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A direct STAT3 inhibitor 6-ethoxydihydrosanguinarine exhibits anticancer activity in gastric cancer

Abstract

Signal transducer and activator of transcription 3 (STAT3) plays a key role in promoting tumor malignant progression. Suppression of hyperactivated STAT3 signaling has emerged as a potential therapeutic strategy for many cancer types. In this study, the effect of 6-ethoxydihydrosanguinarine (6-EDS), a secondary transformation product after dihydrosanguinarine is isolated from *Macleaya* (Papaveraceae), was evaluated on gastric cancer (GC). We demonstrated that 6-EDS inhibited the survival, migration, and invasiveness of GC cells *in vitro*. 6-EDS inhibited STAT3 phosphorylation and transcriptional activity to suppress the mRNA expression of downstream target genes associated with malignant survival, migration, and invasiveness of GC cells. Molecular docking found that 6-EDS directly bound the SH2 domain of STAT3. Molecular dynamics simulation suggested that 6-EDS inhibited the binding of phosphorylated and non-phosphorylated STAT3 to target DNA. A cellular thermal shift assay and microscale thermophoresis further confirmed the direct binding of 6-EDS to STAT3. Site-directed mutagenesis indicated that the S611 residue site in the SH2 domain of STAT3 is critical for 6-EDS binding. *In vivo*, 6-EDS reduced tumor growth in xenografted nude mice by blocking the STAT3 signaling. These findings indicate that 6-EDS, as a direct STAT3 inhibitor, could be a potent anticancer candidate for the therapy of GC.

Key Words: 6-ethoxydihydrosanguinarine, gastric cancer, STAT3, invasion, migration

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1. Introduction

Gastric cancer (GC), a common malignancy of the alimentary system, is a the third leading cause of tumor-related death worldwide [1]. It has the highest annual incidence in East Asian countries [2]. There are approximately 679,000 newly diagnosed GC patients in China each year. Due to its large population base, China actually accounts for the vast majority of GC patients in East Asia [3]. Despite the substantial improvements made in surgical techniques and medication for GC over the past decade, the 5-year survival rate for patients with advanced GC is <15% [4]. Identifying new drugs is still the main strategy for exploring new treatment options for tumors[5], and GC is no exception.

The signal transducer and activator of transcription (STAT) signaling pathway is a family of nucleocytoplasmic shuttling transcription factors responsible for the signal transduction of extracellular cytokines and the activation of gene transcription [6]. This family includes STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 which have relatively conserved homologous functional domains. All seven STAT proteins play central roles in cellular immune responses, cell survival, differentiation, angiogenesis, and motility [7]. STAT3 is the most widely studied member of the STAT family, the most closely related to tumorigenesis, and is a key regulatory molecule in various oncogenic signaling pathways[8]. According to statistics, abnormally activated STAT3 is present in more than 70% of human cancers[9, 10], including the breast, lung, liver, stomach, brain, colon, cervix, and prostate cancers, and acute myeloid leukaemia and multiple myeloma [8]. The protein structure of STAT3 contains an N-terminal domain, a coiled-coil domain, a DNA-binding domain, a linker region, an SH2 domain, and a C-terminal transactivation domain, which is similar to

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other STAT family members [11]. STAT3 exists as an inactive monomer in the cytoplasm. After the tyrosine residues of JAKs are phosphorylated by upstream growth factor receptors (e.g., gp130 and EGFR), the SH2 domain of STAT3 recognizes and binds to these phosphorylated tyrosine docking sites, placing STAT3 near the activated JAKs, and then phosphorylation occurs at Y705 in the C-terminal transactivation domain. Phosphorylation of Y705 causes the SH2 domain to mediate the reverse dimerization of the STAT3 monomer, which further translocates to the nucleus. After entry into the nucleus, STAT3 binds to the common response element in the target gene promoter, thereby promoting coding cell growth (such as cyclin D1), survival (such as survivin) and promoting cell invasion and migration (such as MMPs and vimentin) regulatory factor transcription expression [12]. In addition, an increasing number of studies have shown that constitutive activation of STAT3 promotes tumor-induced immune suppression at several levels [13]. Based on the above characteristics, STAT3 has been widely studied in tumor malignant progression and as a tumor target.

As an herbaceous perennial, *Macleaya cordata* (Wild.) R.Br. belongs to the *Papaveraceae* family and is ubiquitously dispersed in China, North America, and Europe [14, 15]. *M. cordata* is recorded in many ancient books as a traditional Chinese medicine in China. In recent years, studies have reported that it has a variety of pharmacological activities, such as antifungal, antimicrobial, anti-inflammatory, antioxidant and antitumor activities [16-18]. Pharmacological studies have shown that the main biologically active ingredient of *M. cordata* is alkaloids. To date, 147 alkaloids have been identified and/or isolated in *M. cordata*. Most are isoquinoline alkaloids, including sanguinarine, chelidonine, allocryptine, dihydrochelerythrine, oxysanguinarine, and dihydrosanguinarine [19, 20].

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6-ethoxydihydrosanguinarine (6-EDS, Figure 1A) is the product of secondary transformation after dihydrosanguinarine is extracted from *M. cordata*. [21]. The effect and mechanism of 6-EDS on gastric cancer are still unclear. Here, we investigated the effect of 6-EDS in GC.

2. Materials and methods

2.1 Reagents

6-EDS with a purity of ≥ 98% was purchased from BioBioPha Co., Ltd. (Kunming, China).

2.2 Cell culture

Human GC cell lines BGC823, MGC803, and AGS were purchased from ATCC, and were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% FBS and antibiotics and incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

2.3 Cell cytotoxicity assays

Cell cytotoxicity was determined by MTT assay and xCELLigence real time cell analysis (RTCA) assay. For MTT assay, cells were seeded into 96-well plate then treated with 6-EDS. The absorbance was tested at 490 nm by automated microplate reader (BioTek, USA) [22]. For RTCA assay, the cells were seeded into an 16-well E-Plate (ACEA Biosciences, USA), and then, an xCELLigence RTCA HT Instrument (ACEA Biosciences, USA) was used for label-free, real-time and automated monitoring. After 24 h, the cells were treated with 6-EDS. The cell index analysis (15 min interval detection) lasted for 60 h.

2.4 Soft-agar colony formation assay

Soft-agar colony formation analysis was performed as previous described[23].

2.5 High-content analysis

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The Operetta CLS^{TM} high-content analysis system (PerkinElmer, Germany) was used to monitor the movement potential of cells. Cell tracks were recorded in real-time to dynamically determine the migration. Cells were seeded into 96-well plate, and cell movement was recorded overnight in real-time. The paths of the cells were automatically analyzed by the Harmony 4.1 software.

2.6 Annexin V/PI double staining and apoptosis detection

Cells were incubated with 6-EDS for 24 h. Cell apoptosis was detected by AV/PI detection using an AV/PI kit (Dojindo, Japan), flow cytometry was performed using flow cytometer (Beckman, USA) [24].

2.7 Mitochondrial membrane potential measurement

After incubated with 6-EDS for 24 h, cells were collected and stained with JC-1 fluorescent probe. After incubating for 20 min, flow cytometer (Beckman, USA) was used to detect fluorescence.

2.8 DAPI staining

To observe nuclear morphology, cells were fixed and then stained with DAPI (Beyotime, China). The images of stained cells were obtained using a fluorescence microscope (Olympus, Japan).

2.9 Western blot

Western blot was conducted according to the standard method described in the previous article[25]. The primary antibodies used were anti-GAPDH, anti-MMP9, anti-cyclin D1, anti-JAK2 (Santa Cruz Biotechnology, USA); anti-phospho-STAT3 (Y705), anti-phospho-STAT3 (S727), anti-STAT3, anti-phospho-JAK2 (Y1007/Y1008),

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anti-E-cadherin, anti-caspase-9, anti-PARP, anti-caspase-3, anti-PARP, anti-phospho-EGFR (T1068), anti-EGFR (Cell Signaling Technology, USA); anti-c-Myc, anti-MMP2, anti-vimentin, anti-Lamin B1 (Proteintech Group, USA).

2.10 Wound healing assay

Wound healing assay was carried out as previous described[26]. ImageJ software was used for measuring the wound gaps.

2.11 XCELLigence real-time cellular analysis assay for cell invasion

The upper chamber of 16-well CIM-Plate (ACEA Biosciences, USA) was pre-coated with 1 mg/mL Matrigel (BD Biosciences, USA) for 4 h. Cells (8×10^3 cells per well) in serum-free medium were seeded in the upper chambers, and complete medium containing 20% FBS was added to the lower chambers. Then, the RTCA DPlus Instrument (ACEA Biosciences, USA) was used to detect the cell index (15-minute interval detection) for 50 h.

2.12 Invasion assay

Transwell invasion assay was carried out as previous described[26].

2.13 Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was performed in an ABI StepOnePlus™ Real-Time PCR System (ABI, USA) using SYBR® Green Realtime PCR Master Mix (Toyobo Co., Ltd., Japan). Primers are listed in Table 1.

2.14 Isolation of nuclear and cytoplasmic fractionation

Nuclear and cytosolic fractions were separated by the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, China). Western blot was used to detect the expression of STAT3, Lamin B1 and GAPDH.

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2.15 Immunofluorescence assay

Cell or tissue sections were fixed in 4% formaldehyde, and then blocked with 3% BSA/0.2% Triton X-100 in PBS. Subsequently, cells were incubated with STAT3 antibody, and then incubated with Alexa Fluor® 488-conjugated secondary antibody (Abbkine, China). Next, the slides were stained with DAPI (Beyotime Biotechnology, China). Fluorescence images were acquired on Olympus laser scanning confocal microscope.

2.16 Transfection of DNA

The pcDNA3.1-EGFP-STAT3-Myc expression plasmid was purchased from HeBio Co. Ltd. (Shanghai, China). Plasmids were transfected by Lipofectamine® 3000 transfection reagent (Invitrogen, USA).

2.17 Molecular docking

The structure of phosphorylated STAT3 core protein binding to DNA was obtained from RCSB PDB database (PDB code: 6NJS). The wild type of STAT3 was obtained by mutating back to unphosphorylated tyrosine using mutagenesis model of PyMOL. The missing DNA strands are repaired by base pairing and modeled using 3DNA webserve [27]. Then structure was prepared by removing hydrogen assignments at pH 7.0 with academic Mastro [28]. Water molecules near small molecular binding pockets are retained. Ligand-protein docking experiments were mainly conducted using Autodock Vina, the search space at the binding pocket was a rectangle of 22.5 Å ×22.5 Å × 22.5 Å in the X, Y, and Z dimension. The docked poses were clustered using a tolerance of 2 Å root mean-square deviations and evaluated for the final docking structure according to the docking score.

2.18 Molecular dynamic simulation

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Molecular dynamic simulations (MD) were performed using Gromacs 5.1.5 program[29]. The present study constructed 4 systems to estimate the effects of 6-EDS on STAT3-DNA binding, including: (i) wild type STAT3 with DNA (wt-STAT3_DNA), (ii) 6-EDS compound with wild type STAT3 and DNA (6-EDS_wt-STAT3_DNA), (iii) 6-EDS with phosphorylated STAT3 (6-EDS_Y2P-STAT3), and (iv) 6-EDS with phosphorylated STAT3 and DNA (6-EDS_Y2P-STAT3_DNA). Every simulation systems were solved using TIP3P waters and neutralizing by Na^+ and Cl^- ions. The periodic boundary conditions with a minimal distance of 1.0 Å between the molecules and the edge of the box. [30]. The ff99SB force field was used for STAT3 and DNA and GAFF force field was used for 6-EDS topology [31]. Simulation systems were subjected to constant number of particles, volume, and temperature (NVT) equilibration and constant number of particles, pressure, and temperature (NPT) equilibration. Finally, MD was performed for a period of 50 ns with every step of 2 fs. The trajectory information was collected every 2 ps [32].

2.19 Binding free energy calculation

The binding free energies (ΔG_{bind}) were calculated using MM/GBSA model [33]. GROMACS output trajectory was correctly fitted and PBC conditions were removed before running the calculations with gmx_MMPBSA. The ΔG_{bind} is calculated as follows:

$$\Delta G_{bind} = \Delta H - T\Delta S \approx \Delta G_{solv} + \Delta G_{GAS} - T\Delta S \quad (1)$$

$$\Delta G_{GAS} = \Delta E_{int} + \Delta E_{vdw} + \Delta E_{ele} \quad (2)$$

$$\Delta G_{solv} = \Delta E_{SURF} + \Delta E_{GB} \quad (3)$$

Here ΔG_{solv} refers to solvation free energy, ΔG_{GAS} refers to the gas phase energy, $T\Delta S$ refers to changes of the conformational entropy upon binding, ΔE_{int} refers to the bond, angle and

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dihedral energies, usually taken as zero. ΔE_{ele} refers to electrostatic, ΔE_{vdw} refers to van der Waals energies, and ΔG_{solv} refers to the sum of the no electrostatic solvation component (nonpolar contribution, e.g., ΔE_{SURF}) and the electrostatic solvation energy (polar contribution, e.g., ΔG_{PB}). $T\Delta S$ was also omitted in this method dude its intrinsic low prediction accuracy [34, 35].

2.20 Microscale thermophoresis assay

Purified human STAT3 protein was labeled using the Monolith™ NT.115 Protein Labeling Kit (NanoTemper Technologies GmbH, Germany). Cells transfected with EGFP-STAT3 expression plasmid were lysed in RIPA buffer to isolate total protein. Then, sixteen serial dilutions of 6-EDS were prepared ranging from 1.25 mM to 0.038 μM. The labeled STAT3 or cell lysates containing EGFP-STAT3 protein was quantified, and then incubated with 6-EDS for 10 min in the dark. Samples were loaded into standard capillaries in the NanoTemper Monolith™ NT (NanoTemper Technologies GmbH, Germany). The dissociation constant (Kd) was then calculated.

2.21 Drug affinity responsive target stability assay

Cells were harvested and lysed with lysis buffer (Sigma, USA). Lysates were treated with 6-EDS and then incubated with pronase (Sigma, USA) at 37 °C for 30 min. The hydrolyzation of STAT3 was detected by Western blot [36].

2.22 Cellular thermal shift assays

Cells were harvested and lysed with lysis buffer (Sigma, USA). Lysates were then heated at 35-55 °C after incubation with 6-EDS for 30 min. The stabilizing effect of 6-EDS on STAT3 was detected by Western blot[37].

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2.23 Xenograft experiments

Female nude, 5-7 weeks old, were purchased from Vital River Laboratory (Beijing, China), and monitored in a SPF environment. The mice were injected subcutaneously with 6×10^6 BGC823 cells. Before treatment, mice were randomly divided into two groups: Vehicle group (0.8% DMSO, 12% cremophor, and 8% Ethanol in saline; n=7) and 6-EDS-treated group (intraperitoneal injection of 2.0 mg/kg 6-EDS; n=7). The mice were treated 4 times/week for a total of 4 weeks. Caliper measurements were performed twice a week using the following formula: $4\pi/3 \times (\text{width}/2)^2 \times (\text{length}/2)$. Mice were sacrificed when tumors reached 1.5 cm or appeared moribund. Subcutaneous tumors were excised for Western blot, or fixed and then embedded in paraffin for H&E staining and immunohistochemistry.

2.24 Statistical analysis

All experiments were repeated at least three times and the data were presented as the mean± SD. Differences between data groups were evaluated for significance using Student's t-test of unpaired data or one way analysis of variance and Bonferroni post-test. $P < 0.05$ indicate statistical significance.

3. Results

3.1 6-EDS suppresses the growth of GC cells

We investigated the effects of 6-EDS on GC cell lines. The IC_{50} values of 6-EDS in AGS, BGC823, and MGC803 cells were 5.86 μM, 6.43 μM, and 5.48 μM, respectively, at 24 h (Figure 1B). We performed xCELLigence real-time cell analysis (RTCA) to monitor the proliferation capacity of cells. The RTCA assay showed that 6-EDS reduced the growth rate of AGS, BGC823, and MGC803 cells (Figure 1C). Next, a colony formation assay

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demonstrated that 6-EDS markedly decreased the clonogenic ability of AGS, BGC823, and MGC803 cells (Figure 1D). High-content imaging technology was used to track the morphology of cells. The number of tracked cells treated with 6-EDS decreased and the mean roundness of cells treated with 6-EDS showed an increasing trend at 24 h (Figure 1H, I).

3.2 6-EDS induces mitochondrial-mediated apoptosis in GC cells

Apoptosis was further analyzed in AGS, BGC823, and MGC803 cells. AV/PI staining and flow cytometry assays showed that 6-EDS induced apoptosis of AGS, BGC823, and MGC803 cells (Figure 2A). DAPI staining further confirmed that 6-EDS induced chromatin condensation and fragmentation in AGS, BGC823, and MGC803 cells (Figure 2B). Next, the mitochondrial membrane potential (MMP) was measured. The red JC‐1 aggregates represent intact MMP, while green JC‐1 monomers represent damaged MMP. Fluorescence detection showed that 6-EDS decreased the number of red fluorescent cells and increased the number of green fluorescent cells. Therefore, the ratio of red/green JC-1 fluorescence was decreased (Figure 2C), suggesting that 6-EDS induced MMP damage in GC cells. Furthermore, expression of caspase and apoptotic protein was detected by Western blot. The results revealed that 6-EDS induced a decrease in the precursor form of caspase-3 (pro-casp3) and caspase-9 (pro-casp9) and induced cleavage of PARP in AGS, BGC823, and MGC803 cells (Figure 2D). These data suggested that 6-EDS induced mitochondrial-mediated intrinsic apoptosis in GC cells.

3.3 6-EDS suppresses migratory and invasive behavior of GC cells

High-content imaging technology was used to track the movement of AGS, BGC823, and MGC803 cells. The cell migration capacity and displacement area decreased significantly

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with increasing 6-EDS concentration (Figure 3A). The wound-healing and transwell assays were also performed to measure the migratory ability. 6-EDS treatment significantly inhibited the migration of AGS, BGC823, and MGC803 cells (Figure 3B and 3C). Next, we utilized the RTCA instrument to monitor real-time changes on cell invasion by examining the cell index. Results indicated that, under the increasing concentration of 6-EDS treatment, the cell invasion curve decreased significantly (Figure 3D). In the transwell invasion assay, 6-EDS treatment significantly reduced the number of invasive cells (Figure 3E). Therefore, 6-EDS significantly reduced the metastatic capacity of GC cells.

3.4 6-EDS suppresses GC progression by inhibiting STAT3 activity

The mechanism of 6-EDS on inhibiting the malignant progression of GC was further investigated. Western blot analysis showed that 6-EDS down-regulated the expression of several proteins associated with cell malignant proliferation, migration and invasion, and anti-apoptosis, including cyclin D1, c-Myc, MMP2, MMP9, and survivin (Figure 4A). Moreover, 6-EDS reversed the EMT of GC cells, mainly manifested in the down-regulation of vimentin and the up-regulation of E-cadherin expression (Figure 4A). Furthermore, qPCR showed that 6-EDS inhibited the mRNA levels of *MMP2*, *vimentin, c-Myc,* and *cyclin D1* (Figure 4B). These four molecules share a common upstream transcription factor, STAT3 [38]. The expression and activation of STAT3 was therefore investigated. The results suggested that 6-EDS significantly decreased the phosphorylation of Y705 of STAT3 but did not alter the phosphorylation of S727 nor the total protein expression level of STAT3 (Figure 4C). It was further found that the expression and phosphorylation of JAK2, a direct regulatory molecule upstream of STAT3, was not affected by 6-EDS (Figure 4C), suggesting that 6-EDS

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may directly suppress the activation of STAT3. The transcriptional activity of STAT3 was further detected by nucleocytoplasmic separation, and the results showed that 6-EDS inhibited the level of phosphorylated STAT3 in the nucleus and cytoplasm (Figure 4D). Cytokines and growth factors (IL-6, EGF, etc.) promote the phosphorylation and activation of STAT3 through their receptors (gp130, EGFR), thereby promoting the malignant progression of tumors[7]. Further experiments found that exogenous IL-6 stimulation promoted STAT3 activation in GC cells, while pretreatment with 6-EDS overcame the effect of IL-6 (Figure 4E, 4F). Moreover, 6-EDS overcame the promoting effect of IL-6 on the expression of STAT3 target genes (cyclin D1, c-Myc, and vimentin) (Figure 4E). 6-EDS had little effect on EGF-stimulated phosphorylation of EGFR (Figure 4G). However, 6-EDS overcame the activation effect of EGF on STAT3, and further reduced the promotion effect of EGF on the expression of cyclin D1, c-Myc, and vimentin (Figure 4G). These data demonstrated that 6-EDS inhibited the expression of downstream target genes associated with tumor malignant progression by inhibiting STAT3 activity. Next, we determined whether the sensitivity of cells to 6-EDS cytotoxicity was associated with the level of STAT3 expression. GC cells were transfected with the EGFP-STAT3-Myc expression plasmid (Figure 4H). Overexpression of STAT3 significantly antagonized the inhibitory effect of 6-EDS on GC cell proliferation, migration, and invasion (Figure 4I-J). Taken together, the above results suggested that 6-EDS inhibited the malignant progression of GC by directly inhibiting the activity of STAT3.

3.5 6-EDS is an inhibitor that directly binds STAT3

We further investigated the above *in vitro* results *in silico*. The protein structure of STAT3 is shown in Figure 5A. Molecular docking suggested that 6-EDS bound the SH2 domain of the

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STAT3-DNA complex (Figure 5B, 5C), and several water molecules mediate the binding of 6-EDS to STAT3. Two water molecules formed water bridges between the side chain of K591 and the oxethyl group of 6-EDS. One water mediates the binding of S611 and S613 with the 1,3-benzodioxole of 6-EDS via a water bridge. The another 1,3-benzodioxole group formed a hydrogen bond with the side chain of R609. Hydrophobic interactions have been observed between nitrogen methyl group of 6-EDS and the side chain of residues V637 and P639 (Figure 5D). The binding energy of 6-EDS to STAT3 is −6.8 kcal/mol. Next, the MD simulations were performed on the 6-EDS–STAT3 complex. The root mean squared deviation (RMSD) value was used to explore the effects of 6-EDS on the binding stability of STAT3 protein. No significant structural differences were observed in short-term simulations. The 4 systems showed similar fluctuation trend of the protein skeleton, and maintained approximately 0.45 nm in the second half of the simulation (Figure 5E). Previous studies have shown that STAT3 binds to DNA only after phosphorylation, but recent studies have reported that unphosphorylated STAT3 can also bind to DNA[39]. Next, the structure alignment showed that phosphorylated STAT3 and unphosphorylated STAT3 are structurally very similar except for Y705 (Figure 5F). Furthermore, we estimated the effects of 6-EDS on the DNA binding of STAT3. As shown in Table 2, ΔG_{bind} showed that unphosphorylated STAT3 (wt-STAT3_DNA) and phosphorylated STAT3 (Y2P-STAT3_DNA) bind to DNA with similar energies. Interestingly, upon 6-EDS binding, the binding energy of DNA with both systems decreased. The binding energy of phosphorylated STAT3 with DNA decreased from –225.66 to –114.57 kcal/mol. The binding energy of unphosphorylated STAT3 with DNA decreased from –217.85 to –105.36 kcal/mol (Figure 5G). These results indicated that the

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binding of 6-EDS can indeed affect the binding ability of phosphorylated or not of STAT3 and DNA. From the energy decomposition point of view, the binding of 6-EDS mainly affects the van der Waals interaction and electrostatic interaction between STAT3 and 6-EDS. Collectively, *in silico* validation through dynamics simulations of the docked drug-target complex confirmed the *in vitro* results of the effective inhibition of STAT3 by 6-EDS.

To validate the interaction between 6-EDS and STAT3 and to quantify the binding affinity, a microscale thermophoresis (MST) assay was performed. The MST quantification analysis revealed the binding ability of 6-EDS and STAT3 with a dissociation constant (Kd) of 76.42 μM and a signal-to-noise ratio (S/N) of 11.4 (Figure 5H). The binding of small molecules promotes the stability of the target protein. The drug-affinity responsive target stability (DARTS) assay was conducted to detect the stability of STAT3. The results showed that incubation with 6-EDS led to a concentration-dependent decrease in STAT3 proteolysis (Figure 5I). A cellular thermal shift assay (CETSA) was used to further confirm the effect of 6-EDS on STAT3 stability. The results showed that 6-EDS facilitated STAT3 thermotolerance in a temperature gradient (35−55 °C) (Figure 5J). Collectively, these data demonstrated that 6-EDS directly bound STAT3. To further confirm the critical amino acid residues of STAT3 binding to 6-EDS, we performed site-directed mutations on K591, R609, S611, and S613 of the EGFP-STAT3 plasmid according to the molecular docking prediction. The EGFP, EGFP-STAT3, EGFP-STAT3^{K591A}, EGFP-STAT3^{R609A}, EGFP-STAT3^{S611A}, and EGFP-STAT3^{S613A} plasmids were transfected into HEK293T cells respectively and the total protein was extracted and incubated with 6-EDS. The MST assay showed that EGFP-STAT3 binds to 6-EDS but EGFP does not (Figure 5K). In addition, K591A, R609A, S613A

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mutations had no significant effect on the binding effect of STAT3 and 6-EDS. STAT3 of K591A, R609A still bound to 6-EDS, but the Kd value of binding increased significantly, indicating that the binding effect was weakened. In contrast, the S611A mutation inhibited the binding of STAT3 to 6-EDS, suggesting that S611 plays a key role in the binding of STAT3 to 6-EDS. Conclusively, the above results indicated that 6-EDS suppressed the activity of STAT3 and its binding to DNA by directly binding to the SH2 domain.

3.6 6-EDS hinders tumor growth *in vivo*

We next detected the inhibitory effect of 6-EDS on tumor growth *in vivo.* After generation of BGC823 xenograft murine models, 6-EDS (2 mg/kg, *i.p.*) was administered for 4 weeks. After treatment for 4 weeks, the mice were sacrificed (Figure 6A). Consistent with the *in vitro* proliferation data, 6-EDS significantly inhibited the of tumor growth *in vivo* (Figure 6B-E). As shown in Figure 6F, treatment with 6-EDS did not cause obvious changes in the weight of mice. Reduced Ki67 intensity was observed in the 6-EDS-treated xenograft tumors, as shown by immunohistochemical staining (Figure 6G). In addition, the fluorescent immunohistochemistry and Western blot suggested that 6-EDS inhibited the phosphorylation of STAT3 (Figure 6H, 6I). Furthermore, the expression of target genes downstream of STAT3 associated with proliferation, invasion and migration in tumor tissue was detected by qPCR. The results showed that 6-EDS significantly reduced the mRNA expression levels of *MMP2*, *vimentin, cyclin D1*, and *c-Myc* in tumor tissue (Figure 6J). The above results suggested that 6-EDS inhibited tumor progression *in vivo* through STAT3.

Overall, we concluded that 6-EDS inhibited malignant progression of GC mainly by directly binding to the SH2 domain of STAT3, inhibiting the phosphorylation and transcriptional

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activity of STAT3.

4. Discussion

The critical role of STAT3 in malignant tumorigenesis and progression has sparked a rush of targeted drug discovery to screen small molecules that disturb STAT3 activity. Here, we revealed a novel small molecule STAT3 inhibitor, 6-EDS, which has great promise in pre-clinical evaluations for the treatment of GC. 6-EDS showed definable toxicity toward GC cell survival, including promoting cell apoptosis and reducing invasion and migration. Moreover, 6-EDS decreased tumor burden in GC xenografts *in vivo*. 6-EDS exerted the above anti-tumor effects by binding the SH2 domain to inhibit the binding of phosphorylated and non-phosphorylated STAT3 to DNA.

We further demonstrated that 6-EDS inhibited GC cell viability and induced GC cell apoptosis by affecting the mitochondrial apoptosis pathway mediated by caspase-9, which was closely associated with the down-regulation of the STAT3 downstream pro-survival molecule survivin [40]. We used RTCA and a high-content imaging system to demonstrate that 6-EDS significantly inhibited the viability, area, and motility of GC cells. Increased cell roundness indicates decreased cellular activity and reduced migratory capacity [41]. Various cytokines, growth factors, and intracellular signaling pathways promote tumor cell survival and malignant progression by activating STAT3 signaling [11]. We detected IL-6 and EGF signaling upstream of STAT3, and the key regulatory molecule JAK2, demonstrating that 6-EDS down-regulated the expression of downstream invasion, migration and EMT-related target genes by directly inhibiting STAT3 activity. Knockdown of STAT3 has been widely reported to significantly inhibit GC cell growth, EMT and its mediated tumor invasion and

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migration [42, 43]. Our investigation further showed that overexpression of STAT3 significantly antagonized the inhibitory effect of 6-EDS on gastric cancer cell malignant progression, suggesting that 6-EDS inhibits GC malignant progression at least in part by inhibiting STAT3.

Currently, the core strategies for targeting STAT3 include: suppressing STAT3 phosphorylation, binding to the SH2 domain, disrupting STAT3-DNA binding, or suppressing STAT3 transcriptional activity [44]. Targeting the SH2 domain is the main way to screen STAT3 inhibitors, but targeting the SH2 domain to inhibit STAT3 phosphorylation and dimerization may not completely inhibit abnormal STAT3 signal transduction. Recently, studies have reported that STAT3 participates in transcriptional regulation without tyrosine phosphorylation, that is, non-phosphorylated STAT3 dimers can bind to DNA to promote transcription [45, 46]. Inhibition of phosphorylated and non-phosphorylated STAT3 binding to DNA may represent another effective way to eliminate STAT3 signaling. Molecular docking analysis showed that the best region for 6-EDS binding to STAT3 was the SH2 domain. 6-EDS bound to the SH2 domain and inhibited the phosphorylation of Y705, thereby repressing STAT3 dimerization. The dimerization region of STAT3 covers the SH2 domain and the 688-722 aa region of the transactivation domain, in which Y705 resides. The phosphorylation of Y705 is necessary for dimerization[44]. Binding of 6-EDS reduced the dimerization and nuclear entry of STAT3, and also, it may also be detrimental to the phosphorylation of STAT3 through structural changes. The energy dissipation-cum-signaling mechanism suggests that local structural changes or binding to ligands are thermodynamically coupled to the distal activation site[47]. Conformational changes caused by mutation or ligand

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binding can be as large as 20 Å or more, resulting in identifiable functional consequences on kinase activity[48]. The binding of 6-EDS in the SH2 domain may be thermodynamically coupled to the Y705 site through distal residues, thereby affecting the degree of phosphorylation of Y705. Moreover, through MD simulations, it was found that although 6-EDS did not bind to the DNA binding domain, it could reduce the binding free energy of phosphorylated STAT3 and non-phosphorylated STAT3 to target gene DNA. Binding free energy is dominated by electrostatic and hydrophobic interactions. 6-EDS has strong electronegativity and is suitable for binding basic amino acids, such as K591 and R609. With the participation of water molecules, the side chain of S611 formed a hydrogen bond with 6-EDS. As a polar hydrophilic amino acid, S611 has no other interactions, which may be important in recognizing intermolecular binding. Furthermore, we demonstrated the direct binding effect of 6-EDS and STAT3 and key amino acid sites by MST, CETSA, and DARTS assays. Although site-directed mutagenesis showed that the S611 site of STAT3 played a critical role in binding to 6-EDS, the affinity of 6-EDS with STAT3 protein needs to be further improved.

Conclusively, our data reveal that 6-EDS is a novel STAT3 inhibitor and demonstrate that 6-EDS has a significant inhibitory effect on the survival, invasion and migration of GC cells and activates mitochondrial apoptosis. These effects were associated with direct inhibition of STAT3 by 6-EDS. This study is expected to provide an experimental foundation for the antitumor targeted therapy of 6-EDS.

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Conflicts of interest

The authors declare no conflicts of interest.

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4779-4784.

Table 1. Primer sequences for qPCR.

Gene	Primer sequence
MMP2	F: ACGACCGCGACAAGAAGTAT
	R: ATTTGTTGCCCAGGAAAGTG
Vimentin	F: ACTACGTCCACCCGCACCTA
	R: CAGCGAGAAGTCCACCGAGT
Cyclin D1	F: GTCGCTGGAGCCCGTGA
	R: GGATGGAGTTGTCGGTGTAGATG
c-Myc	F: GGCTCCTGGCAAAAGGTCA
	R: CTGCGTAGTTGTGCTGATGT

F, forward; R, reverse.

Table 2. MM/GBSA binding free energy (kcal/mol) of simulation systems.

Energy Component	Systems					
	wt-STAT3_DNA	Y2P-STAT3_DNA	6-EDS_wt-STAT3	6-EDS_Y2P-STAT3	6-EDS_wt-STAT3_DNA	6-EDS_Y2P-STAT3_DNA
ΔE_{vdw}	-63.86 ± 4.44	-66.19 ± 14.26	-40.10 ± 3.30	-29.81 ± 3.60	-46.14 ± 4.81	-35.55 ± 7.94
ΔE_{ele}	-1035.82 ± 96.01	-1270.16 ± 111.34	-18.06 ± 7.89	-15.96 ± 3.77	-1270.52 ± 76.17	-890.26 ± 87.68
ΔE_{GB}	890.92 ± 94.95	1119.66 ± 106.10	19.23 ± 5.46	8.77 ± 3.93	1219.46 ± 71.51	816.27 ± 87.97
ΔE_{SURF}	-9.08 ± 0.47	-8.99 ± 1.92	-5.04 ± 0.30	-3.48 ± 0.45	-7.16 ± 0.46	-5.06 ± 1.23
ΔG_{GAS}	-1099.69 ± 96.42	-1336.34 ± 103.66	-59.17 ± 8.84	-45.77 ± 5.51	-916.66 ± 76.46	-925.78 ± 92.47
ΔG_{solv}	881.83 ± 94.97	1110.68 ± 107.01	14.19 ± 5.32	5.28 ± 3.79	811.31 ± 71.31	811.21 ± 81.27
ΔG_{bind}	-217.85 ± 5.81	-225.66 ± 9.00	-44.98 ± 4.63	-40.49 ± 3.00	-105.36 ± 8.19	-114.57 ± 10.55

Legends for figures

Figure 1. 6-EDS suppresses the growth of GC cells. (A) Chemical structure of 6-EDS. (B) The inhibitory effects of 6-EDS on AGS, BGC823, and MGC803 cells were evaluated by MTT assay. (C) The inhibitory effects of 6-EDS on the viability of AGS, BGC823, and MGC803 cells were monitored by RTCA assay. (D-E) Colony formation assays of AGS,

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BGC823, and MGC803 cells treated with 6-EDS. (F-H) AGS, BGC823, and MGC803 cells were treated with 6-EDS for 24 h. Growth of cells was assessed and imaged at 24 h by using brightfield and digital phase-contrast microscopy on a Perkin-Elmer Operetta microscope (F). The roundness and area of AGS, BGC823, and MGC803 cells was determined at 24 h by Harmony software (G, H). Scale bars, 50 μm. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure 2. 6-EDS induces mitochondrial-mediated apoptosis in GC cells. (A) AGS, BGC823, and MGC803 cells were treated with 6-EDS for 24 h and then detected by annexin V/PI staining and flow cytometry. (B) AGS, BGC823, and MGC803 cells were incubated with 6-EDS for 24 h and then examined by DAPI staining. Red arrows indicate apoptotic cells. (C) AGS, BGC823, and MGC803 cells were incubated with 6-EDS for 24 h, and the cell MMP was detected by JC-1 staining and flow cytometry. (D) AGS, BGC823, and MGC803 cells were treated with 6-EDS for 24 h and then Western blot was performed. *$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$.

Figure 3. 6-EDS suppresses the invasive behavior of GC cells. (A) The movement of AGS, BGC823, and MGC803 cells was analyzed in real time using the Operetta CLS high-content analysis system. After treatment with 6-EDS for 24 h, the cells were imaged with a 20× objective in the DPC channel. Upper panel: Cells were identified using the Find Cells module, and migration was monitored for 12 h using the Track Objects module. Lower panel: Cell displacement was visualized. The current displacement Y was plotted against the current displacement X using the Multiple Graphs module for display. Each point corresponds to the displacement of a cell at a given time point. Scale bars, 50 μm. (B) Wound healing assays were performed in 6-EDS-treated AGS, BGC823, and MGC803 cells. Scale bars, 100 μm. (C)

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Transwell migration assays were performed in 6-EDS-treated AGS, BGC823, and MGC803 cells. Scale bars, 50 μm. (D) AGS, BGC823, and MGC803 cells were cultured in 16-well CIM plates precoated with 250 μg/mL Matrigel for 10–40 h. The invasive capacity of the cells was determined by RTCA impedance sensing. The cell index directly correlates with the invasion capacity of cells. (E) Transwell invasion assays were performed in 6-EDS-treated AGS, BGC823, and MGC803 cells. Scale bars, 50 μm. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure 4. 6-EDS suppresses GC progression by inhibiting STAT3 activity. (A-C) AGS, BGC823, and MGC803 cells were treated with 6-EDS for 24 h and then Western blot or qPCR was performed. (D) AGS, BGC823, and MGC803 cells were treated with 6-EDS for 24 h. The cytoplasmic and nuclear fractions of cells were isolated, and then Western blot was performed. (E-G) AGS, BGC823, or MGC803 cells were pretreated with 6-EDS for 24 h and then stimulated with IL-6 (10 ng/mL) for 10 min or with EGF (100 ng/mL) for 10 min. Western blot or immunofluorescence assays were used to detect the indicated antibodies. Scale bars, 20 μm. (H-K) AGS, BGC823, or MGC803 cells were transfected with EGFP-STAT3-Myc plasmid, and then treated with 6-EDS for 24 h. Western blot, RTCA, wound healing, or transwell invasion assays were used to detect protein expression, cell viability, migration, and invasion. Scale bars, 100 μm (J); 50 μm (K). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure 5. 6-EDS is an inhibitor that directly binds STAT3. (A) The domains and boundaries of the domains of STAT3 protein. (B-C) *In silico* molecular docking analysis of 6-EDS into STAT3. (D) The zoomed view of 6-EDS at the SH2 domain of STAT3. (E) RMSD plot of 4 systems during 50 ns dynamic simulation. (F) The difference between Y705

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phosphorylated STAT3 (magentas stick) and unphosphorylated STAT3 (green stick). the phosphate group is shown as orange-red sticks. (G) ΔG_{bind} of 5 systems during energy calculation. (H) Binding affinity of 6-EDS with purified protein was detected using MST assay. (I) Total protein in AGS, BGC823 or MGC803 cells was incubated with different concentrations of 6-EDS, and then treated with pronase to detect the expression of STAT3. (J) AGS, BGC823 or MGC803 cells were treated with 100 μM 6-EDS or DMSO. CETSA was performed to detect the stabilizing effect of 6-EDS on the STAT3 protein. (K-L) HEK293T cells were transfected with EGFP, EGFP-STAT3, EGFP-STAT3^{K591A}, EGFP-STAT3^{R609A}, EGFP-STAT3^{S611A}, or EGFP-STAT3^{S613A} plasmids, and total protein was extracted and then incubated with different concentrations of 6-EDS. An MST assay was used to detect the binding affinity. $^{**}P < 0.01$; $^{***}P < 0.001$.

Figure 6. 6-EDS hinders tumor growth *in vivo*. (A) Process diagram of xenograft tumor models. (B-C) Tumor volumes of nude mice following 2 mg/kg 6-EDS treatments. (D) Images of xenograft tumors obtained from the sacrificed mice. (E) Weight of the tumors from the sacrificed mice. (F) Body weight of nude mice following 6-EDS treatments. (G) H&E staining and immunohistochemistry staining of representative tumor tissues. Scale bars, 50 μm. (H) Immunofluorescence staining of representative tumor tissues. Scale bars, 20 μm. (I) Western blot assay of representative tumor tissues. (J) qPCR detection of representative tumor tissues. $^{*}P < 0.05$; $^{**}P < 0.01$.