Novel STAT3 Phosphorylation Inhibitors Exhibit Potent Growth-Suppressive Activity in Pancreatic and Breast Cancer Cells

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Abstract

The constitutive activation of signal transducer and activator of transcription 3 (STAT3) is frequently detected in most types of human cancer where it plays important roles in survival, drug resistance, angiogenesis, and other functions. Targeting constitutive STAT3 signaling is thus an attractive therapeutic approach for these cancers. We have recently developed novel small-molecule STAT3 inhibitors, known as FLLL31 and FLLL32, which are derived from curcumin (the primary bioactive compound of turmeric). These compounds are designed to bind selectively to Janus kinase 2 and the STAT3 Src homology-2 domain, which serve crucial roles in STAT3 dimerization and signal transduction. Here we show that FLLL31 and FLLL32 are effective inhibitors of STAT3 phosphorylation, DNA-binding activity, and transactivation in vitro, leading to the impediment of multiple oncogenic processes and the induction of apoptosis in pancreatic and breast cancer cell lines. FLLL31 and FLLL32 also inhibit colony formation in soft agar and cell invasion and exhibit synergy with the anticancer drug doxorubicin against breast cancer cells. In addition, we show that FLLL32 can inhibit the induction of STAT3 phosphorylation by IFNα and interleukin-6 in breast cancer cells. We also show that administration of FLLL32 can inhibit tumor growth and vascularity in chicken embryo xenografts as well as substantially reduce tumor volumes in mouse xenografts. Our findings highlight the potential of these new compounds and their efficacy in targeting pancreatic and breast cancers that exhibit constitutive STAT3 signaling.

Introduction

The American Cancer Society estimated that more than 200,000 new cases of invasive breast cancer and more than 40,000 deaths are expected among women in the United States per year. In women, breast cancer remains the second most fatal form of cancer after lung cancer. Pancreatic cancer is one of the most serious of cancers. According to the American Cancer Society, there are an estimated 42,470 new cases and 35,240 deaths from pancreatic cancer in the United States in 2009. The prognosis is generally regarded as poor and the overall survival rate of all stages is less than 1% at 5 years, with most patients dying within 1 year. There is a critical need to develop more effective treatments for pancreatic and breast cancers.

Signal transducer and activator of transcription 3 (STAT3) is a member of a family of latent cytosolic transcription factors whose activation is contingent on the phosphorylation of a conserved tyrosine residue (Y705) by upstream kinases such as Janus kinase 2 (JAK2; ref. 1). This event promotes the dimerization of STAT3 monomers via their Src homology-2 (SH2) domains, rendering them in a transcriptionally active conformation (2). Persistent activation of the JAK2/STAT3 signaling pathway has been documented in a wide range of human solid and blood cancers and is commonly associated with a worse prognosis (3, 4). Among the cancer-promoting activities ascribed to persistent STAT3 signaling are those involved with cell proliferation, metastasis, angiogenesis, host immune evasion, and resistance to apoptosis (5, 6). Interference with the STAT3 signaling pathway in cancer cells, such as by antisense oligonucleotides and dominant-negative STAT3 mutants, has been shown to result in growth inhibition and the induction of apoptosis (7–10). Although STAT3 serves critical and necessary roles in early embryogenesis, its presence in the majority of normal adult cell types is largely dispensable, making it an attractive target for cancer therapy.
(11–13). Numerous strategies have since been used to inhibit constitutive STAT3 signaling in cancerous cells. The selective inhibition of JAK2 can prevent STAT3 phosphorylation and dimerization, and several JAK2 inhibitors including AG490, WP1066, and SD-1029 have been reported in recent literature (14–16). The necessity of an intact SH2 domain also makes it a rational target for the disruption of STAT3 signaling (17). Peptide-based SH2 inhibitors have previously been described, but they are hindered by limited cell permeability, poor in vivo stability, and the potential for immunogenicity (18, 19). The development of nonpeptide small-molecule SH2 inhibitors, such as Statick, STA-21, and S3I-201, has also been recently reported (17, 20, 21).

In conjunction with our collaborators, we have developed and evaluated a series of novel nonpeptide JAK2/SH2 inhibitors based on the structure of the plant phytochemical known as curcumin. Curcumin is the bioactive component of Curcuma longa and the subject of extensive research due to its broad spectrum of biologically beneficial activities and its relative safety in large dosages (22, 23). The complex chemistry of curcumin allows it to inhibit multiple oncogenic processes, including those associated with the JAK2/STAT3 pathway (24). Poor bioavailability limits the use of curcumin as a cancer therapeutic agent, but it is potentially useful as a lead compound for the development of new JAK2/STAT3 inhibitors (25). In our initial testing, two of our compounds in particular, designated FLLL31 and FLLL32, were found to be especially potent at inhibiting the viability of breast and pancreatic cancer cells that feature constitutively activated STAT3, thus warranting a closer look at their antitumor properties. Here we report our findings on the growth-suppressive activities of FLLL31 and FLLL32, their efficacy in inhibiting constitutive STAT3 signaling in vitro, and the effects of FLLL32 on the processes of tumor growth and angiogenesis in vivo.

Materials and Methods

Cell culture. Pancreatic cancer cell lines (PANC-1, BXPC-3, HPAC, and SW1990), breast cancer cell lines (MDA-MB-231, SK-BR-3, MDA-MB-468, and MDA-MB-453), WI-38 normal human lung fibroblasts, and human embryonic kidney (HEK)-293 cells were purchased from the American Type Culture Collection. These cells were cultured in DMEM supplemented with 10% fetal bovine serum. SUM159 breast cancer cells were obtained from Dr. Max S. Wicha (University of Michigan, Ann Arbor, MI). Normal human mammary epithelial cells (HMEC) and normal human embryonic smooth muscle cells (BdsMC) were purchased from Lonza Walkersville, Inc. SUM159 and HMEC were cultured in Ham’s F12 containing 5% fetal bovine serum (FBS), 5 μg/mL insulin, 1 μg/mL hydrocortisone, and 10 ng/mL epithelial growth factor. The BdsMC were cultured in medium recommended by the protocol from Lonza Walkersville. Human pancreatic duct epithelial cells (HPDE) were provided by Dr. Ming-Sound Tso (Ontario Cancer Institute, University of Toronto, Toronto, Ontario, Canada) and maintained in CnT-07CF epidermal keratinocyte medium supplemented with 0.07 mmol/L CaCl2.

JAK2 and STAT3 SH2 inhibitors. FLLL31, FLLL32, and WP1066 (16), a JAK2 inhibitor, were synthesized in Dr. Pui-Kai Li’s laboratory. JAK2 inhibitors AG490 (15) and SD-1029 (14) and STAT3 SH2 inhibitors S3I-201 (21) and Statick (20) were purchased from Calbiochem. Curcumin was purchased from Sigma-Aldrich.

Western blot analysis. Cancer cells were treated with these agents or DMSO for 24 h. For IFNα and interleukin-6 (IL-6) treatments, MDA-MB-453 breast cancer cells were serum starved for 24 h. The cells were pretreated with FLLL32 (10 μmol/L) for 2 h and 50 ng/mL of IFNα or IL-6 were then added for 30 min before the cells were collected. Membranes were probed with a 1:1,000 dilution of antibodies (Cell Signaling Tech.) against phospho-specific STAT3 (hereafter referred to as P-STAT3; tyrosine 705), extracellular signal-regulated kinase-1/2 (ERK1/2; threonine 202/tyrosine 204), AKT (serine 473), epidermal growth factor receptor (EGFR; tyrosine 1068), p70S6K (threonine 389), protein kinase C-δ (PKC-δ; threonine 505), Src (tyrosine 416), mammalian target of rapamycin (mTOR; serine 2448), cleaved poly(ADP-ribose) polymerase (PARP), cleaved caspase-3, cyclin D, Bcl-2, survivin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Membranes were analyzed using enhanced chemiluminescence Plus reagents and scanned with the Storm Scanner (Amersham Pharmacia Biotech, Inc.).

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JAK2 and other human kinase activity assays. JAK2 kinase activity was assessed with the HTScan JAK2 Kinase Assay Kit per manufacturer’s protocol (Cell Signaling Technologies). The possible effects of FLLL32 on the other 10 purified human protein kinases were determined by using a KinaseProfiler Assay (Reaction Biology Corp.).

STAT3 DNA-binding and STAT3-dependent transcriptional luciferase activity assays. Following treatment with FLLL31, FLLL32, curcumin, or DMSO for 24 h, nuclear extracts of cells were analyzed for STAT3 and STAT1 DNA binding activities in triplicates using TransFactor Universal STAT3- and STAT1-specific kits following the manufacturer’s protocol (Clontech, Inc.). To assess transcriptional activity, MDA-MB-231 cells were stably transfected with the pLucTKS3 luciferase reporter, which contains seven copies of the STAT3 binding site in a thymidine kinase minimal promoter (26). The cells were harvested in reporter lysis buffer after 24 h of treatment. Luciferase activity is expressed as counts per second per microgram of protein. Luciferase activity is presented relative to a pLucTKS3-transfected sample treated with DMSO, arbitrarily set at 100%. The results of the luciferase assay represent the averages from three independent experiments.

Reverse transcription-PCR analysis. RNA was collected from MDA-MB-231 and PANC-1 cells with RNeasy kits (Qiagen) after 24 h of treatment with FLLL31, FLLL32, and...
Curcumin. cDNA was generated from 500-ng samples of RNA using Omniscript RT (Qiagen). Primer sequences and source information can be found in Supplementary Table S1.

**MTT cell viability assay.** Cells were seeded in 96-well plates (3,000 per well) in triplicate and then treated with 0.5 to 5 μmol/L of FLLL31 or FLLL32 or with 0.5 to 30 μmol/L of curcumin (Sigma) for 72 h. MTT (Sigma; 25 μL) was added to each sample and incubated for 3.5 h. Then, 100 μL of N,N-dimethylformamide solubilization solution were added to each well. The absorbance at 450 nm was read the following day. Half-maximal inhibitory concentrations (IC50) were determined using Sigma Plot 9.0 software (Systat Software, Inc.).

**Soft-agar colony formation assay.** A 0.6% agar gel with 10% FBS in DMEM was prepared and added to a six-well culture dish as a base agar. MDA-MB-231 cells (5,000 per well) were plated in 0.4% agar gel with 10% FBS in DMEM supplemented with curcumin, FLLL31, or FLLL32 on top of the base agar and allowed to grow for 2 wk. Colonies were stained with MTT dye. Numbers were normalized as a percentage of colonies formed in DMSO treatment. The results represent the averages from three independent experiments.

**Cell invasion assay.** Cell invasiveness was evaluated with the InnoCyte Laminin-Based 96-Well Cell Invasion Assay per manufacturer’s instructions (EMD Biosciences). In brief, 5 × 10⁴ MDA-MB-231 cells were seeded in a laminin-coated cell culture insert in serum-free DMEM and treated with 1 μmol/L of DMSO, FLLL31, FLLL32, or curcumin. Cells were allowed to migrate/in invade the laminin layer in the presence of DMEM supplemented with 10% FBS as a chemoattractant for 24 h. Invading cells were stained with Calcein-AM and detected with a fluorimeter. The data were normalized as relative fluorescent units (RFU) per 10⁴ cells. Results are from three independent experiments and compared as a percentage of the RFU of the DMSO-treated controls.

**Transfection with constitutive active STAT3.** HEK293 cells were transfected using Lipofectamine 2000 with a vector that encodes constitutive STAT3 (STAT3-C), which is tagged with a FLAG epitope (27). The second day, cells were treated with FLLL32 or DMSO. Twenty-four hours later, cells were harvested for Western blot analysis.

**Mouse xenografts.** All animal studies were conducted in accordance with the standard procedures approved by the Institutional Animal Care and Use Committee of the Research Institute at Nationwide Children’s Hospital. MDA-MB-231 breast cancer cells (1 × 10⁷ in Matrigel) were implanted s.c. into the flank region of 4- to 5-wk-old female athymic nude mice. After tumors developed (12 d), the mice were randomized into two groups and treated with 50 mg/kg FLLL32 or DMSO (five mice per group) i.p. daily for 19 d. Tumor growth was determined by measuring the major (L) and minor (W) diameter with a caliper. The tumor volume was calculated according to the following formula: tumor volume = 0.5236 × L × W².

**Chorioallantoic membrane xenograft assay.** The procedure used was a modification of a previously published procedure (28). Fertile leghorn chicken eggs (CBT Farms) were incubated until 10 DI. The sample size was 8 to 14 embryos from two separate experiments. PANC-1 cells
(250,000 in inert extracellular matrix) were implanted just under the chorioallantoic membrane (CAM). Four and 48 h after tumor implantation, a maximal tolerated dose of 5 mg/kg gemcitabine (ChemieTek), 25 mg/kg FLLL32, or solvent (DMSO in sterile water for injection) was placed onto the chorion. Four days after tumor implantation, CAMs were fixed as described (28). Images were captured at ×6.25 magnification using a bright-field dissecting microscope (Wild M400 Photomakroscop) connected to a cooled camera (QImaging). The number of vessels surrounding the tumor was manually counted, and the size of the tumor determined using NIH ImageJ densitometry software.

**Determination of combinatorial effects.** The ability of doxorubicin, FLLL31, and FLLL32 to act in a synergistic manner with regard to growth inhibition was determined as described by Chou and colleagues (29). CalcuSyn software (Biosoft) was used to determine the combinational index (CI) for each concentration of drug mixture used. A CI value of <1 represents a case where synergism of doxorubicin, FLLL31, and FLLL32 was present. CI values of 1 and >1 represent additive and antagonistic effects, respectively.

**Results**

*The JAK2/SH2 inhibitors FLLL31 and FLLL32.* FLLL31 and FLLL32 are diketone analogues of curcumin (Fig. 1A). The central β-dicarbonyl moiety of curcumin is subject to keto-enol tautomerization, which is hypothesized to influence its target selectivity and by virtue of its biological activity.6 By replacing the two hydrogen atoms on the central carbon of curcumin with geminal dimethyl substituents (FLLL31) or a spiro-cyclohexyl ring (FLLL32), the ability of curcumin to enolize is eliminated. These modifications are also predicted to better interact with key binding sites of JAK2 and the SH2 dimerization domain of STAT3 than the keto-enol form of curcumin (Fig. 1B). In addition, FLLL31 and FLLL32 feature 3,4-dimethoxy substituents to mimic those of dimethoxycurcumin, a curcumin analogue that has been shown to have increased stability, higher plasma...

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6 J. Fuchs et al., unpublished data.
concentration, and greater efficacy against cancer cells than standard curcumin (30, 31).

**FLLL31 and FLLL32 inhibit JAK2 kinase activity.** JAK2 serves as a docking site for the SH2 domain of STAT3 monomers. The subsequent JAK2-mediated phosphorylation of STAT3 activates the transcription factor, promoting its dimerization (32, 33). We evaluated the ability of FLLL31 and FLLL32 to inhibit JAK2 kinase activity. In our comparisons, 5 μmol/L of FLLL31 and FLLL32 significantly inhibited JAK2 kinase activity (P < 0.05) over a DMSO control, resulting in approximately 60% and 75% reductions in activity, respectively (Supplementary Fig. S1). In addition, our compounds were more effective than previously characterized JAK2 inhibitors, such as AG490 and WP1066, and curcumin itself.

**FLLL31 and FLLL32 downregulate STAT3 phosphorylation and DNA-binding activity.** To examine the efficacy of FLLL31 and FLLL32 in inhibiting STAT3 phosphorylation, a panel of breast and pancreatic cancer cell lines known to express high endogenous levels of constitutively activated STAT3 were used. FLLL31 and FLLL32 effectively reduced levels of P-STAT3 in MDA-MB-231 breast and PANC-1 pancreatic cancer cells (Fig. 2). We observed similar results in SK-Br-3, MDA-MB-468, and SUM159 breast cancer cells and in HPAC, BXPC-3, and SW1990 pancreatic cancer cells (Supplementary Figs. S2A–C and S3A–C, respectively). The expression of STAT3 target genes, such as *cyclin D1*, *survivin*, and *Bcl-2* (3, 34, 35), was likewise downregulated, and neither compound had appreciable inhibitory effects on the expression or phosphorylation status of other kinases and transcription factors such as ERK1/2, mTOR, PKC-δ, p70S6K, AKT, and Src (Fig. 2; Supplementary Fig. S2). Increased levels of P-ERK1/2 were observed in a few cancer cell lines on FLLL31 and FLLL32 treatment. Decreases in P-STAT3 were concomitant with increased cleavage of procaspase-3 and PARP, one of the immediate substrates of this effector caspase.

We further examined whether FLLL32 inhibits the activity of other human kinases using a kinase profile assay. FLLL32 exhibits little inhibition (IC₅₀ >100 μmol/L) on tyrosine kinases, containing SH2 or both SH2 and SH3 domains and other protein kinases such as AKT2, cyclin-dependent kinase-2/cyclin D1, EGFR, ErbB2/HER2, and Met (Supplementary Table S2). These results support the specificity of FLLL32 to inhibit STAT3.

To support that induction of apoptosis is through inhibition of endogenous STAT3, we transiently expressed a known constitutively active STAT3, STAT3-C (27), in cancer cells. We rationalize that if FLLL32 induces apoptosis through inhibition of endogenous STAT3, the restoration of STAT3 activity by transfected STAT3-C should rescue FLLL32-mediated apoptosis. We chose to focus on FLLL32 in this experiment instead of FLLL31 because, based on the computer modeling and free binding energy, FLLL32 is more selective for STAT3 (Fig. 1) and is our leading compound to target STAT3. Our results show that the expression of STAT3-C can rescue the induction of apoptosis evidenced by cleaved caspase-3 in HEK293 cancer cells (Supplementary Fig. S4). These results provide additional support that induction of apoptosis by FLLL32 is through the endogenous STAT3 protein in cancer cells.

Furthermore, the induction of apoptosis was limited to the cancer cells because concentrations of FLLL31 and FLLL32 as high as 10 μmol/L were unable to induce caspase-3 cleavage in HMEC or HPDE cells, which represent normal breast and pancreatic cells, respectively (Fig. 2C). Additional normal cell lines, such as WI-38 lung and BdsMC bladder cells, likewise showed no evidence of apoptosis following FLLL31 and FLLL32 treatment (Supplementary Fig. S5). We also found that the STAT3 phosphorylation levels were low, and FLLL32 treatment slightly induced STAT3 phosphorylation levels in WI-38 and BdsMC cells (Supplementary Fig. S5)

![Figure 3](https://example.com/figure3.png)

**Figure 3.** A, FLLL31 and FLLL32 reduce the ability of STAT3 to bind DNA in nuclear extracts isolated from MDA-MB-231 breast cancer and PANC-1 pancreatic cancer cells. B, FLLL31- and FLLL32-mediated decreases in STAT3 DNA-binding activity correlate with increases in STAT1 DNA-binding activity in MDA-MB-231 cells.
but reduced STAT3 phosphorylation levels in HMEC cells (data not shown). The selectivity of our compounds for STAT3 inhibition was evident when compared with closely related proteins, such as STAT1 and STAT2. The addition of IFNα resulted in robust phosphorylation of STAT1, STAT2, and STAT3 (Fig. 2D). Pretreatment of these cells with FLLL32 effectively inhibited phosphorylation of STAT3 specifically but had no effect on the extent of STAT1 and STAT2 phosphorylation. FLLL32 was similarly able to block induction of P-STAT3 in MDA-MB-453 by IL-6 (Supplementary Fig. S6).

We then examined the ability of STAT3 to bind DNA in breast and pancreatic cancer cells following treatment with FLLL31 and FLLL32. Levels of STAT3 DNA-binding activity dropped sharply in MDA-MB-231 and PANC-1 cells following FLLL31 and FLLL32 treatment, decreases that were significantly lower than those elicited by equimolar concentrations of curcumin (Fig. 3A). Similar results were observed in MDA-MB-468 and HPAC (Supplementary Fig. S7A and B). It is again important to note that these effects were specific to STAT3 DNA-binding activity. STAT1 DNA-binding activity was not only unimpaired but actually slightly enhanced by FLLL31 and FLLL32 (Fig. 3B), which may have important consequences given the proapoptotic and antiproliferative activities of STAT1 (36, 37).

**FLLL31 and FLLL32 are potent inhibitors of STAT3-mediated gene transcription.** We next assessed the effects of FLLL31 and FLLL32 on STAT3 transcriptional activity with luciferase assays. Due to its high endogenous levels of P-STAT3, the MDA-MB-231 breast cancer cell line was chosen for stable transfection with pLucTKS3 (Fig. 4A). We found that both FLLL31 and FLLL32 reduced luciferase activity in a dose-dependent fashion following 24 hours of treatment (Fig. 4B). Furthermore, the extents of these reductions were significantly greater than those induced by comparable dosages of curcumin ($P < 0.05$).

To further confirm these observations, we then looked at the expression of several STAT3 target genes, such as cyclin D1, Bcl-2, Bcl-x, survivin, and VEGF (34, 35). In MDA-MB-231 (Fig. 4C) and PANC-1 (Fig. 4D) cancer cells, FLLL31 and FLLL32 visibly reduced the expression of the STAT3 target genes over a DMSO control and equimolar concentrations of curcumin.

**FLLL31 and FLLL32 inhibit cell viability and cell invasion and generate synergy with doxorubicin.** After establishing the efficacy of FLLL31 and FLLL32 as selective JAK2/STAT3 inhibitors, we compared their growth-suppressive activities to those produced by previously reported inhibitors, including the aforementioned JAK2 inhibitors WP1066, SD-1029,
and AG490 and the STAT3 SH2 inhibitors Stattic and S3I-201. The IC50 values calculated for each compound and cell line are summarized in Supplementary Table S3. In our comparisons, FLLL31 and FLLL32 showed better growth-suppressive activity relative to the other compounds, performing similarly with respect to one another in each of the cell lines tested.

We next looked at the effect that FLLL31 and FLLL32 had on the anchorage-independent growth and invasive capacity of the MDA-MB-231 cell line. We assessed anchorage-independent growth with soft-agar colony formation assays (38). As expected, FLLL31 and FLLL32 were both found to inhibit the formation of MDA-MB-231 colonies, each reducing overall colony numbers by approximately 40% over that of curcumin (Fig. 5A).

We also determined what effects FLLL31 and FLLL32 had on the invasive capacity of MDA-MB-231 over curcumin, which had comparatively minimal effect (Fig. 5B).

We also examined whether FLLL31 or FLLL32 could act in an additive or synergistic manner with conventional chemotherapeutic drugs, such as doxorubicin. We found that 100 to 400 nmol/L dosages of doxorubicin acted in a synergistic fashion with a 5,000 nmol/L dose of FLLL31 or FLLL32 (Fig. 5C and D, respectively) in the MDA-MB-231 breast cancer cell line, resulting in a significant decrease in cell viability over either agent acting alone (P < 0.05).

**FLLL32 suppresses tumor growth and vascularization in vivo.** Mouse xenograft experiments were then done to gauge the antitumor effects of our compounds in an in vivo system. We shifted our focus to FLLL32 for these experiments due to its higher predictive binding energy to JAK2 and STAT3 SH2 than FLLL31 (data not shown). The administration of FLLL32 resulted in significantly reduced tumor burdens in the MDA-MB-231 xenografted mice relative to their DMSO-treated counterparts (Fig. 6A). Western blots done with tumor tissue samples harvested from these mice also showed decreases in total levels of STAT3 phosphorylation.

We next examined whether FLLL32 would affect tumor growth and vascularity in a chicken embryo CAM human tumor xenograft model (ref. 28; Fig. 6B–D). It was found that gemcitabine, the standard chemotherapeutic agent for treatment of pancreatic cancer, reduced both tumor volume (Fig. 6C) and tumor vascularity (Fig. 6D) as compared with untreated embryos, but these decreases were statistically insignificant. FLLL32 administration resulted in a significant reduction in both tumor volume (Fig. 6C) and tumor vascularity (Fig. 6D) in the CAM xenograft assay.

**Discussion**

In the United States alone, breast and pancreatic cancers are estimated to have caused approximately 40,170 and 35,240 deaths, respectively, for the year ending in 2009 (40). The present work was prompted by the following
observations: a large percentage of pancreatic and breast cancers feature aberrantly activated STAT3 (3); approaches that selectively target constitutive STAT3 signaling have been shown to be effective at inhibiting cancer-associated processes and cancer cell viability (34, 41); and curcumin, a natural plant polyphenol, displays some capacity for inhibiting STAT3 without the dose-limiting toxicities inherent to other cancer therapeutic agents (22, 42, 43). Herein we investigated the effects of two novel curcumin-based compounds, designated FLLL31 and FLLL32, against breast and pancreatic cancers in both in vitro and in vivo systems.

FLLL31 and FLLL32 differ with respect to other curcumin analogues in that they were designed to preferentially interact with both the SH2 domain of STAT3 and its upstream activator, the JAK2 kinase. As our data can attest, this strategy produced potent and selective inhibitors of the STAT3 signaling pathway. The merits of targeting STAT3 as a potential cancer therapy have been thoroughly investigated, and a growing body of evidence has shown that the inhibition of constitutively active STAT3 leads to impaired survival and proliferation (3, 34). The ability of curcumin to inhibit the JAK2/STAT3 pathway was one of many factors that made it a desirable choice for a lead compound. In addition to its antiproliferative and proapoptotic effects, curcumin also has antioxidant and anti-inflammatory properties (44). Although we have yet to evaluate FLLL31 and FLLL32 in these other regards, their close structural similarity to curcumin suggests that they may share its pleiotropic nature.

Figure 6. A, comparison of tumor volumes over time in mouse xenografts versus MDA-MB-231 breast cancer cells. The mice were given daily i.p. dosages of 50 mg/kg FLLL32 or DMSO. Tumor samples from mice were analyzed with P-STAT3 and one representative sample is shown. B, effect of FLLL32 on vascularity and tumor growth in CAMs. The pictures show blood vessel density around xenografted tumors (t). C, relative tumor sizes of CAM xenografts. D, relative CAM blood vessel density. Data are from seven to nine individual embryos and two separate experiments. *, P > 0.05; **, P < 0.01, one-way ANOVA with Neuman-Keuls post-test. UnTx, untreated.
In this report, we have shown that FLLL31 and FLLL32 are effective JAK2/STAT3 inhibitors, and in our analysis, each had higher efficacy against pancreatic and breast cancer cell viability than currently available commercial JAK2 and STAT3 inhibitors as well as curcumin. Moreover, FLLL31 and FLLL32 are selective for their intended targets, JAK2 and STAT3. FLLL31 and FLLL32 did not inhibit STAT1 DNA-binding activity or the phosphorylation of mTOR, p70S6K, ERK1/2, AKT, EGFR, and Src. FLLL32 also had little effect on the kinase activity of five tyrosine kinases containing SH2 and/or SH3 domains and five other protein kinases. The ability of FLLL31 and FLLL32 to generate synergy with doxorubicin in breast cancer cells suggests that a combination of FLLL31 or FLLL32 with doxorubicin may be more effective to treat cancer patients than either drug alone. In addition, the capacity of FLLL32 to inhibit tumor growth and vascularity in a chicken embryo CAM model using PANc-1 pancreatic cancer cells suggests that FLLL32 may have the potential to inhibit tumor angiogenesis in cancer patients. Furthermore, FLLL32 is capable of suppressing tumor growth in the MDA-MB-231 breast cancer (Fig. 6A) and PANc-1 pancreatic cancer (data not shown) xenografts in mouse tumor models. These results provide supportive evidence that FLLL32 may be effective in suppressing pancreatic and breast tumor cell growth in cancer patients with constitutive STAT3 signaling.

Although both FLLL31 and FLLL32 inhibit JAK2 and STAT3, FLLL31 is better by eliminating the enol form, thus enhancing effective inhibition concentration, while FLLL32 gains additional binding interactions and is more selective to STAT3 and JAK2 than FLLL31. FLLL32 has a docking free energy −8.5 kcal/mol of binding to STAT3 SH2, compared to FLLL31 with a docking free energy −8.1 kcal/mol. For binding to JAK2, FLLL32 has a docking free energy −10.3 kcal/mol compared to FLLL31 with a docking free energy −9.6 kcal/mol. To our knowledge, FLLL32 is the first small molecular inhibitor that was designed from curcumin to target both JAK2 and STAT3 SH2 domain. The basic requirements for potential chemopreventive and therapeutic agents require them to be effective against tumor cells in vitro and in mouse tumor models in vivo, relatively nontoxic to the normal cells, and have better bioavailability. Our present investigation of FLLL32 suggests that this small molecule meets many of these criteria, and its further evaluation and optimization may yield even more effective antitumor agents in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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