GYY4137, a hydrogen sulfide (H\textsubscript{2}S) donor, shows potent anti-hepatocellular carcinoma activity through blocking the STAT3 pathway

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Abstract. GYY4137, a hydrogen sulfide (H\textsubscript{2}S) donor, exhibits anticaner activity by a combination of cell cycle arrest and promoting apoptosis, and inhibits tumor growth, however, the precise mechanisms involved remain unclear. In this study, we discovered that GYY4137-mediated suppression of cell proliferation in human hepatocellular carcinoma (HCC) cell lines and tumor growth in a subcutaneous HepG2 xenograft model may be due to directly targeting the signal transducer and activator of transcription 3 (STAT3) pathway. We found that GYY4137 suppressed STAT3 activation by reducing p-STAT3 (Y705) levels effectively in HepG2 and Bel7402 cells. Altered expression levels of STAT3-regulated downstream proteins including Bcl-2, cyclin D1, Mcl-1, survivin, vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α) may contribute to the inhibition of G1/S cell cycle transition and angiogenesis. Increased cleaved caspase-9, caspase-3 and poly(ADP-ribose) polymerase (PARP) cleavage may induce cell apoptosis in HepG2 and Bel7402 cells. In vivo, GYY4137 significantly inhibited tumor growth in the subcutaneous HepG2 xenograft model by inhibiting STAT3 activation and its target gene expression. These results suggest that GYY4137-mediated suppression of HCC growth may be due to the inhibition of the STAT3 pathway.

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide, with the average annual incidence on the rise both in China and elsewhere (1). The increasing knowledge in the molecular pathogenesis of HCC, as well as the introduction of molecular targeted therapies in oncology, has created an encouraging trend in the management of this malignancy. Most HCC treatments are designed to abrogate signaling pathways related to cancer cell proliferation, cell survival, angiogenesis, invasion and metastasis (2,3). Signal transducer and activator of transcription 3 (STAT3), a major transducer to mediate the signal from interleukin-6 (IL-6) to the nucleus, may be involved in oncogenesis, cell proliferation, angiogenesis, immune evasion and apoptotic resistance (4-8). IL-6 induces STAT3 phosphorylation at tyrosine residue 705 through Janus-activated kinase (JAK) (9). STAT3 phosphorylation results in homodimerization or heterodimerization of STAT3, enabling nuclear localization and DNA binding, and regulating downstream genes involved in controlling cell cycle progression and programmed cell death (e.g., Bcl-2, cyclin D1, Mcl-1 and survivin) (10), and in the regulation of angiogenesis [e.g., vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α)] (4). The inhibition of aberrant STAT3 activation by genetic or pharmacological approaches has repeatedly been demonstrated to result in growth inhibition, apoptosis in vitro (11,12), as well as tumor growth and metastasis inhibition in vivo (12-14) in HCC. STAT3 is significantly correlated with the prognosis of HCC patients (8,15), indicating that IL-6/STAT3 signaling pathway might be a therapeutic target.

The potential biological significance of hydrogen sulfide (H\textsubscript{2}S) has attracted growing interest in recent years. A number of studies have investigated the role of H\textsubscript{2}S in triggering cell death and evidence has been presented that this gas can affect cancer cell survival using sulfide salts as donor agents (16,17). The slow-releasing H\textsubscript{2}S donor, GYY4137, exhibits anti-cancer activity by releasing H\textsubscript{2}S down slowly (18). It exhibits anticaner activity by a combination of cell cycle arrest and promoting apoptosis, inhibits tumor growth (18), however, the precise mechanism(s) involved remain unclear. Recent study reported that GYY4137 exhibited anti-inflammatory effect in vivo and in vitro (18,19). We propose that the anticancer activity of GYY4137 may be relative with its suppression of IL-6/STAT3 pathway.

We therefore sought to investigate whether GYY4137 is efficacious for treatment of HCC with a particular focus on its suppression of IL-6/STAT3 pathway. We found that GYY4137 blocked IL-6-induced STAT3 cascade leading to the suppression of cell growth, induction of cell apoptosis and...
cell cycle arrest. GYY4137 also inhibited tumor growth and STAT3 activation in a subcutaneous xenograft model with HepG2 cells in vivo. These results suggest that GYY4137 may be a candidate for HCC therapy through blocking constitutive STAT3 signaling.

Materials and methods

Materials. GYY4137 was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). STAT3, p-STAT3 (Y705), p-STAT3 (S727), JAK, p-JAK2, Bcl-2, Mcl-1, cyclin D1, survivin, HIF-1α, VEGF, cleaved-poly(ADP-ribose) polymerase (PARP), cleaved-caspase-9 and cleaved-caspase-3 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA), β-actin antibody was purchased from Sigma (St. Louis, MO, USA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), RNase and sodium-orthovanadate and propidium iodide (PI) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell lines and cultures. HCC cell lines HepG2, and Bel7402, and hepatocellular LO2 cells were obtained from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). All cells were cultured in a humidified atmosphere with a 5% CO₂ incubator at 37°C.

Cell viability assay. HepG2, Bel7402 and LO2 cells were incubated in triplicate in a 96-well plate at a density of 1x10⁴ cells with 100 µl culture medium per well in the presence or absence of indicated concentrations of GYY4137 for 24, 48 and 72 h. After which, cell viability was determined by the MTT dye uptake method as described earlier (20).

Western blot analysis. HepG2 and Bel7402 cells were cultured in 6-well plates at a density of 5x10⁵ cells/ml in a CO₂ incubator overnight and treated with GYY4137. The total protein was prepared as previously described (21). The equalized amounts of proteins from each sample were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 1% (w/v) bovine serum albumin (BSA) for 2 h, and then incubated with the primary antibody overnight at 4°C. Primary antibodies were STAT3, p-STAT3 (Y705), p-STAT3 (S727), JAK2, p-JAK2 (Y1007/1008), Bcl-2, Mcl-1, cyclin D1, survivin, HIF-1α, VEGF, cleaved-PARP, cleaved-caspase-9, cleaved-caspase-3 (Cell Signaling Technology) and β-actin (Sigma). The membranes were incubated with secondary antibody conjugated with IgG horseradish peroxidase (HRP) for 1 h at room temperature and immune complexes were detected by the enhanced chemiluminescence system. β-actin served as a loading control.

Cell cycle analysis. HepG2 and Bel7402 cells were incubated in 6-well plates in the presence of different concentrations of GYY4137 for 24 h. Thereafter, treated cells were fixed and incubated with RNase and propidium iodide (PI) in PBS. Cell cycle distribution was analyzed with a FACScan laser flow cytometer (FACScalibur, Becton-Dickinson, Franklin Lakes, NJ, USA). The data were analyzed using the software CELL Quest.

Apoptosis assay. Apoptosis was measured with caspase-3/7 assay (Promega, Madison, WI, USA) according to the manufacturer’s protocol. Briefly, cells were seeded into a 96-well plate. After the treatment, 100 ml of Apo-One Caspase-3/7 reagent was added to each well and was incubated at 37°C for 30 min. The fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Enzyme-linked immunosorbent assay (ELISA). For the measurement of VEGF, 5x10⁵ U266 cells/well were seeded in 12-well plates and grown to 75-80% confluence, then cells were switched to fresh serum-free medium in the presence or absence of rosiglitazone and ATRA and incubated for another 12 h. Cell-free culture supernatants were harvested and assayed for secreted VEGF using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA).

Animal experiments. Male BALB/c-nu/nu mice (4-6 weeks old, Slac Laboratory Animal, Shanghai, China) were kept in the animal facilities at the Nanjing Medical University and maintained under specific pathogen-free conditions. All animal procedures were conducted according to the guidelines approved by the China Association of Laboratory Animal Care. Cultured HepG2 cells (5x10⁶) suspended in 0.2 ml PBS were injected into the right flank of mice. The mice were kept in a pathogen-free environment, 5-7 days later when the tumor volume reached 100 mm³, the mice were divided into four groups (control group and three-dose GYY4137 groups) in a manner to equalize the mean tumor among the four groups (n=7 each). GYY4137 at 50, 20 and 10 mg/kg dose suspended in 0.5% carboxymethyl cellulose (CMC) was given as gavage to mice daily for 4 weeks, and mice of control group were given 0.1 ml 0.5% CMC solution. The tumor size was measured in two orthogonal directions using calipers every three days, and the tumor volume (mm³) was estimated using the equation length × (width)² x 0.5. Four weeks later, the mice were sacrificed and the tumors were resected. Part of tumor tissues were homogenized and subjected to western blot assay. The primary antibodies were STAT3, p-STAT3 (Y705), p-STAT3 (S727), Bcl-2, Mcl-1, cyclin D1, survivin, HIF-1α, VEGF (Cell Signaling Technology) and β-actin (Sigma).

Immunohistochemical (IHC) analysis. Immunohistology analysis was carried out using paraffin section. Paraffin section were incubated in a blocking solution (10% donkey serum + 5% non-fat dry milk + 4% BSA + 0.1% Triton X-100) for 10 min and then hydrated sections were incubated at 4°C overnight with anti-p-STAT3 (Y705) and p-STAT3 (S727) or anti-STAT3 antibody, respectively. After washing with PBS, the sections were incubated with diluted (1:200) biotinylated secondary antibody for 30 min. Subsequently, the slides were washed again in PBS and incubated for 30 min with the preformed avidin-horseradish peroxidase macromolecular complex. Development of peroxidase reaction was achieved by incubation in 0.01% 3,3-diaminobenzidine tetrahydrochlo-
ride (DAB) in PBS containing 0.01% hydrogen peroxide for approximately 5 min at room temperature. Sections were then washed thoroughly in tap water, counterstained in haematoxylin, dehydrated in absolute alcohol, cleared in xylene and mounted in synthetic resin for microscopic examination.

Statistical analysis. Statistical difference was analyzed by two-way Student’s t-test. P<0.05 was considered to indicate statistical significance. The values are expressed as the mean ± SD. Three or more separates experiments were performed.

Results

GYY4137 inhibits IL-6-induced STAT3 phosphorylation in HCC cells. To examine whether the antitumor activity of GYY4137 may be due to the inhibition of IL-6/STAT3 pathway, we evaluated the effects of GYY4137 on IL-6-induced STAT3 phosphorylation in human HCC cells. HepG2 and Bel7402 cell lines with higher IL-6 and phosphorylated STAT3 were treated with GYY4137 for 24 h. As shown in Fig. 1A, IL-6 significantly activated STAT3 phosphorylation at Y705 both in HepG2 and Bel7402 cells. GYY4137 inhibited the IL-6-induced p-STAT3 (Y705) in a dose-dependent manner, whereas it had no effect on p-STAT3 (S727) and total STAT3 (Fig. 1A). Furthermore, inhibition was evident as early as 4 h after treatment and lasted for 24 h (Fig. 1B) in HCC cell lines. These findings indicated that GYY4137 inhibited IL-6-induced STAT3 activation in human HCC cells.

GYY4137 suppresses constitutive JAK2 activation and STAT3 downstream gene expression. JAK2 is considered as one of the most common activators of STAT3. To explore the mechanism of the inhibitory effects on p-STAT3 (Y705), the levels of JAK2 phosphorylation were evaluated. P-JAK2 (Y1007/1008) was suppressed in HepG2 and Bel7402 cell lines with GYY4137 treatment, whereas basic JAK2 was not detectable (Fig. 2A). To further investigate whether GYY4137 would affect STAT3 downstream genes, we used western blot analysis to examine the expression of Bcl-2, Mcl-1, cyclin D1 and survivin. As shown in Fig. 2B, GYY4137 treatment downregulated the expression of these proteins both in HepG2 and Bel7402 cell lines.

GYY4137 causes cell cycle arrest, suppresses cell viability and induces cell apoptosis. To examine the growth inhibitory effect of GYY4137, we first assessed cell cycle distribution by flow cytometric analysis. As shown in Fig. 3, GYY4137 increased the number of cells in the G0/G1 phase in HepG2 and Bel7402 cells. The results are consistent with the inhibitory effect of GYY4137 on cyclin D1 in these cells.

Since IL-6/STAT3 targets certain genes such as Bcl-2, Mcl-1 and survivin involved in anti-apoptosis, we next examined the effect of GYY4137 on apoptosis induction. As shown in Fig. 4A, GYY4137 promoted the cleavage of pro-apoptotic 116 kDa full-length PARP in an 89 kDa fragment, a process known to impair genomic integrity before apoptosis. We also observed that the levels of cleaved caspase-9 and caspase-3 were increased by treatment with GYY4137 in a dose-dependent manner (Fig. 4A). Flow cytometric data revealed that treatment with 200 and 400 µM of GYY4137 increased the number of cells in the subG1 phase (13-20%), indicating that the HCC cells were undergoing apoptosis.
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MTT assay showed that GYY4137 inhibited tumor cell viability time- and dose-dependently (Fig. 4C), while it showed weak inhibition on normal hepatocellular LO2 cells only after 400 µM GYY4137 treatment for 72 h (data not shown).

Figure 2. Effects of GYY4137 on constitutive JAK2 activation and STAT3 downstream gene expression. (A) HCC cells were treated with the indicated concentrations of GYY4137 for 24 h, after which whole-cell extracts were processed for western blot analysis using anti-p-JAK2 and anti-JAK2 antibodies. (B) HCC cells were treated with the indicated concentrations of GYY4137 for 24 h, after which whole-cell extracts were processed for western blot analysis using anti-Bcl-2, anti-Mcl-1, anti-cyclin D1 and anti-survivin antibodies. β-actin served as a loading control.

Figure 3. Effects of GYY4137 on cell cycle distribution in HepG2 and Bel7402 cells. (A) HepG2 and Bel7402 cells were treated with GYY4137 (50–400 µM) for 24 h, stained with PI and analyzed with a FACSCalibur flow cytometer. (B) Quantification of PI staining is presented as percentages corresponding to cell cycle distribution. Each value represents the mean ± SD of triplicate wells. Statistical difference was analyzed by Student's t-test, *P<0.05, **P<0.01 compared to control (0 µM of GYY4137).
shown). These results showed that GYY4137 was able to induce apoptosis in HCC cells through regulating the caspase pathway.

**GYY4137 inhibits expression of VEGF and HIF-1α in HCC cells.** A low oxygen level is a characteristic feature of solid tumors and a negative prognostic factor for cancer patient survival. The response of cancer cells to hypoxia not only drives neo-angiogenesis, but also enhances cancer cell survival and malignant phenotype development. Hypoxia is known to increase the expression of HIF-1α, a major regulator of pro-angiogenic signaling. Since HIF-1α is the major transcriptional modulator of angiogenic factors such as VEGF, we evaluated the effect of GYY4137 on hypoxia induced expression of HIF-1α and VEGF in HCC cells. The cells were treated with various concentrations of GYY4137 under hypoxia mimicking condition induced by 100 µM CoCl₂ for 6 h. As shown in Fig. 5A, GYY4137 suppressed the expression of HIF-1α, which was increased after exposure to hypoxia. To further confirm the effect of GYY4137 on hypoxia-induced VEGF, an immediate downstream target gene of HIF-1α, VEGF protein level secreted to media was measured by ELISA in HepG2 cells. A notable increase of VEGF was observed under the hypoxic condition, whereas the treatment of GYY4137 suppressed VEGF production dose-dependently (Fig. 5B).

**GYY4137 inhibits tumor growth in a subcutaneous HepG2 xenograft model.** To determine the antitumor activity of GYY4137 in vivo, we evaluated its effect in a nude mouse xenograft model of HepG2 cells. GYY4137 was administered for 4 weeks. After 4 weeks, the mice were sacrificed and the tumor mass was dissected. The high dose of GYY4137 (50 mg/kg/d) inhibited tumor growth significantly (P<0.05) after administration for 13 days, and the low dose (10 mg/kg)
of GYY4137 exhibited no significant inhibitory effect on tumor growth (Fig. 6A). Body weight of the mice was observed during administration of GYY4137. There was no significant weight loss in mice treated with GYY4137 of middle and high dose groups, while significant weight loss was detected in mice treated with GYY4137 of low dose or saline groups (Fig. 6B).

To confirm whether the inhibition of GYY4137 on xenograft tumor growth was mediated by blocking STAT3 signaling, we investigated the expression levels of p-STAT3 and STAT3 in xenograft tumor of mice treated with GYY4137 by IHC analysis. As shown in Fig. 7A and B, the level of p-STAT3 (Y705) was decreased in xenograft tumors of mice treated with GYY4137 compared with the model xenograft tumors, whereas the level of STAT3 had no significant change in the model group or the GYY4137 treated group. In concert with the decreased expression of p-STAT3, a clear downregulation in the target gene expression of STAT3, such as cyclin D1, Mcl-1, HIF-1α and VEGF, were found in the xenograft tumor tissue of the GYY4137-treated mice, in a GYY4137 dose-dependent manner (Fig. 7C). Results in vivo further confirmed that the antitumor effect of GYY4137 was partly mediated by blocking the STAT3 signaling pathway.

**Discussion**

Clinical studies have indicated that constitutive STAT3 activation plays a critical role in tumor formation and development in a variety of primary human cancers and cell lines, including HCC. Therefore, STAT3 signaling has emerged as an important therapeutic target for anticancer drug development (12,22). Aberrant activation of STAT3 may promote tumor cell proliferation through the upregulation of cyclin D1 and may suppress apoptosis through the upregulation of Bcl-2, Mcl-1 and survivin (10). Therefore, suppression of constitutively activated STAT3 not only induces apoptosis in cancer cells but also overcomes chemoresistance and radioresistance (23). The aim of this study was to investigate whether GYY4137 exerts its anticancer effect through inhibiting STAT3 signaling in HCC. We found that GYY4137 had anti-HCC effects and the underlying mechanism was associated with the inhibition of STAT3 signaling that led to the suppression of cancer cell proliferation by induction of cell cycle arrest, increased apoptosis and inhibition of angiogenesis *in vitro* and *in vivo*.

Phosphorylation of STAT3 at Y705 enables its dimerization, nuclear translocation, DNA binding and gene transcription (23), whereas phosphorylation of another conserved STAT3 residue, S727, enhances STAT3 transcriptional activity (24). Cooperation of tyrosine and serine phosphorylation is necessary for the full activation of STAT3 (25). Here, we showed that GYY4137 could inhibit IL-6-induced STAT3 activation in HCC cells by blocking JAK2 phosphorylation. STAT3 was constitutively phosphorylated at Y705 and S727 in HCC and GYY4137 inhibited phosphorylation of Y705 among HCC.
cells and HepG2 xenografts of nude mice that exhibited different molecular or genetic characteristics. The inhibition was evident as early as 4 h after treatment and lasted for 24 h, suggesting that the actions of GYY4137 are relatively rapid and prolonged. Moreover, we showed that GYY4137 downregulated STAT3 mediated downstream gene products involved in controlling cell cycle progression and programmed cell death (e.g., cyclin D1, Mcl-1, Bcl-2 and survivin) in vitro and in vivo. Constitutively active STAT3 is associated with the induction of resistance to apoptosis possibly through the expression of Bcl-2 and cyclin D1 (26-28). Overexpression of Mcl-1 is found in a great percentage of HCC. Targeting Mcl-1 is a promising novel approach in HCC therapy (29). Therefore, downregulation of the expression of cyclin D1, Mcl-1, Bcl-2 and survivin was likely correlated with the ability of GYY4137 to facilitate apoptosis in HCC cells.

Figure 6. Effects of GYY4137 on tumor growth of nude mice bearing HepG2 xenograft tumors. (A) Tumor volume and (B) mouse body weight changes were determined every week during delivery period, statistical difference was analyzed by Student's t-test, the values are expressed as the mean volume of xenograft tumors ± SEM. *P<0.05, **P<0.01 and ***P<0.01 compared to vehicle mice; +P<0.05 and ++P<0.01 compared to normal mice.

Figure 7. Effects of GYY4137 on STAT3 signaling in the xenografted HepG2 tumors of nude mice. (A) The xenografts in mice of model group and GYY4137 treatment groups were subjected to immunohistochemical analysis using p-STAT3 (Y705) or STAT3 antibodies. Positive cells are brown, negative cells are blue, magnification x200. (B) The xenograft tumor tissue in mice of model group and GYY4137 treatment groups were subjected to western blot analysis to determine the protein of anti-p-STAT3 (Y705) and anti-STAT3. (C) The xenografts in mice of the model group and GYY4137 treatment groups were subjected to western blot analysis to determine the protein of anti-cyclin D1, anti-Mcl-1, anti-HIF-1α and anti-VEGF.
Apoptosis is a process of programmed cell death that serves as a major mechanism for the precise regulation of cell numbers and as a defense mechanism to remove unwanted cells (30). Mitochondria mediate apoptotic signaling via activation of the cell death initiator procaspase-9 (30,31). Activated caspase-9 in turn cleaves executioner caspase-3. The activated caspase-3 then cleaves PARP, a 116 kDa nuclear protein related to the process of programmed cell death (32). In addition, we observed that the antitumor activity of GYY4137 was also associated with the induction of apoptosis in HCC cells as indicated by in vitro evidence of increased cleaved caspase-9, caspase-3 and PARP cleavage. These results indicated that GYY4137 inhibited cell growth by regulating cell cycle progression and induced apoptosis in HCC cells.

VEGF and HIF-1α are prominent transcriptional targets for STAT3 among the angiogenesis factors (10,33). HIF-1α activation appears to be a very early event in carcinogenesis and this protein is expressed before histological evidence of angiogenesis or invasion (34,35). In regards to HCC, overexpression of HIF-1α has been reported, which has been associated with a poor prognosis (36). Recently, both HIF-1α and VEGF were identified to be involved in the malignant transformation of dysplastic liver nodules and additionally a hypoxia-independent overexpression of HIF-1α has been shown to be involved in a model of mouse hepatocarcinogenesis (37). Suppression of HIF-1α and STAT3 activity was demonstrated to inhibit the growth in HCC, ultimately leading to a reduction of tumor growth and vascularization in vivo (38). In the present study, we found that GYY4137 effectively inhibited VEGF and HIF-1α expression in HCC cells and HepG2 xenografts of nude mice, which may be related with its inhibition of STAT3 activation. Therefore, downregulation of VEGF and HIF-1α by GYY4137 may be of great significance in suppressing tumor cell invasion and metastasis.

In conclusion, GYY4137 blocks the STAT3 signaling pathway. The inhibition of STAT3 (Y705) phosphorylation triggered downregulation of gene products related to cell survival, cell cycle progression, thus in turn triggered cell apoptosis and cell cycle arrest. Importantly, GYY4137 inhibited antitumor effect in the HepG2 xenograft nude mouse model. These results suggested that GYY4137 is a potential candidate for treatment of HCC.

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References