A novel imidazopyridine analogue as a phosphatidylinositol 3-kinase inhibitor against human breast cancer

Hyunseung Leea,1, Guang-Yong Lia,1, Yujeong Jeongb, Kyung Hee Junga, Ju-Hee Leea, Kyungrok Hamb, Sungwoo Hongb,⇑, Soon-Sun Honga,⇑

aDepartment of Biomedical Sciences, College of Medicine, Inha University, 3-ga, Sinheung-dong, Jung-gu, Incheon 400-712, Republic of Korea
bDepartment of Chemistry, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 305-701, Republic of Korea

Abstract

Potentiation of anti-breast cancer activity of an imidazopyridine-based PI3Kα inhibitor, HS-104, was investigated in human breast cancer cells. HS-104 shows strong inhibitory activity against recombinant PI3Kα isoform and the PI3K signaling pathway, resulting in anti-proliferative activity in breast cancer cells. It also induced cell cycle arrest at the G2/M phase as well as apoptosis. Furthermore, oral administration of HS-104 significantly inhibited the growth of tumor in SkBr3 mouse xenograft models. Therefore, HS-104 could be considered as a potential candidate for the treatment of human breast cancer.

Keywords:
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1. Introduction

Breast cancer is one of the major causes of premature death in women. If not treated early, breast cancer may metastasize and spread to almost all parts of the body such as the lymph nodes, bones, liver, lungs, and brain, often resulting in death. It is usually treated with chemotherapy first, then surgery and possibly radiation therapy. With best treatment and dependent on staging, 5-year relative survival rate varies from 98% to 23%, with an overall survival rate of 85%. Despite its high 5-year survival rate, the recurrence rate of breast cancer is relatively high, at 20–30%, depending on stage [1]. Moreover, it is still the most common malignancy in women in the United States and is the second-leading cause of cancer death in women, with about 39,800 deaths recorded annually [2].

Recently many therapeutic targets have been identified to treat cancers, and the phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway has been considered as one of the most attractive targets for many cancers. It regulates various cellular processes such as proliferation, growth, apoptosis, and cytoskeleton rearrangement [3]. The PI3K-Akt-mTOR pathway is frequently hyperactivated in breast cancer by multifarious mechanisms. First, in breast cancer, mutations of PIK3CA (encoding p110α) that occur in one-third of patients are site specific (located in the helical and kinase domains) and more frequent in tumors expressing hormone receptors and HER2 [4,5]. Second, loss of function of the tumor suppressor phosphatase and tensin homolog (PTEN) is a general event in breast cancer [6], resulting in increased PI3K-pathway activity, metastasis, and poor survival [4,7,8]. Third, HER2 overexpression and amplification have been observed in 20% in breast cancer patients [9], and promote PI3K signaling through HER2/HER3 heterodimerization [10]. Taken together, over 70% of human breast cancers have a deregulated PI3K pathway [11]. Moreover, aberrant activation of the PI3K pathway results in resistance to anti-HER2 and other anti-cancer agents. In this regards, targeting of PI3K signaling can be considered as a promising strategy for the treatment of PI3K-Akt-mTOR pathway-dependent breast cancer. Several PI3K pathway inhibitors are currently under early clinical development [12–15], and we recently developed a new imidazopyridine-based PI3K inhibitor, HS-104 (full name: N-(5-(3-(5-methyl-1,2,4-oxadiazol-3-yl)imidazo[1,2-a]pyridin-6-yl)pyridin-3-yl)benzenesulfon-amide) [16].

Herein, we report studies on the pharmacologic action and anti-tumor activity of HS-104 against breast cancer with hyperactivated-PI3K signaling pathway.

2. Materials and methods

2.1. Preparation of HS-104

HS-104 was synthesized in our previous study [16]. High performance liquid chromatography analysis for checking purity of synthesized HS-104 was performed on Waters HPLC equipped with a reverse phase column and by HRMS. "H NMR
2.2. Cell lines

The human breast cancer cell lines SkBr3, T47D and MCF-7 were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). T47D and MCF-7 cells were cultured in Roswell Park Memorial Institute Media 1640 (RPMI-1640), and SkBr3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cultures were maintained at 37°C in a CO2 incubator with 10% CO2.

2.3. In vitro PI3K assay

An active PI3K (100 ng) was preincubated with HS-104 or LY294002 for 5 min in kinase reaction buffer (25 mM MOPS pH 7.0, 5 mM MgCl2 and 1 mM EGTA) and 10 µg L-α-phosphatidylinositol. Before addition of L-α-phosphatidylinositol, it was sonicated in water for 20 min to facilitate micelle formation. The reaction was started by the addition of 10 µM ATP and was run for 180 min. To terminate the kinase reaction, the same volume of Kinase-Glo® Max buffer (Promega) was added. After 10 min, the plates were then read on a GloMax plate reader for luminescence detection.

2.4. Cell viability assay

Cell viability was confirmed using an established 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based assay. Briefly, SkBr3, T47D and MCF-7 cells were plated at a density of 3–5 x 103 cells/well in 96-well plates for 24 h. Then, the medium was removed, and cells were treated with either DMSO as a control or various concentrations of HS-104, tamoxifen and LY294002. After the cells were incubated for 48 h, 100 µL MTT solution (2 mg/mL) was added to each well for another 4 h at 37°C. The formazan crystals that formed in DMSO (200 µL/well) by constant shaking for 5 min. The plate was then read on a microplate reader at 540 nm. Three replicate wells were used for each analysis.

2.5. Western blotting

Total protein was extracted from cells with lysis buffer containing 1% Triton X-100, 1% Nonidet P-40, and the following protease and phosphatase inhibitors: apro tinin (10 µg/mL), leupeptin (10 µg/mL) [ICN Biomedicals, Asse-Relegem, Belgium], phenylmethylsulphonyl fluoride (1.72 mM), NaF (100 mM), NaVO3 (500 mM) and NaN3-DVS (500 mg/mL) (Sigma–Aldrich). The proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto nitrocellulose membranes. Immunostaining of the blots was performed using the primary antibodies, followed by the secondary antibody conjugated to horseradish peroxidase and detection by enhanced chemiluminescence reagent (Amersham Biosciences). Antibodies to poly (ADP-ribose) polymerase (PARP), cleaved caspase-3, -8 and -9, and G3PDH antibodies were from Cell Signaling Technology.

2.6. Immunofluorescence microscopy

SkBr3 cells were plated on 18-mm cover glasses in RPMI-1640 medium at approximately 70% confluence for 24 h. Then, cells were incubated in the presence or the absence of 5 µM HS-104 for 6 h. The cells were washed twice with PBS and fixed in acetone/methanol (1:1, 10 min, −20°C). Cells were blocked in 1% horse serum/PBS for 30 min at room temperature, then incubated with primary antibody in a humidified chamber. After two washes with PBS, the cells were incubated with mouse fluorescein-labeled secondary antibody (Dianova, Germany) (1:100 in 1% horse serum/PBS, at room temperature, protected from light) for 20 min at 37°C. To stain the nucleus, 4,6-diamidino-2-phenylindole (DAPI) was used. The slides were washed twice with PBS, and then covered with DABCO (Sigma–Aldrich). Confocal laser scanning microscopy was performed at 488 nm and at 568 nm.

2.7. Cell cycle analysis

SkBr3, T47D and MCF-7 cells were plated in 100-mm-diameter culture dishes. The next day, cells were treated with 5 µM HS-104. The floating and adherent cells were collected and fixed in cold 70% ethanol at 4°C overnight. After washing, the cells were subsequently stained with 50 µg/mL propidium iodide (PI) and 100 µg/mL RNase A for 1 h in the dark and subjected to flow cytometric analysis to determine the percentage of cells at specific phases of the cell cycle (sub-G1, G0/G1, S and G2/M). Flow cytometric analysis was performed using a FACS Calibur flow cytometer (BD Biosciences) equipped with a 488 nm argon laser. Events (approximately 20,000) were evaluated for each sample and the cell cycle distribution was analyzed using Cell Quest software (BD Biosciences). All the experiments were performed three times.

2.8. Annexin V assay

For flow cytometric analysis of apoptosis, SkBr3, T47D and MCF-7 cells were treated with HS-104 for 24 h. 5 x 103 cells were removed from the culture, washed twice with cold PBS, and double stained with Annexin V-fluorescein isothiocyanate (FITC) and PI (BD Biosciences) in Annexin-binding buffer, followed by analysis on a FACS Calibur flow cytometer (BD Biosciences) equipped with a 488-nm argon laser. To avoid nonspecific fluorescence from dead cells, live cells were gated using forward and side scatter.

2.9. DAPI staining and TUNEL assay

SkBr3 cells were plated on 18-mm cover glasses in RPMI-1640 medium at approximately 70% confluence for 24 h. The cells were then treated with HS-104 at 10 µM for 24 h. They were fixed in ice-cold 1% paraformaldehyde, washed with PBS and then permeabilized with a 0.2% Triton X-100/0.1% sodium citrate solution. Slides were blocked in 1% horse serum/PBS for 1 h, then incubated with primary antibody in a humidified chamber and then stained with DABCO (Sigma–Aldrich).

Fig. 1. Enzymatic characterization of HS-104. (A) Chemical structure of HS-104. (B) Inhibition of PI3Kcy by HS-104 and LY294002 using the Kinase-Glo assay. (C) The putative binding mode of HS-104 with the PI3Kcy homology model, based on PI3Kγ crystal structure. Each value is the mean ±SD from triplicate samples.
PBS and then stained with 2 μg/mL DAPI for 20 min at 37 °C. The stained cells were examined under a fluorescence of nuclear fragmentation. Terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) was performed following the manufacturer’s protocol (Chemicon).

2.10. Tumor xenograft studies

To establish SkBr3 tumor xenograft in mice, the SkBr3 cell line was grown in culture, detached by trypsinization, washed and resuspended in PBS. 6 weeks old athymic BALB/c nude mice (Orient Bio, Gyeonggi, Korea) were injected with 5 × 10⁶ cells in right flank of each mouse to initiate tumor growth. After tumor volume reached 30–50 mm³, the mice were randomly divided into two groups, each having six mice. Mice in control group were fed with 0.4 mL vehicle (DMSO: PEG400: saline = 5:40:55) by oral gavage daily and the second group with 20 mg/kg dose of HS-104 in 0.4 mL daily for 24 days. Body weight and tumor size were recorded twice per week. The tumor size was calculated by 0.5 × long axis × (short axis)². Animal care and experimental procedures were conducted in accordance with the Guide for Animal Experiments by the Korean Academy of Medical Sciences, and the protocols used to this study were approved by Institutional Animal Care and Use Committee in Inha University Hospital (Approval No.: 100222-55).

2.11. Immunohistochemistry

After being blocked with normal goat serum (Vector Laboratories) for 1 h, frozen tissue sections were incubated for 1 h at room temperature in dilutions of 1:100 of cleaved caspase-3, p-Akt (Ser473), and p-mTOR (Ser2448) antibodies. The sections were visualized by an avidin–biotin peroxidase complex solution using an ABC kit (Vector Laboratories). The sections were washed with PBS and developed with a diaminobenzidine tetrahydrochloride substrate for 15 min and then counterstained with hematoxylin.

![Fig. 2. HS-104 specifically blocks the PI3K-Akt-mTOR pathway. (A) SkBr3, T47D and MCF-7 were treated with various concentration of HS-104 for 6 h. The protein levels of Akt, mTOR, GSK3β, p70S6K, 4E-BP1 and their phosphorylated form was analyzed by Western blot. (B) Three breast cancer cell lines were treated with or without 5 μM HS-104 for 6 h. The phosphorylation of Akt, p70S6K and 4E-BP1 was detected by confocal microscopy. Pictures were taken at 400-fold magnification. A representative experiment from three independent experiments is shown.](image-url)
3. Results

3.1. HS-104 inhibits PI3Kα

HS-104 was found to be a potent ATP-competitive inhibitor against PI3Kα (Fig. 1A) [16]. Kinase inhibition activity of HS-104 was tested against class I PI3K using an ATP depletion (Kinase-Glo) assay and showed a highly potent inhibitory activity against PI3Kα in a dose-dependent manner (IC50 < 10 nM). In this assay, LY294002 inhibited p110α with an IC50 of 1 μM, showing approximately 100-fold less potency than HS104 as shown in Fig. 1B. The binding affinity of HS-104 for PI3Kα was also tested in a high-throughput binding assay (KINOMEscan, Ambit Biosciences), which revealed high binding affinity with an associated Kd value of 1.2 nM. Docking simulations were conducted to obtain energetic and structural insight into the binding modes, and Fig. 1C shows the lowest-energy binding mode of HS-104 in the ATP-binding site of PI3Kα. Three dimensional (3D) coordinates in the X-ray crystal structure of PI3Kα in the resting form (PDB code: 2RD0) were selected as the receptor model for docking simulations [17]. After removing the solvent molecules, hydrogen atoms were added to each protein atom. We used the AutoDock program for docking studies between HS-104 and HS-104 because the outperformance of its scoring function over those of the others had been shown in several target proteins [18]. HS-104 appeared to be in close contact with residues Val851, Tyr836, Asp810, and Lys802, which belong to the hinge region, the gatekeeper site, DFG pocket and catalytic lysine region, respectively. Hydrogen-bond formation by the backbone amide nitrogen of Val851 was observed in stabilizing the inhibition by donating a hydrogen bond to the imidazole group of HS-104. It is noted that the nitrogen of pyridyl group forms a hydrogen bond with the side-chain hydroxyl group of Tyr836. Another stable hydrogen bond is also established between the sulfonamide group and the ammonium group of catalytic Lys802. These three hydrogen bonds seem to play a critical role of anchor for binding of HS-104. HS-104 may be further stabilized in the ATP-binding site via the hydrophobic interactions among its nonpolar groups with the side chains of Val851, Tyr836, Asp810, and Lys802. Thus, the overall structural features derived from docking simulations indicated that the binding affinities of HS-104 for PI3Kα stemmed from multiple hydrogen bonds and hydrophobic interactions established simultaneously in the ATP-binding site.

3.2. HS-104 blocks the PI3K-Akt-mTOR pathway in breast cancer cells

The PI3Kα inhibitory activity of HS-104 was assessed in breast cancer cells with different modulations of the PI3K-Akt cascade, namely mutated p110α (MCF-7, T47D) or amplification of HER2 (SkBr3). The phosphorylation of Akt and its substrate, mTOR, and phosphorylation of downstream factors including GSK3β, p70S6K and 4E-BP1 were effectively suppressed in all three breast cancer cells, indicating complete suppression of the PI3K pathway (Fig. 2A). HS-104 also strongly suppressed phosphorylation of Akt, p70S6K and 4E-BP1 in SkBr3 cells, as demonstrated by confocal fluorescent microscopy (Fig. 2B).

3.3. HS-104 inhibits cell proliferation and induces cell cycle arrest in breast cancer cells

To investigate the inhibition of growth by HS-104 in breast cancer cells, we examined the effect of HS-104 on cell proliferation in SkBr3, T47D and MCF-7 cells. As shown in Fig. 3A, HS-104 inhibited the growth of all three breast cancer cell lines in a dose-dependent manner. The IC50 values of HS-104 were 4.8 μM for SkBr3, 1.1 μM for T47D, and 1.2 μM for MCF-7. Especially, anti-proliferative activity of HS-104 was more effective than tamoxifen, a most common drug for breast cancer and LY294002, a well-known PI3K inhibitor. In addition, to examine the mechanism responsible for HS-104-mediated cell growth inhibition, cell cycle distribution was evaluated using flow cytometric analysis. As shown in the Fig. 3B, HS-104 induced a time-dependent increase in the G2/M phase. After 12 h treatment with HS-104, there were increase of the G2/M phase above 25%, 16% and 32% in the SkBr3, T47D and MCF7 compared with control.
3.4. HS-104 induces apoptosis in breast cancer cells

Because HS-104 successfully demonstrated its ability to inhibit both the PI3K-Akt-mTOR signaling pathway and cell proliferation, we further determined whether HS-104 induced apoptosis in the breast cancer cells. First, TUNEL and DAPI staining were carried out to investigate whether cell death was originated from the apoptosis of SkBr3 cells (Fig. 4A). TUNEL-positive apoptotic cell were predominantly located in HS-104 treated cells. And increased number of fragmented nuclei was seen after treatment with 5 µM HS-104 by DAPI staining. The percentage of apoptotic cells was measured by the number of Annexin V-positive cells and sub-G1 DNA peak analysis following HS-104 treatment. Exposure to 5 µM HS-104 increased apoptosis with 38%, 40% and 25% in SkBr3, T47D and MCF-7 cells, respectively (Fig. 4B). HS-104 treatment also led to the increase of the percentage of sub-G1 in breast cancer cells (Fig. 4C) and cleavage of PARP and caspase-3 in a dose-dependent manner (Fig. 4D).

3.5. HS-104 is an orally available PI3K inhibitor with antitumor activity

To determine whether HS-104 could inhibit tumor growth in vivo, we established a SkBr3 mouse xenograft models. HS-104 was administered orally with a daily dose of 20 mg/kg when tumors reached an average volume of 30–50 mm³. Administration of HS-104 for 24 days significantly suppressed the growth of tumor. The average tumor volume of HS-104-treated mice was reduced by about 50% compared to that of control mice (Fig. 5A and B). In addition, the average tumor weight of HS-104-treated mice was reduced by 60% compared to control (Fig. 5C). To assess general toxicity, we measured the body weight of tumor-bearing mice...
mice. The same dose of HS-104 showed no significant change on the body weight of mice (Fig. 5D), suggesting low toxicity of HS-104 at the test dosage and conditions.

To evaluate the in vivo effects of HS-104 on intracellular signaling, six mice per each group were sacrificed after 24 days of treatment, and tumors were excised for analysis by immunohistochemistry. Phosphorylation of Akt and mTOR were suppressed in tumors from HS-104-treated mice (Fig. 5E), which reflected the in vitro results obtained from HS-104 treated SkBr3 cell line. Furthermore, an increased cleaved caspase-3 staining and TUNEL positive cells in the tumors from HS-104-treated mice revealed that HS-104 induced apoptosis in vivo. These data suggest that, in the SkBr3 mouse xenograft model, administration of HS-104 leads to tumor regression through the inhibition of PI3K pathway and induction of apoptosis.

4. Discussion

In recent years, it is evident that many human tumors carry somatic missense mutations in PIK3CA at high frequency. Specifically, in breast cancer, PIK3CA mutation (~32.5%), HER2 amplification (~15–30%), and PTEN deficiency (~30%) are frequently reported to activate the PI3K pathway, leading to cell proliferation and survival.

To evaluate the inhibitory effect of HS-104 against PI3Kα, we carried out the in vitro kinase assay (Fig. 1B). Its inhibitory activity against PI3Kα was approximately 100-fold stronger than LY294002, a well-known PI3K inhibitor. Moreover, in anti-cancer activity using cell lines, HS-104 was superior to LY294002 or tamoxifen, a most common drug for breast cancer. Further binding mode analyses and docking simulations indicated that HS-104 is
stabilized in the ATP-binding site of PI3Kα through the formation of three hydrogen bonds and hydrophobic interactions with nonpolar residues. As shown in the Fig. 2, HS-104 effectively blocked PI3K-Akt-mTOR signaling pathway in the breast cancer cells. Interestingly, feedback activation of Akt was not observed in the HS-104-treated breast cancer cells, unlike many other PI3K pathway inhibitors.

Recently, it is reported that 4E-BP1 is significant in cell proliferation, and p70S6K is critical for cell size regulation [19]. GSK3β is also involved in regulating cell proliferation [20]. The activation of Akt, p70S6K and GSK3β could be completely inhibited by HS-104 treatment. In addition, the phosphorylation of 4E-BP1, which is another key substrate of mTOR, was efficiently inhibited by HS-104 treatment (Fig. 2). Based on these mechanistic studies, we further investigated the activity of HS-104 on cancer cell growth. At the concentration of less than 5 μM, HS-104 potently inhibited the growth of three breast cancer cell lines. T47D and MCF-7 cells with mutated p110α were more sensitive to HS-104 than SkBr3 cells with wild-type p110α, indicating that proliferation of T47D and MCF-7 cells appears to be more dependent on PI3K pathway than SkBr3 cells. In this study, breast cancer cells treated with HS-104 showed cell cycle arrest during the G2/M phase, resulting in reduced cell proliferation (Fig. 3). Akt has been reported to function as a G2/M initiator [21,22]. The phosphorylation of Akt blocks G2 arrest induced by DNA-damaging agents and radiation [21]. The suppression mechanism of Akt on G2 arrest is not well-understood, although it has been observed that Akt-mediated G2 checkpoint inhibition is associated with suppression of Chk2 activation, and up-regulation of cyclin B and Cdc2 expression [21,22]. Furthermore, Akt promotes cell cycle progression at the G2/M transition through WEE1H1 inactivation in mammalian cells [23]. Presently, inhibition of the Akt phosphorylation by HS-104 treatment was involved in accumulation of inactive-phospho-cdc2, which may be due to the increase of Chk2 activation, leading to subsequent G2 arrest.

The PI3K-Akt-mTOR pathway is also considered to mediate anti-apoptotic signals in tumor cells [24]. One of the targets identified with direct implications for regulating cell survival is the proapoptotic Bcl-2-family member BAD. When it is not phosphorylated, BAD inhibits Bcl-2 and other anti-apoptotic Bcl-2 family members by direct binding to them [25]. Once phosphorylated by Akt, however, pro-apoptotic activity of BAD is neutralized [26]. GSK3β is also a key target of PI3K signaling leading to prevention of apoptosis. [27]. It is critical downstream element of PI3K-Akt cell survival pathway whose pro-apoptotic activity is inhibited by Akt-mediated phosphorylation. From this study, activation of pro-apoptotic protein and disturbance of cell cycle regulation by the PI3K-Akt pathway inhibitor, HS-104, seemed to induce apoptotic effect of human breast cancer cells.

Many breast cancers are sensitive to hormones, especially the female hormone estrogen that encourages the growth of breast cancer, which makes it possible to treat them by drugs that block the estrogen. Therefore, estrogen receptor (ER) has been a therapeutic target for breast cancer treatment. For example, tamoxifen which is an antagonist of the ER, has been used as a popular breast cancer treatment for more than 25 years. Although tamoxifen was strongly antitumor activity against breast cancer xenograft without critical toxicity. HS-104 also induced apoptosis presumably via PI3K-Akt-mTOR signaling pathway in breast cancer cells. Based on the results, HS-104 can be considered as a potential anti-breast cancer therapeutic candidate for further development.

References


