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# Bazedoxifene exhibits growth suppressive activity by targeting interleukin-6/glycoprotein 130/signal transducer and activator of transcription 3 signaling in hepatocellular carcinoma

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The interleukin (IL)-6/glycoprotein (GP)130/signal transducer and activator of transcription (STAT)3 pathway is emerging as a target for the treatment of hepatocellular carcinoma. IL-6 binds to IL-6R, forming a binary complex, which further combines with GP130 to transduce extracellular signaling by activating STAT3. Therefore, blocking the interaction between IL-6 and GP130 may inhibit the IL-6/GP130/STAT3 signaling pathway and its biological effects. It has been reported that bazedoxifene acetate (BAZ), a selective estrogen receptor modulator approved by the US Food and Drug Administration, could inhibit IL-6/GP130 protein-protein interactions. Western blot, immunofluorescence staining, wound healing and colony formation assays were used to detect the effect of BAZ on liver cancer cells. Cell viability was evaluated by MTT assay. Apoptosis of cells was determined using the Annexin V-FITC detection kit. Mouse xenograft tumor models were utilized to evaluate the effect of BAZ in vivo. Our data showed that BAZ inhibited STAT3 phosphorylation (P-STAT3) and expression of STAT3 downstream genes, inducing apoptosis in liver cancer cells. BAZ inhibited P-STAT3 induced by IL-6, but not by leukemia inhibitory factor. BAZ inhibited P-STAT1 and P-STAT6 less significantly as elicited by interferon-α, interferon-γ and IL-4. In addition, pretreatment of BAZ impeded the translocation of STAT3 to nuclei induced by IL-6. BAZ inhibited cell viability, wound healing and colony

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formation in vitro. Furthermore, tumor growth in HEPG2 mouse xenografts were significantly inhibited by daily intragastric gavage of BAZ. Our results suggest that BAZ inhibited the growth of hepatocellular carcinoma in vitro and in vivo, indicating another potential strategy for HCC prevention and therapy.

**KEYWORDS**
bazedoxifene, glycoprotein 130, hepatocellular carcinoma, interleukin-6, signal transducer and activator of transcription 3

## 1 | INTRODUCTION

Human hepatocellular carcinoma (HCC) is the second leading cause of cancer death in men worldwide in less developed countries, while it is the sixth cause of death in males of developed countries. Worldwide, 782 500 new cases of HCC were diagnosed in 2012 and it was responsible for 745 500 deaths that year.[1] Numbers of new cancer cases and deaths estimated by the American Cancer Society indicate that incidence rates of liver cancer in the USA have increased by approximately 3% per year in women and 4% in men.[2] In China, liver cancer is the third leading cause in selected cancer deaths and accounted for approximately 15% of all estimated deaths during 2015.[3] Due to a lack of incipient symptoms, HCC is often noticed and diagnosed at a late stage when potentially curative therapies, such as chemotherapy, chemoembolization, ablation and proton-beam therapy, are less effective. Moreover, resection of even very early tumors is linked to a recurrence rate of approximately 60% in 5 years, indicating a poor prognosis accompanied by a low 5-year survival rate in the majority of patients with HCC.[4] Aberrant modulation of cell proliferation and apoptosis is frequently associated with the development and progression of HCC. Despite plenty of studies that have contributed to the exploration of HCC, the exact pathophysiological process that accounts for the initiation and progression of HCC remains obscure, indicating that it may be promising and essential to study the molecular mechanisms involved with an aim to develop novel effective therapies.

The interleukin (IL)-6/glycoprotein (GP)130/signal transducer and activator of transcription (STAT)3 pathway has been emerging as a target for the treatment of HCC.[5-7] Previous studies have provided abundant supporting evidence that HCC involves the dysregulation of proteins, especially STAT3.[8] STAT3 can be phosphorylated and activated by the Janus kinase (JAK1-JAK3) family of tyrosine kinases, which plays a pivotal role in the development of both solid and hematopoietic malignancies. STAT3, an important member of the STAT family, is persistently activated in a wide variety of human malignancies. Indeed, activated STAT3 is necessary not only for oncogenesis and tumor progression, but also for invasion of HCC in vitro and in vivo.[9,10] Levels of cytokines were found to be elevated in HCC, especially IL-6.[11,12] IL-6 binds to IL-6R to form a binary complex and further combines with GP130 to transduce extracellular signaling. Therefore, blocking the interaction between IL-6 and GP130 may inhibit the IL-6/GP130/STAT3 signaling pathway and its biological effects.[13] The excessive expression of IL-6 contributes to the pathological progression in HCC through STAT3 activation, such as in hepatocarcinogenesis,[14,15] self-renewal of liver tumor-initiating cells,[16] minimal hepatic encephalopathy[11] and cancer cachexia.[17] GP130 is a co-receptor of the IL-6 receptor and defined as an oncogene that is involved in the progression of hepatocellular tumors.[18] Activation of GP130 induces the phosphorylation of JAK1 and JAK2 in response to the extracellular signaling through cytokines and growth factors. The protein family of JAK includes four mammalian members of tyrosine kinases, which are related to signaling transduction. Three of them, JAK1, JAK2 and TYK2, are expressed in various tissues, but JAK3 is expressed mainly in cells of the hematopoietic system.[19] IL-6-induced phosphorylation of STAT3 is more associated with JAK1 and JAK2 activity. TYK2 catalytic activity is required for major signaling events downstream of IL-12 and IL-23 but does not appear to contribute significantly to signaling events downstream of IL-6.[20] Phosphorylation of JAK1/2 results in phosphorylation of STAT3 on tyrosine-705, triggering the homodimerization of STAT3, which facilitates nuclear translocation of STAT3 followed by binding to DNA and the activation of STAT3 downstream target genes. Lack of GP130 expression in hepatocytes reduces tumor progression, but not HCC initiation in the diethylnitrosamine model. Targeting GP130 in hepatocytes may be a potential strategy to inhibit the growth of HCC.[21] However, it has never been reported whether the growth or activity of liver cancer cells could be inhibited by impeding the binding of IL-6 to GP130.

It has been shown that inhibition of persistent activation of the STAT3 signaling pathway results in decreased proliferation and increased apoptosis in human cancer cells, indicating that STAT3 may be a viable molecular target for cancer therapy.[22] In recent years, several novel STAT3 inhibitors have been reported to inhibit the growth of cancer cells, such as FLLL32, LLL12 and LY5.[23-27] However, their clinical application is limited by poor bioavailability and side-effects. The absorption, distribution, metabolism, excretion and toxicity properties of the compounds should be thoroughly studied before clinical use. Therefore, there is an urgent need for better approaches to this aggressive disease.

Bazedoxifene acetate is a third-generation selective estrogen receptor modulator (SERM) that has been approved by the US Food and Drug Administration (FDA) in 2013 for the prevention

and treatment of postmenopausal osteoporosis.[28-30] It has been reported that bazedoxifene acetate is a novel inhibitor of IL-6/GP130 protein-protein interaction, determined by the computational strategy for fragment-based drug design through combining multiple ligand simultaneous docking and repositioning.[31] However, there are still no reports of the effects of bazedoxifene on human liver cancer cells in vitro or in vivo.

## 2 | MATERIALS AND METHODS

This investigation has been conducted in accordance with the ethical standards according to national and international guidelines and has been approved by the institutional review board of Tongji Hospital.

### 2.1 | Human liver cancer cell lines

Human liver cancer cell lines (Hep3B, HEPG2, SSMC 7721, HUH-7) were purchased from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM)/high glucose supplemented with 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA) and 1% penicillin/streptomycin (Sigma, St Louis, MO, USA). All human liver cancer cell lines were cultured in a humidified 37° incubator with 5% $CO_2$.

### 2.2 | IL-6/GP130 inhibitor

Small molecular IL-6/GP130 inhibitor bazedoxifene was purchased from Cayman Chemical (Ann Arbor, MI, USA). Bazedoxifene was dissolved in sterile dimethyl sulfoxide (DMSO; Sigma) to make 20 mmol/L stock solution, stored at −20° until use.

### 2.3 | Cell viability

MTT cell viability assay kits were purchased from Promoter Biotechnology (Wuhan, China). SSMC 7721 and HUH-7 were seeded in 96-well plates (5000 cells/well). They were cultured with DMEM/high glucose containing 10% FBS and 1% penicillin/streptomycin. After 24 hours, cells were treated with 5-30 μmol/L of bazedoxifene at 37°C for 24 hours in triplicate. MTT viability assay was performed according to manufacturer's protocol (Promoter Biotechnology). The absorbance (A) was read at 570 nm. After subtraction of the absorbance of the blank group, results of the DMSO group were set arbitrarily to 100%. Cell viability was calculated as follows: (A of bazedoxifene group − A of blank group) / (A of DMSO group − A of blank group) × 100%.

### 2.4 | Colony formation

Cells were planted in six-well cell culture plates and were treated with or without bazedoxifene (20, 25 μmol/L) for 2 hours. Then, the treated cells were determined, stained with trypan blue (Promoter Biotechnology) and counted. Viable cells of low cell density (5000/plate) were then seeded on 10-cm plates and grown for 3 weeks. Cells were washed with phosphate-buffered saline (PBS) three times and fixed with cold methanol for 15 minutes. Then, cells were stained with 0.5% crystal violet in 25% methanol for 15 minutes.

### 2.5 | Western blot

Human liver cancer cells were treated with bazedoxifene (10, 15, 20, 25 μmol/L) or DMSO. After 12 hours, cells were collected. For stimulation experiments, such as IL-6 (25 ng/mL), leukemia inhibitory factor (LIF; 25 ng/mL), interferon (IFN)-γ (50 ng/mL), IFN-α (25 ng/mL) and IL-4 (25 ng/mL) (PeproTech, Rocky Hill, CT, USA), Hep3B cells were serum-starved for 12 hours and pretreated with bazedoxifene (10, 15, 20, 30 μmol/L) or DMSO for 2 hours. Then, cells were stimulated with cytokines for another 30 minutes and the cells were collected. IL-6, LIF, IFN-α, IL-4 and IFN-γ were purchased from Cell Signaling Technology (Danvers, MA, USA). The cells were washed by ice-cold PBS buffer and lysed in a modified RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecylsulfate) containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail. The lysates were spun at 138000 *g* for 20 minutes at 4°C and the cells were collected. Protein samples were transferred onto polyvinylidene difluoride membranes and probed with antibodies (Cell Signaling Technology). Antibodies (Cell Signaling Technology) against phospho-specific STAT3 (Tyrosine 705, #9131), phospho-specific JAK2 (#3776) phospho-specific JAK1 (#3331), JAK1 (#3332), JAK2 (#3230) phospho-independent STAT3 (#4904), Cleaved Caspase-3 (Asp175, #9661), Survivin (#2803), Bcl-2 (#2876) and glyceraldehyde 3-phosphate dehydrogenase (#2118) were used. Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The target proteins were determined by an enhanced chemiluminescence western blot kit.

### 2.6 | Immunofluorescence staining

Hep3B cells were seeded on glass cover slips on six-well plates and grown for 12 hours. The next day, the cells were cultured in serum-free medium for 12 hours, and pretreated with bazedoxifene for 2 hours. Then, 25 ng/mL IL-6 or LIF was added for another 30 minutes. Cells were fixed with ice-cold methanol at room temperature for 20 minutes. After washing in PBS, the cells were permeabilized and blocked with 5% normal goat serum and 0.3% Triton X-100 in PBS buffer for 1 hour. Then, the cells were incubated with primary antibodies against total STAT3 proteins (1:200 dilution; Cell Signaling Technology) at 4°C overnight. The cells were washed with PBS containing 0.1% Tween-20, and incubated with Cy3-conjugated anti-rabbit secondary antibody (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature for 1 hour. The cells were mounted with Vectashield HardSet mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Images were captured by fluorescent microscope.

### 2.7 | Wound healing

HUH-7, 7721 and HEPG2 cell lines were seeded in six-well cell culture plates with DMEM/high glucose containing 10% FBS. When cells grew to a confluence of 100%, we scratched the monolayer along the marked line using pipette tips and plates were washed once to remove non-adherent cells. After washing, cells were treated with bazedoxifene (DMSO, 10, 15 μmol/L) for 2 hours. After that, the medium was removed and fresh medium supplemented with 10% FBS was added. Cells were allowed to migrate into the scratched area for an additional 24-36 hours without bazedoxifene, then images were captured.

### 2.8 | Annexin V-PI assay

Apoptosis was determined by fluorescence activated cell sorting (FACS) analysis using the Annexin V-FITC detection kit (KeyGEN BioTECH, Nanjing, China) as described by the manufacturer. HUH-7, 7721 and HEPG2 cell lines were plated in six-well tissue plates ($4 \times 10^5$ cells/well) and incubated overnight. Proliferating cells were treated with or without bazedoxifene for 12 hours. Cells were trypsinized and centrifuged at 720 *g* for 5 minutes. After washing twice with PBS, the cells were then harvested and incubated with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide dye (PI) following the manufacturer's protocol before evaluation by flow cytometry (FACS Caliber; BD Biosciences Franklin Lakes, NJ, USA). CellQuest software was used to analyze apoptosis.

### 2.9 | Mouse xenograft tumor model

Human liver cancer cells, HEPG2 ($10^7$ cells in 100 μL of sterile PBS and matrigel), were injected s.c. into the right flank region of female athymic nude mice (4-6 weeks of age, 20-22 g). Three days after injection, the mice were randomized into control and treatment groups: (i) 5% DMSO and 10% Solutol added 85% hydroxypropyl B cyclodextrin (HPBCD) as vehicle control; and (ii) 5 mg/kg of bazedoxifene (dissolved in 5% DMSO, 10% Solutol and 85% HPBCD). Bazedoxifene was administrated through intragastric gavage once a day for 20 days. Tumor growth was determined by measuring the length (L) and width (W) of the tumor every other day with a caliper. Tumor volume was calculated using the following formula: volume = $(\pi/6) \times L \times W^2$, where L is the largest superficial diameter and W is the smallest superficial diameter. At the end of treatment, the mice were killed and tumors were harvested. All animal studies and protocols were approved and carried out by the Institutional Animal Use and Care Committee of Tongji Medical College, Tongji Hospital, Huazhong University of Science and Technology.

### 2.10 | Statistical analysis

Data were presented as the mean ± SD for at least three independent experiments. Statistical analyses were performed with SPSS software (version 13.0). The significant differences between any two groups were evaluated by one-way ANOVA. The value of $P < 0.05$ was considered statistically significant.

## 3 | RESULTS

### 3.1 | Structure of bazedoxifene and the selective inhibitory effect on STAT3 phosphorylation induced by IL-6

Bazedoxifene is known as a selective estrogen receptor modulator (Figure 1A). In a previous study, we reported bazedoxifene as a novel inhibitor of IL-6/GP130 protein-protein interactions using multiple ligand simultaneously docking and drug repositioning approaches. The Hep3B liver cancer cell line, which is considered estrogen receptor negative,[32] was used to examine whether bazedoxifene is capable of inhibiting IL-6-induced STAT3 phosphorylation. Our results showed that IL-6 induced STAT3 phosphorylation on tyrosine 705 in Hep3B cancer cells. The induction of STAT3 phosphorylation by IL-6 was inhibited with the treatment of bazedoxifene without significant inhibition of total STAT3 (Figure 1B). We further investigated whether bazedoxifene could inhibit the phosphorylation of STAT3

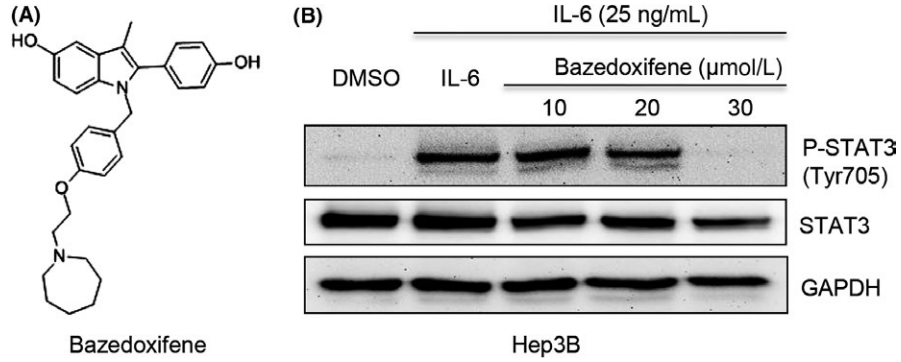


**FIGURE 1** The structure of bazedoxifene and the inhibitory effect on signal transducer and activator of transcription (STAT)3 phosphorylation induced by interleukin (IL)-6. A, The structure of bazedoxifene. B, Hep3B cells were pretreated 2 hours by bazedoxifene (10, 20, 30 μmol/L) and stimulated by IL-6 (25 ng/mL) 30 minutes. Bazedoxifene inhibited IL-6-mediated induction of STAT3 phosphorylation in a dose-dependent manner in Hep3B liver cancer cell lines. DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

induced by LIF (Figure 2A), as well as the phosphorylation of STAT1 induced by IFN-γ (Figure 2B), IFN-α (Figure 2C) and P-STAT6 induced by IL-4 (Figure 2D) in Hep3B liver cancer cells. The results showed that the phosphorylation of STAT3, STAT1 and STAT6 were much less significantly inhibited with treatment of bazedoxifene in Hep3B cells; however, 30 μmol/L of bazedoxifene significantly inhibited the phosphorylation of STAT3 induced by IL-6. These data indicate that bazedoxifene may have a relatively selective inhibitory effect on phosphorylation of STAT induced by different cytokines.

### 3.2 | Bazedoxifene inhibits STAT3, JAK1 and JAK2 phosphorylation and expression of STAT3 downstream genes and induces apoptosis in HEPG2, 7721 and HUH-7 cancer cell lines

We examined the effects of bazedoxifene on HEPG2, 7721 and HUH-7 liver cancer cell lines. Persistent STAT3, JAK1 and JAK2 phosphorylation in these liver cancer cells was inhibited by treatment with bazedoxifene for 12 hours. Bazedoxifene had minimal effect on the overall expression of STAT3, JAK1 and JAK2 in HEPG2, 7721 and HUH-7 cells (Figure 3). Then, we detected the expression of STAT3 downstream target genes, such as Survivin and Bcl-2, in these cells. The expression of Survivin was decreased after treatment with bazedoxifene in HEPG2, 7721 and HUH-7 liver cancer cells as examined by western blot assay (Figure 3). As to the expression of Bcl-2, it was decreased with the treatment of bazedoxifene in HEPG2 and HUH7 cell lines, but not significantly in 7721 cells (Figure 3B). Furthermore, we explored whether bazedoxifene had the potential to induce apoptosis in liver cancer cells. Our results showed that bazedoxifene promoted the cleavage of caspase-3, which is one of the hallmarks of apoptosis in these liver cancer cells (Figure 3).

### 3.3 | Bazedoxifene inhibits cell viability, wound healing and colony formation in liver cancer cells

The results suggested that bazedoxifene effectively inhibited activation of STAT3, suppressed the expression of its downstream target genes and induced apoptosis in human liver cancer cells in which STAT3 has been documented to be persistently activated. Therefore, we further examined whether bazedoxifene could affect STAT3-dependent cell migration, which may lead to invasion and metastasis. MTT assays were used to examine whether bazedoxifene could suppress cell viability. In HEPG2, 7721 and HUH-7 liver cancer cells, treatment with bazedoxifene for 24 hours resulted in a dramatic decrease

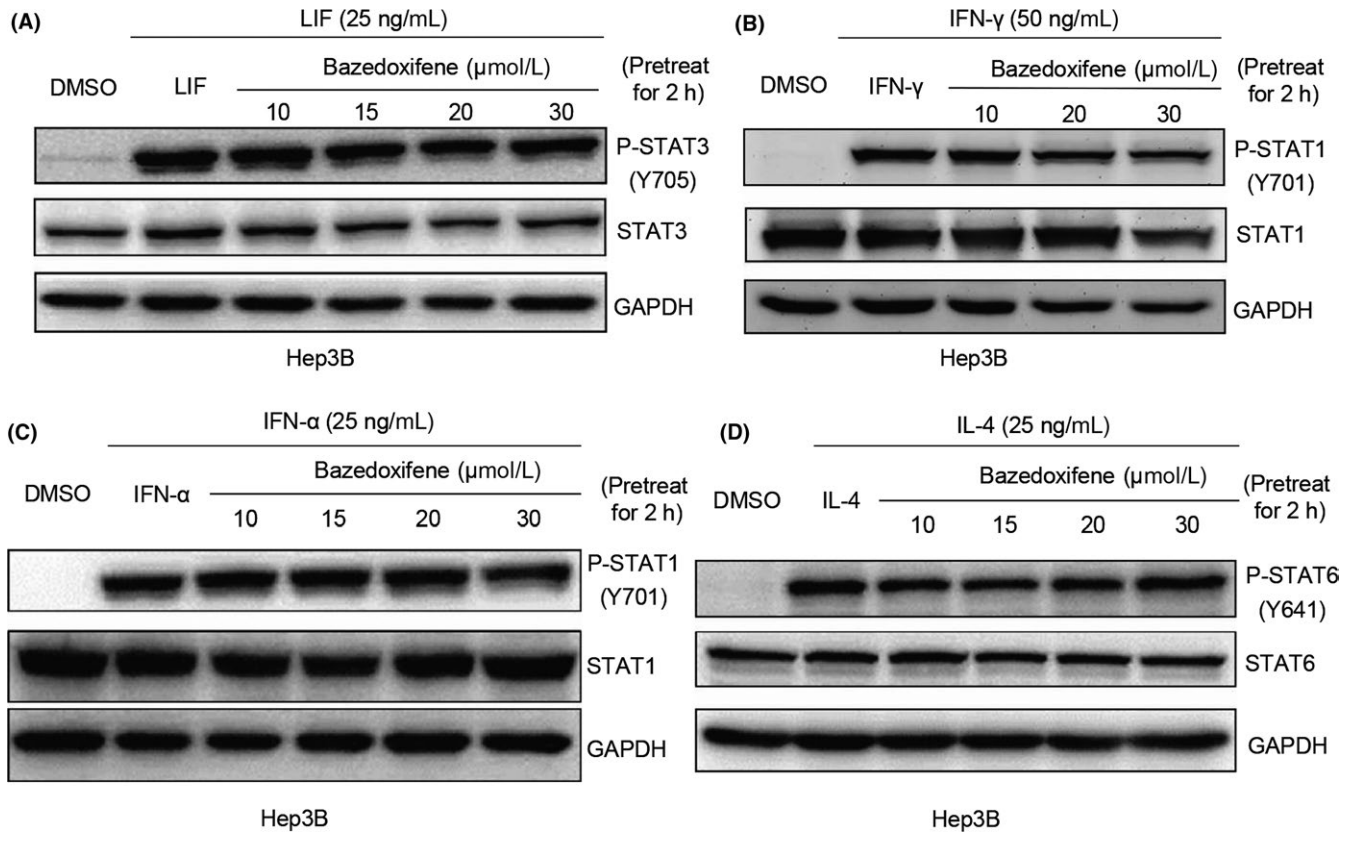


**FIGURE 2** Bazedoxifene does not significantly inhibit signal transducer and activator of transcription (STAT) phosphorylation induced by cytokines except for interleukin (IL)-6. Hep3B cells were pretreated for 2 hours by bazedoxifene and stimulated by cytokines for another 30 minutes as indicated. Bazedoxifene (10, 15, 20, 30 μmol/L) did not significantly inhibit the phosphorylation of STAT3 induced by leukemia inhibitory factor (LIF) (25 ng/mL) (A), and did not significantly inhibit the phosphorylation of STAT1 and STAT6 induced by interferon (IFN)-γ (50 ng/mL) (B), IFN-α (25 ng/mL) (C) and IL-4 (25 ng/mL) (D). DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

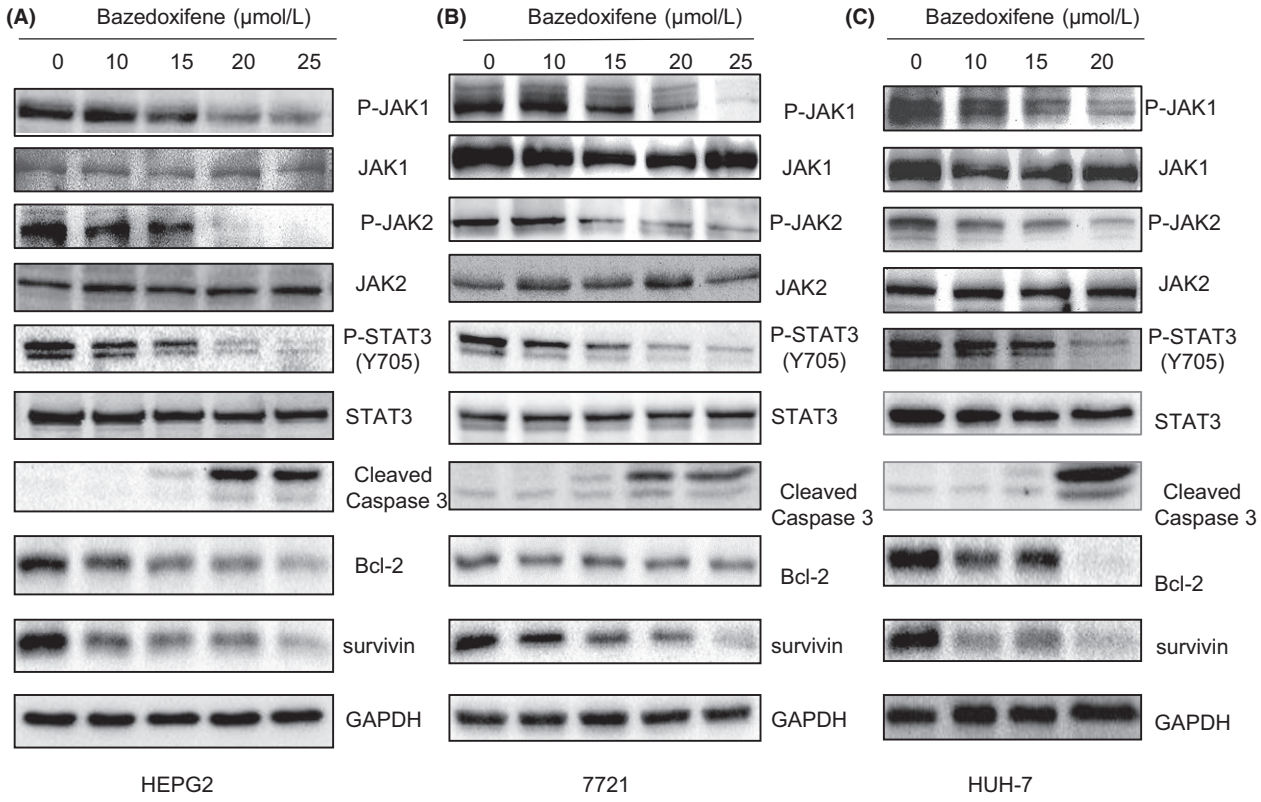


**FIGURE 3** Bazedoxifene inhibits signal transducer and activator of transcription (STAT)3 phosphorylation and expression of STAT3 downstream genes, and induces apoptosis. HEPG2 (A), 7721 (B) and HUH-7 (C) cancer cell lines exhibit constitutively phosphorylated STAT3. Bazedoxifene (10, 15, 20, 25 μmol/L) inhibited the phosphorylation of STAT3 (Tyr 705), Janus kinase (JAK)1 and JAK2, induced apoptosis as evidenced by increased cleaved caspase-3. The expression of STAT3-mediated genes was decreased by bazedoxifene except for the expression of Bcl-2 in 7721 cells. GAPDH, glyceraldehyde 3-phosphate dehydrogenase

of cell viability in a dose-dependent manner (Figure 4A, $^*P < 0.05$). We next evaluated the effect of bazedoxifene on cell migration through wound healing assay in HUH-7, 7721 and HEPG2 liver cancer cells. As our data attests, treatment with 10 and 15 μmol/L of bazedoxifene caused a concentration-dependent suppression on wound healing (Figure 4B, $P < 0.05$). A colony formation assay was performed to investigate the proliferation and regeneration potential of liver cancer cells after treatment with bazedoxifene (20 and 25 μmol/L). We demonstrated that bazedoxifene markedly inhibited colony formation in 7721, HUH-7 and HEPG2 liver cancer cell lines (Figure 4C).

### 3.4 | Bazedoxifene suppresses the nuclear translocation of STAT3 induced by IL-6 without inhibition on nuclear translocation of STAT3 induced by LIF in Hep3B liver cancer cells

As previously reported, the majority of STAT3 were latent and localized in cytoplasm. The phosphorylation-dependent translocation of STAT3 from the cytoplasm to nuclei has been proven to be of importance for STAT3 to exhibit its biological effects as a transcription factor. To detect the inhibitory effect of bazedoxifene on STAT3 translocation, Hep3B cells were cultured in serum-free medium overnight on six-well plates. Cells were pretreated with 20 μmol/L of bazedoxifene or DMSO for 2 hours followed by 25 ng/mL of IL-6 or LIF for another 30 minutes. In Hep3B cells treated with IL-6, STAT3 was phosphorylated and translocated into the nucleus. However, most STAT3 was retained in the cytoplasm when pretreated with bazedoxifene (20 μmol/L) (Figure 5A). We further found that the phosphorylation of STAT3 was inhibited with treatment of bazedoxifene by immunofluorescence staining (see Figure S1). Therefore, these results suggest that the inhibition of STAT3 phosphorylation caused by treatment of bazedoxifene may consequently lead to transcriptional dysfunction of STAT3 in liver cancer cells by diminishing nuclear translocation. On the other hand, bazedoxifene did not inhibit STAT3 nuclear translocation promoted by the treatment of LIF (Figure 5B), suggesting that bazedoxifene may be relatively selective in inhibition on STAT3 nuclear translocation.

### 3.5 | Bazedoxifene induces apoptosis in 7721, HUH-7 and HEPG2 liver cancer cell lines

Induction of apoptosis by bazedoxifene was further evaluated by Annexin V-PI assay of apoptotic cells. HUH-7, 7721 and HEPG2 cell lines were treated with bazedoxifene (20 and 25 μmol/L) for 12 hours and then stained with Annexin V dye/PI dye. Flow cytometry analysis revealed that bazedoxifene prominently facilitated apoptosis compared with the DMSO control group ($^*P < 0.05$) (Figure 6).

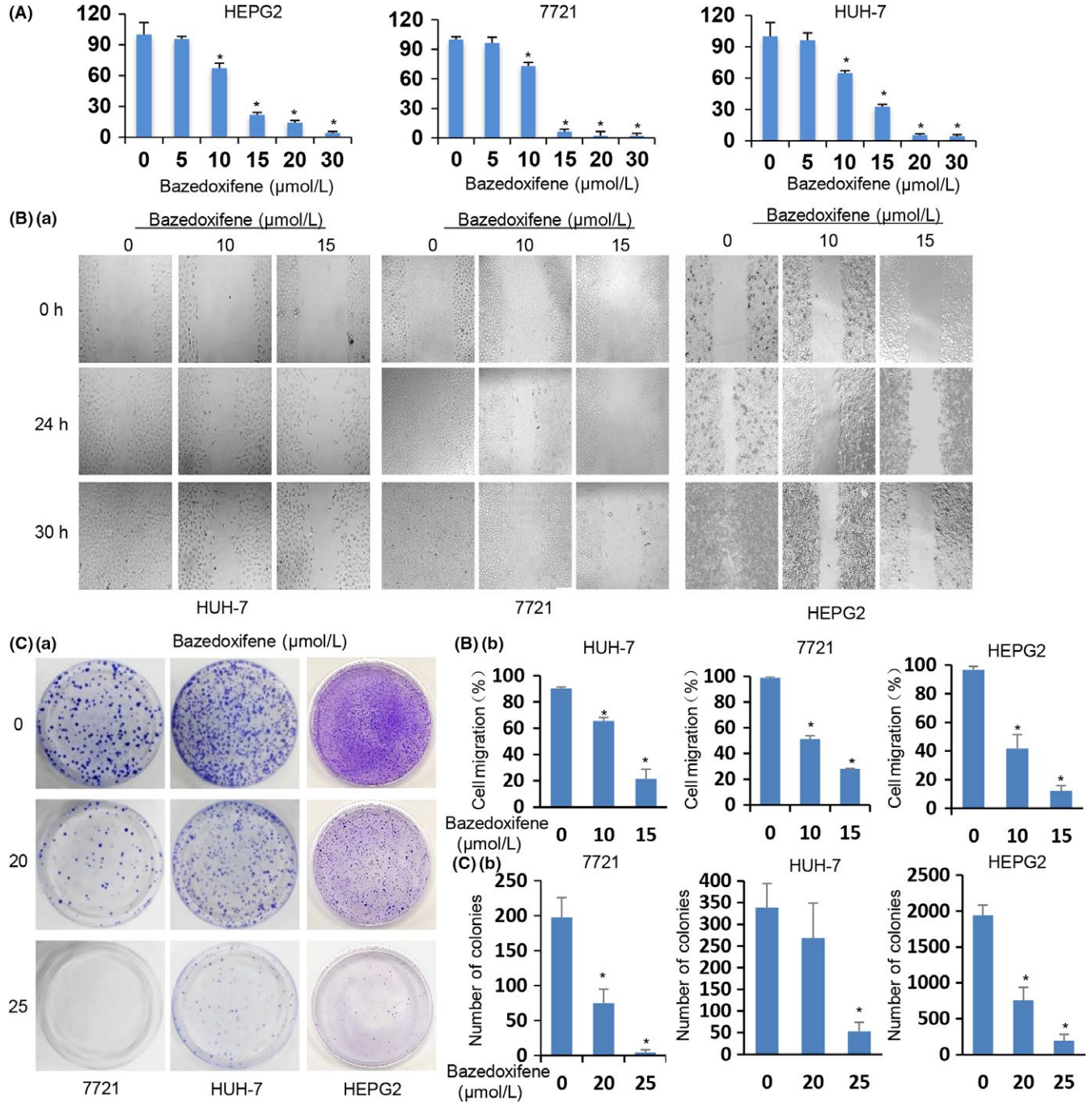


**FIGURE 4** Bazedoxifene inhibits cell viability, wound healing and colony formation in liver cancer cells. A, HEPG2, HUH-7 and 7721 liver cancer cells were treated with or without bazedoxifene (5, 10, 15, 20, 30 μmol/L) at 37°C for 24 h. Bazedoxifene inhibited cell viability (*$P$ < 0.05). B,a, HUH-7, 7721 and HEPG2 liver cancer cells were treated with or without bazedoxifene (10, 15 μmol/L) for 2 hours, cells were allowed to migrate into scratched area for an additional 24-36 hours without bazedoxifene. B,b, Cell migration was assessed by using Image J software (*$P$ < 0.05). C,a, 7721, HUH-7 and HEPG2 liver cancer cells were treated with or without bazedoxifene (20, 25 μmol/L). B,b, The number of colonies was counted after 3 weeks (*$P$ < 0.05). Colony formation was inhibited by bazedoxifene

### 3.6 | Bazedoxifene inhibits tumor growth in HEPG2 mouse xenograft model

To determine whether bazedoxifene may have a therapeutic potential for treatment of liver cancer in vivo, we next detected the effects of bazedoxifene on HEPG2 liver cancer cells in nude mice xenograft models. As shown in Figure 7, bazedoxifene significantly suppressed (*$P$ < 0.05) tumor growth as evidenced by decreased tumor volume (Figure 7A,C) and tumor weight (Figure 7D) of HEPG2 mouse xenografts, accompanied by decreased expression of P-STAT3 and increased cleaved caspase-3 (Figure 7B). The bodyweight of the mice between the two groups had no significant difference over the

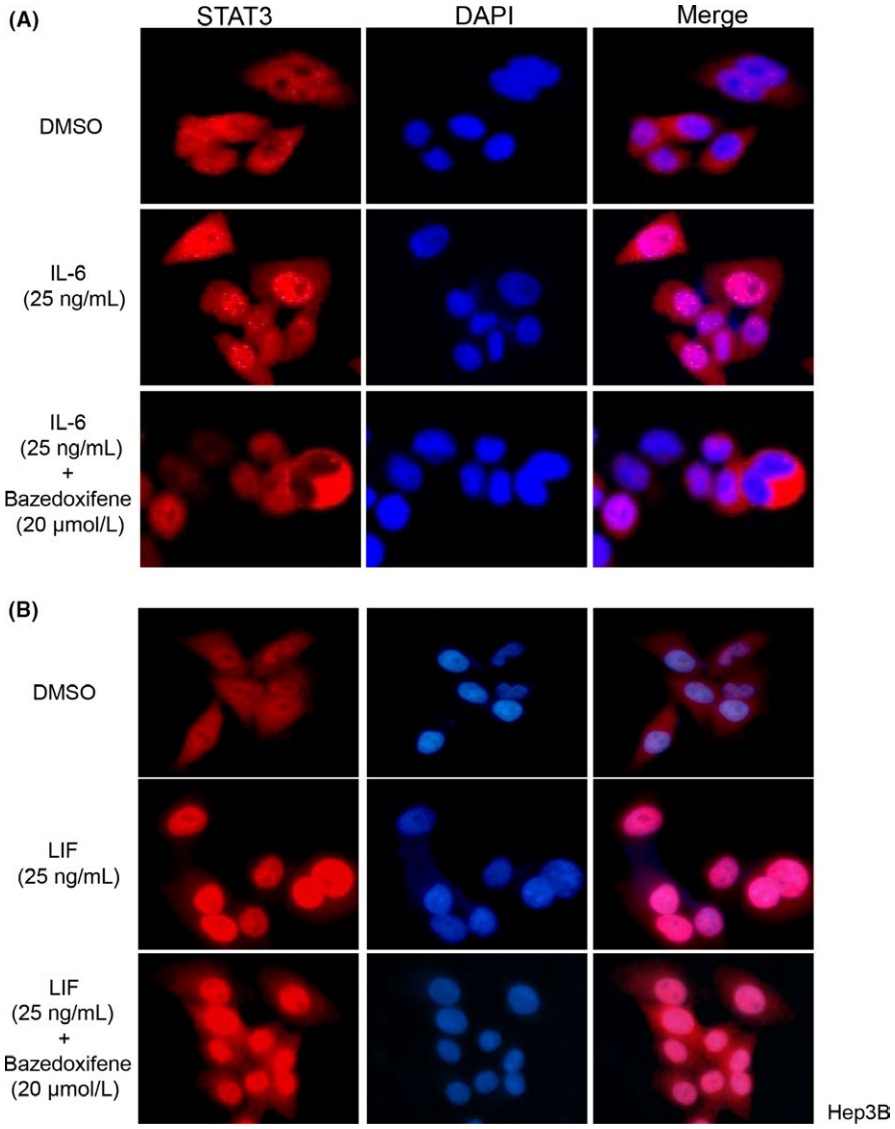


**FIGURE 5** Bazedoxifene suppresses the translocation of signal transducer and activator of transcription (STAT)3 to nuclei induced by interleukin (IL)-6 A, without inhibition on nuclear translocation of STAT3 induced by leukemia inhibitory factor (LIF) B, in Hep3B cells. Hep3B liver cancer cells were pretreated with bazedoxifene (20 μmol/L) for 2 hours, followed by IL-6 (25 ng/mL) or LIF (25 ng/mL) for 30 min, and then processed for STAT3 nuclear translocation detection by immunofluorescence staining as described in Materials and Methods. DMSO, dimethyl sulfoxide

course of treatment (Figure 7E). These results indicate that bazedoxifene resulted in the suppression of tumor growth in mice, suggesting that bazedoxifene may be a potent compound in suppressing HCC.

## 4 | DISCUSSION

Many genetic alterations and critical molecular signaling pathways have been identified as having important roles contributing to the development and progression of HCC. STAT3 is frequently activated in many types of solid and blood cancers, including liver cancer and contributes to cancer progression.[17] In STAT3 knockout mice models, it has been shown that STAT3 was required for tumorigenesis in intestinal, skin and liver tissue.[33-35] In previous studies, constitutively activated STAT3 had been reported in a large proportion of cancers, where it contributed to cell growth, apoptotic resistance, angiogenesis and metastatic potential.[5,35] Impediment of constitutive activation of STAT3 not only induced apoptosis in cancer cells, but also overcame chemoresistance and radioresistance.[36] Selectively targeting activated STAT3 signaling has been shown to be effective in inhibiting cancer-associated processes and cancer cell viability.[37]

Interleukin-6 is a pleiotropic cytokine secreted in response to acute stimulation of inflammation. IL-6 released from T cells can induce proliferation and differentiation of B cells, as well as stimulate antibody production. Recent studies have revealed that the expression of IL-6 in primary liver cancer was dramatically elevated and is positively correlated with morbidity and progression.[6,11,14,38] IL-6 exerts its biological effects through binding to two subunits of signal transducing receptor GP130 and IL-6R, resulting in dimerization of GP130 and IL-6R and subsequently activation of the Janus kinases/STAT3 pathway. Recent studies reported that some novel agents were designed to target these pathways such as LLL12, LY5 and FLLL32.[23,27,39,40] However, few of them have been approved for use in clinical treatment. Celecoxib, a cyclooxygenase-2 inhibitor, was approved by the FDA for the treatment of arthritis. It was reported to exert an inhibitory effect on the STAT3 signaling pathway by targeting the STAT3 SH2 domain.[41] Moreover, these inhibitors targeting the STAT3 signaling pathway may have side-effects due

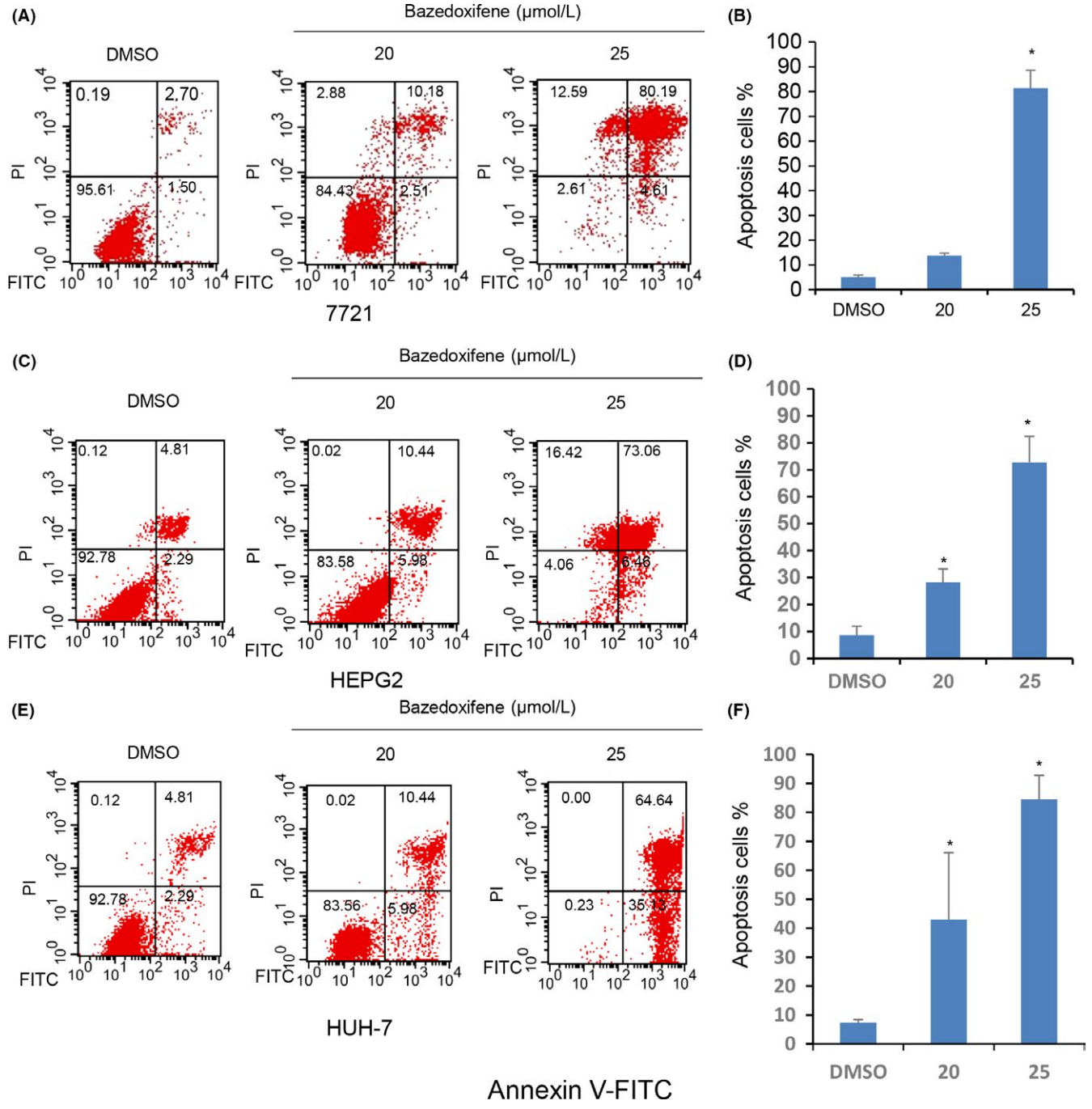


**FIGURE 6** Bazedoxifene induces apoptosis in 7721, HEPG2 and HUH-7 liver cancer cell lines. Bazedoxifene (20, 25 μmol/L) induces apoptosis in 7721, HEPG2 and HUH-7 liver cancer cell lines. A,C,E, Representative histograms of 7721, HEPG2 and HUH-7 cells cultured for 12 hours in dimethyl sulfoxide (DMSO) or bazedoxifene (20, 25 μmol/L) after Annexin V/PI staining and flow cytometric analysis. B,D,F, Bazedoxifene led to a dose-dependent, significant promotion of apoptosis in 7721, HEPG2 and HUH-7 cells. All results were representative of at least three independent experiments (*$P < 0.05$). FITC, fluorescein isothiocyanate

to their multiple inhibition on other upstream signaling of STAT3,[42] suggesting that inhibition of IL-6/GP130 interaction may be another effective strategy in prevention or treatment of cancer. The purpose of this present work is to characterize a novel IL-6/GP130 inhibitor, bazedoxifene, as potential therapy for HCC, and to contribute to further studies investigating other kinds of disease in which the IL-6/GP130/STAT3 signaling pathway plays a significant role.

Bazedoxifene is an estrogen receptor modulator (SERM) that has been approved by the FDA for the prevention and treatment of postmenopausal osteoporosis. In this paper, we found that bazedoxifene suppressed both the constitutive and IL-6-induced phosphorylation of STAT3 in human liver cancer cell lines. We noted that bazedoxifene inhibited STAT3 phosphorylation induced by IL-6 in Hep3B, PANC-1 and SUM159 cell lines which were considered estrogen

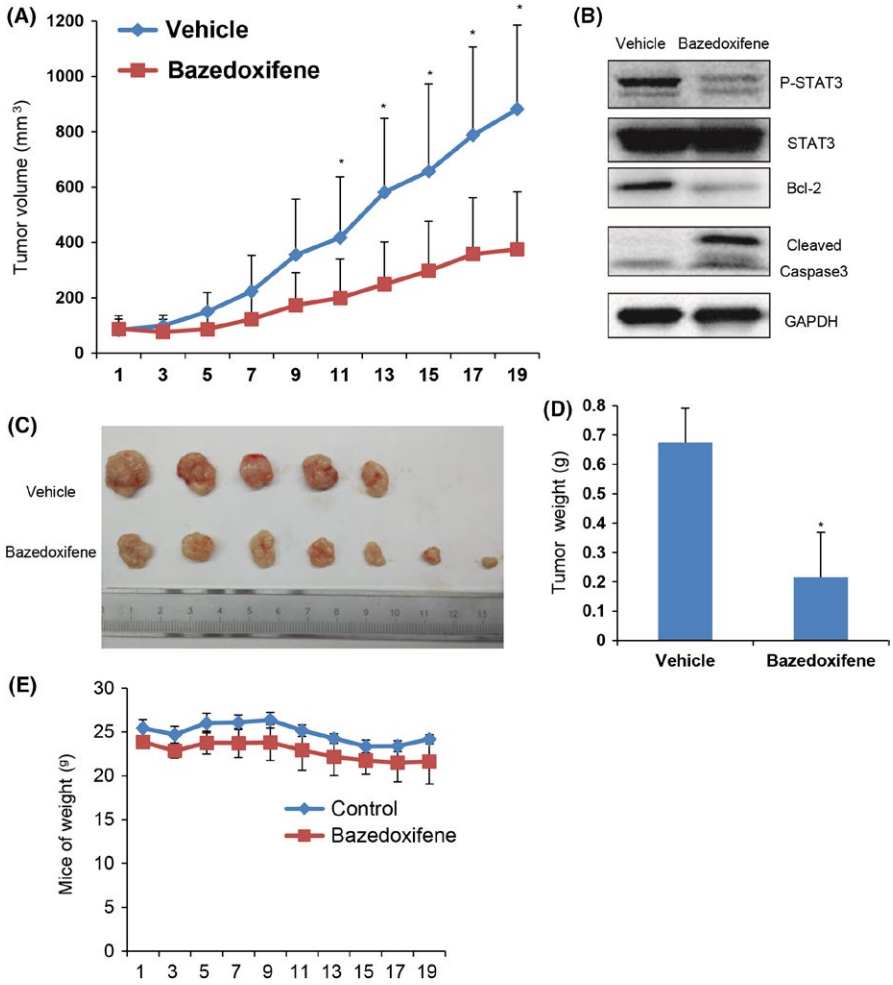


**FIGURE 7** Bazedoxifene inhibits tumor growth in HEPG2 mouse xenograft model. HEPG2 mouse xenografts were established in female athymic nude mice. The mice were randomized to two groups. Bazedoxifene was administrated 5 mg/kg through intragastric gavage once a day for 20 days. Tumor volume was measured every other day and tumor weight was calculated after 20 days. A,C,D, Bazedoxifene significantly (*$P < 0.05$) impaired HEPG2 tumor cell growth as assessed by serial volume measurements and tumor weight. B, Western blot suggested that bazedoxifene inhibited signal transducer and activator of transcription (STAT)3 phosphorylation, decreased the expression of Bcl-2 and promoted the cleavage of caspase-3 in vivo. E, The bodyweight of the mice between the two groups had no significant difference over the course of treatment. GAPDH, glyceraldehyde 3-phosphate dehydrogenase

receptor negative,[31] suggesting that inhibition on STAT3 phosphorylation induced by IL-6 of bazedoxifene may not be dependent on estrogen receptor. Administration of our compound resulted in an inhibition of STAT3 nuclear translocation, reduced expression of STAT3-mediated genes and decreased cell viability accompanied by facilitated apoptosis. Bazedoxifene has been proven to be a novel agent targeting the IL-6/GP130 interaction[31] by binding to the D1 domain of GP130 through spots Ile83, Phe36, Tyr94 and Asn92.[43] IL-6 elicited homodimerization of GP130 by 32F6,[44] differs from LIF-induced heterodimerization of GP130 with the LIF receptor by B-R3.[45] Moreover, IFN-γ, IFN-α and IL-4 trigger downstream signaling by binding to their receptors. Different binding sites and receptors may account for the result, as shown in Figure 2, that bazedoxifene had no significant effect on P-STAT3 activated by LIF, P-STAT1 induced by IFN-γ and IFN-α, and P-STAT6 elicited by IL-4. This indicates that bazedoxifene could be emerging as a relatively selective inhibitor targeting IL-6/GP130, thus rendering inhibition of P-STAT3.

Moreover, the target of bazedoxifene to the interface of the D1 domain of GP130[31] which results in the disturbance of the formation of heterogeneous complex may contribute to the inhibition of constitutive activation of STAT3 by bazedoxifene. It has been reported that the serum levels of IL-6 are elevated in patients and STAT3 is constitutively activated in HCC cell lines.[6,46] Indeed, the interface-dependent dimerization of IL-6R and its co-receptor GP130 plays a crucial role in the activation of the downstream pathway. Despite the different levels of IL-6 in HCC cell lines, the excessive levels of IL-6R and GP130 are documented.[47] Hence, the susceptibility of HCC cell lines under an insult of IL-6 may be relatively higher. STAT3 activation could in turn lead to increased expression of IL-6, forming a positive feedback, resulting in constitutive activation of STAT3.[6,46-48] The interruption of D1 domain of GP130 by bazedoxifene possibly abolishes the positive feedback, thus decreasing the IL-6/GP130 signal transduction-related expression of constitutive P-STAT3 in HCC cell lines. In addition, phosphorylated STAT3 dimerizes and translocates from the cytoplasm to the nucleus where the dimer binds to specific DNA elements to regulate the downstream genes.[31] This transient transcriptional function of STAT3 is strictly modulated by nucleopore transportation and dephosphorylation but in HCC cells malfunction happens. As shown in our results, bazedoxifene could suppress the expression of P-STAT3, P-JAK2 (Figure 3) and P-STAT3 in nuclei in HCC cell lines (Figure S2). The diminished putative feedback leads to rebalance between nucleus and cytoplasma: more inactivated STAT3 in cytoplasma while less P-STAT3 in nucleus.

The expression of Bcl-2 was decreased significantly by bazedoxifene in HEPG2 and HUH7 cell lines, but not in 7721 cells, which indicates that different human liver cancer cell lines may respond differently to bazedoxifene. Our present work did not set up more experiments to explore this subject. Nevertheless, it may be an important point for a future study. The properties of bazedoxifene were further supported by impaired tumor growth in nude mice implanted with HEPG2 cells. These results suggest that bazedoxifene may be used for the treatment of liver cancers in which the IL-6/GP130/STAT3 signaling pathway is constitutively activated.

Traditional prevention and treatment of osteoporosis includes estrogen; however, it is also associated with an increased risk of some types of cancer (e.g. breast cancer and endometrial cancer). Bazedoxifene is used for the prevention and treatment of postmenopausal osteoporosis instead of estrogen, attenuating the risk of these malignancies. Our work indicated that bazedoxifene exhibited an inhibitory effect on liver cancer in vitro and in vivo, providing evidence for the expansion of its clinical indications, to be used not only in postmenopausal women for osteoporosis, but also in patients with liver cancer. Owing to the specific inhibition of the IL-6/GP130 interaction, without significant effect on other signaling pathways, bazedoxifene may have higher selectivity than previously developed inhibitors targeting STAT3. More importantly, bazedoxifene has already been approved by the FDA as a safe and effective drug. Therefore, bazedoxifene may be utilized as a IL-6/GP130/STAT3 inhibitor prior to other agents in the clinical treatment of liver cancer. Additionally, considering bazedoxifene as a pharmacological template, we could preliminarily establish a basis for the development of IL-6/GP130/STAT3 inhibitors with better bioavailability and less side-effects. On the other hand, although these data suggest a significant effect of bazedoxifene in vitro and in animals, further investigation needs to be performed in humans with HCC to study the merits of bazedoxifene thoroughly. Furthermore, whether the inhibitory effect in vivo of bazedoxifene is dependent on the IL-6/GP130 signaling pathway is still unclear in our work. We believe it will be a matter for future studies to elucidate in detail.

Bazedoxifene exerted significant inhibitory effects on STAT3 phosphorylation, nuclear translocation and expression of STAT3-mediated downstream genes in human liver cancer cell lines. Bazedoxifene also showed significant anti-tumor activity in nude mice implanted with HEPG2 cells. Our novel research may provide new insights into cytokine-induced (especially IL-6) HCC and may also help with the expansion of clinical indications for bazedoxifene and the development of novel therapeutic agents for cancer treatment.

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## CONFLICT OF INTEREST

The authors declare no competing interests for this article.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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