KRAB-KLF8 (12 μg), and pSPAX2 (8 μg) and pMD2G (4 μg) packaging system (Addgene) and following the manufacturer’s instructions. For transient
fection, the viral particle-containing HEK293FT cell-culture medium was added to MDA-MB-231 cells in culture and incubated for 48 h at 37 °C. Five nanograms per mL tetracycline was added to the culture and further incubated for 24 h. Infected cells were used in a BioCoat invasion chamber assay, as previously published (7, 8, 22), in the presence or absence of different concentrations of BP-1-102.

Cytokine Assay. Cytokine analysis was performed using a Human Cytokine Array Kit and following the manufacturer’s (R&D Systems) instructions. Briefly, following treatment of cells with 10 μM BP-1-102 for 48 h, 1-mL samples of conditioned culture medium or, in the case of tumors, 500 μg of tumor tissue lysates in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% (vol/vol) Nonidet P-40, 150 mM NaCl, 2 mM EDTA, 0.1% SDS), were mixed with a mixture of biotinylated detection antibodies. The mixture was incubated with the array membrane for antibody binding on the membrane. The membrane was processed for signal development using streptavidin-HRP and chemiluminescent detection reagents and exposed to X-ray films, which were then processed. The relative changes in cytokine levels between samples were analyzed by quantitation of pixel density in each spot of the array with ImageJ (National Institutes of Health).

Fluorescence Polarization Assay. This was conducted as previously reported (1, 16). Full-length Stat3 and its peptide probe, the interleukin (IL)-6R/gp130 peptide, have been previously reported (1, 16). Stat1 and Stat5 were purchased from SignalChem. The peptides, GpYDKPHVHL-NH2 and GpYLVDKWH-NH2, used for Stat1 and Stat5 fluorescence polarization (FP), respectively, were purchased from CanPeptide.

NMR and Isothermal Titration Calorimetry Studies for Effect of BP-1-102 on IL-6R/gp103 pYTrp Peptide Binding to Stat3 SH2 Domain. The 1D-1H Carr-Purcell-Meibom-Gill (CPMG) R2 relaxation filter experiment was conducted at 298K on a Bruker 600-MHz magnet equipped with a cryoprobe (23). Fifty micromolar gp130 phospho-tyrosine peptide ([pY904, Ac-pY-LPQTV-NH2]) was dissolved in 20 mM sodium phosphate (pH 3.2), 0.2% DMSO-d6, and 90% (vol/vol) D2O. Binding of pY904 to monomeric BP-1-102 was at least 10 μM as judged by a 1D-1H NMR solubility test of 200 μM BP-1-102 at varying concentrations of DMSO-d6 (0.2–40% vol/vol). For the isothermal titration calorimetry (ITC) experiments, the same buffer conditions used in the NMR experiment were maintained for 150 μM titrant (pY904 or BP-1-102) and 10 μM STAT3 in the sample cell of a Microcal VP-ITC calorimeter. Titration of Stat3 ligand-precomplexed samples used equimolar amounts of pY904 or BP-1-102 in the sample cell. Titrant solution (290 μL) was injected at 10-μL increments into a sample cell containing 1.4 mL Stat3 at 298K. The heat flow of the titration reactions was measured at each addition of a pY904 or BP-1-102 aliquot to free or ligand-complexed Stat3. Origin software (Microcal) was used to generate the final ITC figures (Fig. S1 D and E). The area under each titration peak in Fig. S1 D and E (Upper) was integrated (Lower) and plotted against the corresponding ligand:Stat3 molar ratio. The y intercept of the lower panel plots represents the directly measured enthalpy change (ΔH).

Phospho-Kinase Profiling. Phospho-kinase profiling assay was performed using a Human Phospho-Kinase Array Kit according to the manufacturer’s instructions (R&D Systems). Briefly, DMSO or BP-1-102–treated cells were washed twice with ice-cold PBS, and whole-cell lysates were prepared using the lysis buffer provided in the kit. Array membranes were blocked with array buffer 1 and incubated overnight at 4 °C with 400 μg of cell lysate diluted with array buffer 1. The membranes were washed with wash buffer, incubated with the detection antibodies (provided in the kit), and subjected to detection using streptavidin-HRP, according to the instructions. After washing, the membranes were developed with Pierce chemiluminescence reagents (Thermo Fisher Scientific). The developed images were analyzed based on the pixel density of each spot of the array by scanning and quantifying, and expressed as the fold change compared with the control group.

Mice and In Vivo Tumor Studies. Six-week-old female athymic nude mice were purchased from Harlan and maintained in institutional animal facilities approved by the American Association for Accreditation of Laboratory Animal Care. All mice studies were performed under an Institutional Animal Care and Use Committee (IACUC)-approved protocol. Athymic nude mice were injected s.c. in the left flank area with 1 × 106 human breast cancer MDA-MB-231 or non–small-cell lung cancer A549 cells in 100 μL PBS. After 5–10 d, tumors of a 30–100 mm3 volume were established. Animals with established tumors were grouped so that the mean tumor sizes in all groups were nearly identical and then given BP-1-102 (in 0.05% DMSO in water) at 1 or 3 mg/kg (i.v.) every 2 or every 3 d or 3 mg/kg (oral gavage, 100 μL) every day for 15 or 20 d. Animals were monitored every day, and tumor sizes were measured with calipers and body weights were taken every 2 or 3 d. Tumor volumes were calculated according to the formula \( V = \frac{a \times b^2}{2} \), where \( a \) is the smallest super- critical diameter and \( b \) is the largest super-critical diameter. For each treatment group, the tumor volumes for each set of measurements were statistically analyzed in comparison with the control (untreated) group using a paired t test.

Plasma and Tumor Tissue Analysis. BP-1-102 concentrations in mouse plasma and tumor tissue lysates were assayed using a validated analytical procedure via HPLC (Prominence UHPLC; Shimadzu Scientific Instruments) and LC/MS/MS (API 4000 linear ion trap mass spectrometer; MDS Sciex). The mass spectrometer was operated in a product ion-scanning mode. BP-1-102 solution diluted in methanol was infused directly into the MS source at a flow rate of 10 μL/min. Tuning was evaluated in both positive and negative MS modes using both turbo ion spray and atmospheric pressure chemical ionization sources. The chromatography used a Phenomenex Kinetex C18 2.1 × 50 mm, 1.7 μm UHPLC column, with a flow rate of 0.300 mL/min using 5 mM ammonium acetate (in water) and 5 mM ammonium acetate (in acetonitrile) as mobile phases A and B, respectively.

In Vivo Toxicity Studies. Six-week-old female athymic nude mice were purchased from Harlan and maintained in institutional animal facilities approved by the American Association for Accreditation of Laboratory Animal Care. All mice studies were performed under an IACUC-approved protocol. Groups of three or four healthy mice were administered BP-1-102, i.v. at 1 or 3 mg/kg every 2 or 3 d, as was pursued in the antitumor efficacy studies, for 21 d and monitored for up to 42 d. Body weights were measured every 2 or 3 d. Following completion, mice were euthanized and surgery was performed to expose the abdominal organs, which were subsequently removed for visual examination and imaging by a digital camera.


Fig. S1. Characterization of Stat3 binding to BP-1-102 compared with the interaction with the tyrosine-phosphorylated IL-6 receptor/glycoprotein (gp)130 peptide. (A) Surface plasmon resonance analysis of the binding of increasing concentrations of BP-1-102 to full-length Stat3. (B and C) Fluorescence polarization assay of the binding to 10 nM 5-carboxyfluoresceinyl-labeled IL-6R/gp130 peptide (GpYLPTQV-NH₂) (B), IFN-γ receptor peptide (GpYDKPHVL-NH₂) (C, i), or erythropoietin receptor peptide (GpYLVLDKW-NH₂) (C, ii) probe of a fixed amount of purified His-Stat3 (200 nM), Stat1 (120 nM), or Stat5 (105 nM), respectively, in the presence of increasing concentrations (0–100 μM) of BP-1-102. Inhibition of FP signal is represented as percent inhibition (of control) vs. concentration of BP-1-102. (D and E) Isothermal titration calorimetry measurements of the binding of (D) pY904 to Stat3 in the absence (Left) and presence (Right) of BP-1-102, or (E) BP-1-102 to Stat3 in the absence (Left) and presence (Right) of pY904. The area under each titration peak (D and E, Upper) is integrated (Lower) and plotted against the corresponding pY904:Stat3 or BP-1-102:pY904 molar ratio. The red line is the fit of the binding isotherm to extract the thermodynamic parameters using a one-site binding model. (F) Overlay of a section of the 1D 1H R2 CPMG NMR spectra of 50 μM pY904 alone (black tracing), in the presence of 2 μM Stat3 (red tracing), and together with 100 μM BP-1-102 (blue tracing) at 0.1% DMSO. The CPMG pulse train used a 3-ms interpulse delay and a total R2 relaxation time of 72 ms, which completely attenuated the Stat3 signal. (G) The 1D 1H NMR spectra of 200 μM BP-1-102 at various concentrations of DMSO (0.2–40%) showing broadened signal at reduced DMSO. The apparent concentration of monomeric BP-1-102 is reduced to 10 μM under completely aqueous conditions. Data are representative of two or three independent determinations.
Fig. S2. Effects of BP-1-102 on the induction of Stat3 phosphorylation, intracellular localization, and transcriptional activity. (A) EMSA analysis using hSIE probe of Stat3 DNA-binding activity in nuclear extracts of equal total protein prepared from the designated tumor cells treated or untreated with 20 μM BP-1-102 for 30 min. (B) Immunoblots of pY705Stat3 and Stat3 in whole-cell lysates of equal total protein prepared from the designated malignant cells treated or untreated with 15 μM BP-1-102 for the indicated times. (C) Cytosolic extracts of equal total protein were prepared from 24-h BP-1-102–treated or untreated MDA-MB-231, Panc-1, or DU145 cells transiently transfected with the Stat3-dependent (pLucTKS3, TKS3) or Stat3-independent [pLucSRE, serum response element (SRE)] luciferase reporter and analyzed for luciferase activity using a luminometer. (D and E) Immunoblotting analysis of lysates of nuclear (Nuc), cytosolic (Cyto), or mitochondrial fractions of equal total protein prepared from the designated tumor cells untreated or treated with 15 μM BP-1-102 for 24 h and probing for pY705Stat3 or Stat3. (F) EMSA analysis using hSIE probe of Stat3C DNA-binding activity in nuclear extracts of equal total protein prepared from normal NIH 3T3 fibroblasts transfected with or without Stat3C vector and treated or untreated with 15 μM BP-1-102 for the indicated times. Positions of protein–DNA complex or proteins in the gel are labeled; control (0) or (−) represents extracts or lysates prepared from 0.05% DMSO-treated cells. Data are representative of two to four independent determinations. Values are the mean and SD of three independent determinations each performed in triplicate. For each transfection, luciferase activity was normalized to transfection efficiency, with β-galactosidase activity as an internal control. **P < 0.01.
Fig. S3. BP-1-102 selectively suppresses proliferation, survival, migration, and invasion in vitro of Stat3-dependent malignant cells. (A–C) Tumor cells harboring aberrantly active Stat3 (MDA-MB-231, DU145, Panc-1, and NIH 3T3/v-Src) or cells that do not (NIH 3T3/v-Ras and mouse thymus stromal epithelial cells, TE-71) (A) and growing in culture were untreated (dash lines) or treated once with 15 μM BP-1-102 (solid lines) for 24–96 h and the number of viable cells was assessed each day by trypan blue exclusion/phase-contrast microscopy and plotted; (B) were seeded as single-cell cultures treated once with 0–15 μM BP-1-102 for 24 h and allowed to culture until large colonies were visible, which were stained with crystal violet and photographed (Upper) or enumerated and plotted (Lower); or (C) were cultured and wounded and treated once with 0–15 μM BP-1-102 for 16 h and allowed to migrate into the denuded area. Cultures were imaged and are represented as photomicrographs. Visualization was done at 10× magnification by light microscopy. (A) (Insets) Bar graphs of viable cell numbers at day 0 (open bars) and day 1 for BP-1-102–treated cells (filled bars). (D) BioCoat migration/invasion chamber assay and the effect of BP-1-102 on the invasiveness of MDA-MB-231 cells. Images were captured under light microscopy with a digital camera. Data are representative of three or four independent determinations. Values are the mean and SD of four independent determinations, each performed in triplicate. *P < 0.05, **P < 0.01.
Fig. S4. Time-course study of effects of BP-1-102 on focal adhesion kinase (FAK) and paxillin phosphorylation. Immunoblotting analysis of whole-cell lysates prepared from MDA-MB-231 cells treated with 10 μM BP-1-102 for the indicated times and probing for FAK, phospho-FAK, paxillin, phospho-paxillin, or β-actin. Positions of proteins in the gel are shown. Data are representative of three independent determinations.

Fig. S5. Transcriptional induction of KLF8 by Stat3 and the effect of BP-1-102. (A) Normalized luciferase reporter activity in cytosolic extracts of equal total protein prepared from normal NIH 3T3 fibroblasts transiently transfected with the wild-type KLF8 promoter-driven luciferase reporter, pLucKLF8, or mutant KLF8 promoter-driven reporter, pLucKLF8-253T/G, together with v-Src and β-galactosidase expression vectors (for internal control), and the effect of 16-h treatment with BP-1-102. (B) KLF8 promoter analysis showing (i) the nucleotide sequences for the three putative Stat3 binding sites, 1, 2, and 3, and (ii) the EMSA analysis of Stat3 DNA-binding activity to the labeled oligonucleotide sequence probes incorporating each of the three putative Stat3 binding sites, compared with the binding to the high-affinity si-inducible element probe using nuclear extract preparations of equal total protein containing activated Stat3 from NIH 3T3/v-Src. (C and D) BioCoat migration/invasion chamber assay of MDA-MB-231-K8ikd cells harboring inducible KLF8 shRNA and the impact of induced KLF8 knockdown (I) on the BP-1-102 effect on cell (C) migration and (D) invasion, compared with the wild-type, uninduced (U) cells. Data are representative of three independent determinations. Values are the mean and SD of two or three independent determinations each performed in triplicate. *p < 0.05, **p < 0.01.

Fig. S6. Effect of BP-1-102 on the activation and localization of NF-κB/p65RelA. (A) Immunofluorescence imaging/confocal microscopy of Stat3 colocalization with p65RelA in MDA-MB-231 cells growing in culture and treated with or without 5–25 μM BP-1-102 for 2 or 16 h, fixed, and stained with anti-Stat3 antibody and secondary Alexa Fluor 546 antibody (red), anti-p65RelA and secondary Alexa Fluor 488 antibody (green), or DAPI nuclear staining (blue). Images were captured using a Leica TCS SPS laser-scanning confocal microscope. (B) Immunoblot immunoassay to the MDA-MB-231 cells treated with or without 15 μM BP-1-102 and probing for RelA or IκB. (C) Immunoblotting analysis of whole-cell lysates of MDA-MB-231 cells stimulated with G-CSF in the presence or absence of BP-1-102 and probing for pY705Stat3, Stat3, pRelA, RelA, and β-actin. Data are representative of three independent studies. Blots were scanned and quantified by ImageQuant analysis, and numbers represented as percentage of control (100%) are shown in parentheses.
**Fig. S7.** Evaluation of toxicity of BP-1-102 in mice. (A and B) Plots of body weight against days of treatment for mice bearing human breast (MDA-MB-231) tumors and treated with BP-1-102 via (A) i.v., 1 or 3 mg/kg or vehicle (0.1% DMSO in PBS) every 2 or 3 d or (B) oral gavage, 3 mg/kg or vehicle (0.1% DMSO) every day for 14 d. Mice were weighed daily or every 2 d. (C and D) Healthy mice were treated with BP-1-102 via i.v., 1 or 3 mg/kg or vehicle (0.1% DMSO in PBS) every 2 or 3 d for 21 d and observed for 42 d and (C) weights, measured daily or every 2 d, are plotted, or (D) images, captured by a digital camera of the internal organs exposed following surgery at the end of the study, are shown. Control (Con or 0) represents 0.05 or 0.1% DMSO-treated mice. Data are representative of four to six mice in each group. Values are the mean and SD of replicates of four to six mice in each group. (C) The arrow indicates the last treatment day.

**Fig. S8.** In vivo treatments with BP-1-102 and effects on Stat3 activity, Stat3-regulated genes, cytokine production, and factors that promote tumor motility, migration, and invasiveness. (A–C) Tumor lysates prepared from control (Con) human breast tumor xenografts or residual tumor (T1–T4) tissues from mice treated with BP-1-102 (1 or 3 mg/kg) via i.v. or oral gavage were subjected to (A, Upper) Stat3 DNA-binding activity/EMSA analysis, or (A, Lower; B and C) immunoblotting analysis probing for pY705Stat3, Stat3, c-Myc, Cyclin D1, Bcl-xL, Survivin, VEGF, pFAK, FAK, phospho-paxillin, paxillin, E-cadherin, KLF8, epithelial–stromal interaction (EPSTI)1, pRelA, RelA, or β-actin (D) Analysis of soluble intercellular adhesion molecule (sICAM), macrophage migration-inhibitory factor (MIG)/glycosylation-inhibiting factor (GIF), Serpine 1, and IL-1RA levels in tumor tissue lysates treated via i.v. Positions of Stat3–DNA complexes or proteins in the gel are labeled; control (Con or 0) represents tumor tissue lysates prepared from 0.05% DMSO-treated mice. Data are representative of three or four independent determinations. Blots were scanned and quantified by ImageQuant analysis, and numbers represented as percentage of control (100%) are shown in parentheses. Values are the mean and SD from replicates of 7–10 tumor-bearing mice in each group. **P < 0.01.
Fig. 59. Model for BP-1-102–mediated molecular changes in malignant cells harboring aberrantly active Stat3 that contribute to antitumor cell effects and the antitumor response. BP-1-102 attenuates aberrant Stat3 signaling and consequently suppresses nuclear Stat3–NF-κB cross-talk and the induction of KLF8, c-Myc, Cyclin D1, Survivin, VEGF, and Bcl-xL. In malignant cells harboring aberrantly active Stat3, BP-1-102 further suppresses FAK and paxillin phosphorylation, EPSTI1 expression, and sICAM, G-CSF, MIF/GIF, Serpine 1, and IL-1RA production, and enhances E-cadherin expression. The modulation of these events would contribute to BP-1-102–induced inhibition of tumor cell growth, survival, motility, migration, invasion, and tumor growth in vivo.

Table S1. Phospho-kinase profile for BP-1-102 effects in MDA-MB-231 cells using a kinase array kit

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Change relative to untreated control, 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>p38α-T180/Y182</td>
<td>0.8</td>
</tr>
<tr>
<td>Erk1/2-T202/Y204, T185/Y187</td>
<td>1.0</td>
</tr>
<tr>
<td>JNK pan-Y183/Y185, T221/Y223</td>
<td>1.4</td>
</tr>
<tr>
<td>MEK1/2-S218/S222, S222, S226</td>
<td>0.9</td>
</tr>
<tr>
<td>MSK1/2-S376/S360</td>
<td>1.1</td>
</tr>
<tr>
<td>Akt-S473</td>
<td>1.0</td>
</tr>
<tr>
<td>Akt-T308</td>
<td>0.9</td>
</tr>
<tr>
<td>GSK-3αβ, S21/S9</td>
<td>0.9</td>
</tr>
<tr>
<td>AMPKα1-T174</td>
<td>0.9</td>
</tr>
<tr>
<td>AMPKα2-T172</td>
<td>1.1</td>
</tr>
<tr>
<td>RSK1/2-S380/S386/S377</td>
<td>1.0</td>
</tr>
<tr>
<td>RSK1/2-S221/S227</td>
<td>1.0</td>
</tr>
<tr>
<td>Pyk2-Y402</td>
<td>1.1</td>
</tr>
<tr>
<td>PLCγ1, Y783</td>
<td>1.0</td>
</tr>
<tr>
<td>p70 S6 kinase-T389</td>
<td>1.0</td>
</tr>
<tr>
<td>p70 S6 kinase-T421</td>
<td>1.0</td>
</tr>
<tr>
<td>p70 S6 kinase-T229</td>
<td>1.0</td>
</tr>
<tr>
<td>Src-Y419</td>
<td>1.2</td>
</tr>
<tr>
<td>Lyn-Y397</td>
<td>0.8</td>
</tr>
<tr>
<td>Lck-Y394</td>
<td>0.9</td>
</tr>
<tr>
<td>Yes-Y426</td>
<td>1.1</td>
</tr>
<tr>
<td>Fgr-Y412</td>
<td>0.8</td>
</tr>
<tr>
<td>Fyn-Y420</td>
<td>1.3</td>
</tr>
</tbody>
</table>