Original Paper

Inhibition of Sonic Hedgehog Signaling Pathway by Thiazole Antibiotic Thiostrepton Attenuates the CD44+/CD24- Stem-Like Population and Sphere-Forming Capacity in Triple-Negative Breast Cancer

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Key Words

Thiostrepton • Triple-negative breast cancer • Sonic hedgehog • Breast cancer stem cell

Abstract

Background/Aim: Triple-negative breast cancer (TNBC) represents a particular clinical challenge because these cancers do not respond to endocrine therapy or other available targeted agents. The lack of effective agents and obvious targets are major challenges in treating TNBC. In this study we explored the cytostatic effect of thiazole ring containing antibiotic drug thiostrepton on TNBC cell lines and investigated the molecular mechanism. **Methods:** Cell viability was measured by MTT assay. Cell surface marker was monitored by FCM. Western blot was applied to assess the protein expression levels of target genes. **Results:** We found that thiostrepton remarkably suppressed the CD44+/CD24- stem-like population and sphere forming capacity of TNBC cell lines. Notably, we showed for the first time that thiostrepton exerted its pharmacological action by targeting sonic hedgehog (SHH) signaling pathway. Thiostrepton repressed SHH ligand expression and reduced Gli-1 nuclear localization in TNBC cell line. Furthermore, the downstream target of SHH signaling undergone dose-dependent, rapid, and sustained loss of mRNA transcript level after thiostrepton treatment. Finally, we showed that SHH ligand was essential for maintaining CD44+/CD24- stem-like population in TNBC cell line. **Conclusion:** We conclude that thiostrepton suppresses the CD44+/CD24- stem-like population through inhibition of SHH signaling pathway. Our results give a new

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insight into the mechanism of thiostrepton anti-tumor activity and suggest thiostrepton as a promising agent that targets hedgehog signaling pathway in TNBC.

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Introduction

Breast cancer is a highly heterogeneous malignancy that can be grouped into major subtypes. Triple-negative breast cancer (TNBC) is a distinct clinical subtype and accounts for ~15% – 20% of breast cancer patients [1]. It is defined as tumors that lack expression of estrogen (ER), progesterone (PR), and human epidermal growth factor receptor 2 (HER2). Gene expression analysis demonstrates that the molecular signature of TNBC generally overlaps with basal-like breast cancer (BLBC), with concordance rates of 70 – 90% [2]. TNBC often associates with more aggressive behavior, poorer prognosis, and a shorter median time to relapse and death than other forms of breast cancer. Despite the progress achieved in breast cancer therapeutic innovations the TNBC still represents a particular clinical challenge because these cancers do not respond to endocrine therapy or other available targeted agents [3]. The lack of effective agents and obvious targets are major challenges in treating patients with TNBC.

Thiostrepton, a thiazole ring containing antibiotic, originally isolated from *Streptomyces azureus*, has been used in veterinary medicine in mastitis caused by gram-negative organisms and in dermatologic disorders. Despite of its antibiotic action, thiostrepton has been shown to induce cell death in a variety of human cancer cell lines. In melanoma cells, thiostrepton efficiently inhibits cell growth and induces apoptosis [4]. In breast cancer cells thiostrepton induces cell cycle arrest and cell death through down-regulating FOXM1 expression [5]. Wang et al. reported that micelle-encapsulated thiostrepton reduced the growth rates of MDA-MB-231 breast cancer xenografts and HepG2-luc liver cancer xenografts [6]. Although thiostrepton has been shown to inhibit cell growth and suppress the expression of FOXM1 in breast cancer, the mechanism of its anticancer activity and its cellular targets remain undetermined.

TNBC is poorly differentiated basal-like adenocarcinomas and display breast cancer stem cell (BCSC) phenotype CD44+/CD24- [7]. The population of CD44+/CD24- cell has been demonstrated to have tumor-initiating properties and associate with stem cell-like characteristics in breast cancer [8]. Tumors with a high percentage of CD44+/CD24- cells develop significantly more distant metastases than did other tumors. Studies have revealed that the sonic hedgehog (SHH) signaling pathway mediates progression from a non-invasive phenotype to an invasive phenotype in breast cancer. In a clinical breast cancer sample analysis, the expression of SHH and Gli-1 were significantly associated with increased risk of metastasis, breast cancer-specific death and a basal-like phenotype [9]. Moreover, SHH signaling pathway plays a key role in mammary stem/progenitor cell self-renewal by directly activating the expression of BMI-1, a central regulator of self-renewal in cancer stem cells [10, 11]. Given the results of clinical analysis and the observation that TNBC often displays BCSC phenotype, inhibition of SHH signaling pathway is expected to be an attractive pharmacological target in TNBC [12, 13].

Here, we show that the thiazole ring containing antibiotic drug thiostrepton decreases the CD44+/CD24- stem-like population through inhibition of SHH signaling pathway. For the first time, we provide evidence that inhibition of SHH signaling pathway is a pharmacological action of thiostrepton. Using *in vitro* suspension cell culture systems, we demonstrate that thiostrepton effectively suppresses the proliferation of breast cancer stem cell of TNBC cell lines. Furthermore, we show that SHH ligand plays an important role in maintaining CD44+/CD24- population of TNBC cell line, suggesting the crucial role of SHH signaling in TNBC. Our results provide evidence for using the antibiotic drug thiostrepton as a potential effective agent that targets SHH signaling pathway in TNBC treatment.

Materials and Methods

Cell viability and colony formation assay

MDA-MB-231, BT549, MCF-7, and T47D cells were grown in DMEM media (Invitrogen) and 10% fetal bovine serum (Hyclone). SUM149 cells were grown in HAM'S/F12 media and 5% fetal bovine serum, supplemented with insulin and hydrocortisone. Cells were cultured in a humidified incubator at 37°C with 5% CO₂. Thiostrepton (Sigma) was dissolved in DMSO at a stocking concentration of 10mM. MTT assay was used to measure cell viability as described previously [14]. Morphologic change was monitored by phase-contrast microscopy. For colony formation assay, 5×10^3 cells were plated in triplicate in 6-cm plate. Twenty-four hours later, treatment was initiated. After 10 days, cells were fixed and stained with crystal violet. The number of colonies was counted using Adobe Photoshop.

Flow cytometric analysis

After treatment of thiostrepton for 24h, cells were trypsinized, washed and suspended in 100 µL PBS containing prejugated primary antibodies: CD44-FITC (1:50, eBioscience); CD24-PE(1:20, eBioscience) and incubated for 30 min on ice. Unstained cells were used for negative control. Cells that only stained with CD44-FITC were used to regulate compensation and set the CD24-PE gate. Cells that only stained with CD24-PE were used to regulate compensation and set CD44-FITC gate. For analysis, cells were suspended in 500 µL of PBS buffer and fluorescence was measured using flow cytometer (Beckman).

Mammosphere culture

Mammosphere culture was performed as previously described [15]. Briefly, cells were seeded in ultra-low-attachment six-well plates (Corning, USA) and cultured in suspension (1000 cells/mL) in serum-free DMEM/F12 media, supplemented with B27 (1:50; Invitrogen), 20 ng/mL human recombinant epidermal growth factor (Preprotech), 20 ng/ml basic fibroblast growth factor, 4 µg/ml heparin and 5 µg/mL insulin (Sigma). 5% matrigel (BD) was added to culture medium to prevent aggregation. The number of Mammospheres were photographed and counted 10 d after thiostrepton treatment using inverted microscope. The diameters of the mammospheres were calculated using the Image pro plus 6.0 software.

Immunofluorescence assay

To study Gli-1 subcellular localization by immunofluorescence assay, MDA-MB-231 cells were seeded on coverslips placed in 35 mm dishes and treated with thiostrepton at 5 or 10 µM for 24 h, after which cells were harvested by fixing with 4% paraformaldehyde. Fixed cells were treated with anti-Gli-1 antibody (1:200, Abcam) and then the fluorochrome-tagged secondary antibody (1:200, Alexa fluor 546-conjugated anti-rabbit). Following incubation in PBS with propidium iodide, coverslips were mounted on slides.

Plasmids and Transfections

The human SHH over-expression construct was purchased from Santa Cruz Biotechnology (sc-400225-ACT). For transfections, cells were seeded to a confluency of 60% and incubated for 6 h with a mix of antibiotic-free transfection reagents containing Lipo2000 (Invitrogen) and the plasmid DNA. Fresh medium was then added to replace the transfection reagent and cells were allowed to grow overnight before use.

Small interfering RNA and Western blot

Two human SHH siRNA oligos and control siRNA oligos were purchased from Genepharma Inc. Western blot analysis was used to confirm the knockdown of SHH expression in MDA-MB-231 cells. Cell pellets were resuspended in ice-cold RIPA lysis buffer and Western blot analyses were performed as previously described [14]. Membranes were incubated with antibodies against sonic Hedgehog ligand (Cell Signaling Technology, Inc.), Gli-1(Cell Signaling Technology, Inc.), Nanog (Epitomic, Inc.), and GAPDH (Epitomic, Inc.) at 4°C overnight. Protein bands were visualized with enhanced chemiluminescence.

Statistical methods

The data of this article are represented as mean $\pm$ SD taken over a minimum of three independent experiments. Statistical significance was measured using standard t tests for two paired samples or two-way ANOVA. P values < 0.05 were considered to be statistically significant.

Results

Thiostrepton effectively inhibits proliferation of triple-negative breast cancer cell lines

Thiostrepton has been reported to have cytostatic effect on breast cancer cells [5, 6]. We were interested to examine whether the effect was different among different subtype of breast cancer. Three TNBC cell lines: SUM149, MDA-MB-231, BT549 and two ER-positive (ER+) breast cancer cell lines: MCF-7, T47D were treated with increasing dose of thiostrepton from 0 to 20 μM over a time course of 24 h. After 24 h treatment TNBC cell lines showed obvious morphologic change (Fig. 1A). MTT assay showed that after 24 h treatment there was an obvious reduction in proliferation in all of cell lines tested at dose of $\geq 10\mu\text{M}$. Notably the three TNBC cell lines were significantly more sensitive to thiostrepton treatment than two ER+ cell lines (Fig. 1B). Since ER+ cell lines were slow growing cells, we extended the treatment for 7 days and observed that at the dose of 5 μM , 10 μM , and 15 μM TNBC cell lines were also significantly more sensitive than ER+ cell lines. Nevertheless at a concentration of 20 μM the cell viability of all the cell lines tested displayed sharply reduction and there was no significant difference between TNBC cell lines and ER+ cell lines (Fig. 1B). The IC50 value of ER+ cell lines were nearly or above two-fold higher than TNBC cell lines after 24 h treatment, whereas after 7 days treatment the difference of IC50 value between ER+ and TNBC cell lines was diminished (Table 1). These results indicate that thiostrepton induces faster cell death in TNBC cell line and it needs higher concentration and longer time to inhibit the proliferation of ER+ cell lines. Drug sensitivity was also tested by colony formation assay. Cells were plated at low density and treated continuously with 5 μM or 10 μM of thiostrepton for 10 days. As shown by the quantification of the colony formation assay, treatment of thiostrepton resulted in dramatically inhibition of colony formation both in TNBC cell lines and ER+ cell lines (Fig. 1C). It suggests that after long term treatment ER+ cell lines are just as sensitive as TNBC cell lines or at least that the stem-like cells within these populations may have similar sensitivity. Nevertheless, the different results between MTT assay and colony assay may be due to the reason that the higher density of more differentiated ER+ cell line are potentially less sensitive to thiostrepton. Taken together, these findings suggest that thiostrepton is a potent agent that effectively targets TNBC cell lines.

Thiostrepton reduces the CD44+/CD24- stem-like population in TNBC cell lines

Previous study has shown that TNBC often has a greater stem cell-like phenotype and a higher proportion of CD44+/CD24- breast cancer stem cell population [7]. We analyzed the two surface makers in TNBC cell lines. In consistent with previous study, MDA-MB-231 and SUM149 cells were enriched for CD44+/CD24- cells, with more than 90% and 70% respectively expressing this surface phenotype (Fig. 2A). Based on the results that TNBC cells were highly sensitive to thiostrepton treatment, we then investigated if thiostrepton had effect on the CD44+/CD24- stem cell-like population in TNBC cells. SUM149 and MDA-MB-231 cells were treated with thiostrepton at 10 μM and 20 μM concentrations for 24 h, after which cells were treated for stem cell marker expression. *In vitro* thiostrepton treatment of TNBC cells caused a significant decrease in the proportion of CD44+/CD24- cells in a concentration dependent manner (Fig. 2A). In the meantime, CD24+ cells were increased to 45.1% and 22.0% at the dose of 20 μM respectively in SUM149 and MDA-MB-231 cells (Fig.

Table 1. The IC50 value of different cell line by treatment with Thiostrepton. Breast cancer cell lines were treated with increasing dose of thiostrepton from 0 to 20 μM over a time course of 24 h or 7 days. IC50 values were calculated from the growth-inhibitory curves

Cell line	24h IC50 (μM)	7 Days IC50(μM)
SUM149	3.82	2.90
BT549	4.74	3.03
MDA-MB-231	6.32	3.14
MCF-7	12.25	5.14
T47D	13.58	3.96

Fig. 1. The effect of thiostrepton on the proliferation of breast cancer cell lines. (A) The morphologic change of MDA-MB-231, BT549, and MCF-7 cells following 24h treatment with 0, 10, 20 μmol/L thiostrepton. (B) MDA-MB-231, SUM149, BT549, MCF-7, and T47D cell lines were treated with thiostrepton (0 - 20 μmol/L) for 24 h or 7 days. Cell viability was determined by MTT assay. (C) The sensitivity of cells to thiostrepton was tested by colony-forming assay. Cell lines were treated continuously with 0, 5, 10 μM thiostrepton for 10 days; media was changed every 3 days. Each experiment was carried out in triplicate. Columns, mean; bars, ±SD. * $P < 0.01$, ** $P < 0.01$, *** $P < 0.001$. Representative wells were shown.

2B). The phenomenon that CD24⁻ cells were induced into CD24⁺ cells following thiostrepton treatment indicated that CD24⁻ cells were sensitive to thiostrepton treatment.

Thiostrepton suppresses mammosphere formation of TNBC cell lines

To determine whether the loss of the stem cell marker expression actually reflected loss of stemness associated with differentiation, we employed *in vitro* suspension culture system to examine the effects of thiostrepton on mammosphere formation. The TNBC cell lines were seeded in suspension culture media. Primary mammospheres were grown in suspension for 7 to 10 days, after which primary mammospheres were dissociated into single cells and seeded in non-adherent culture system for more 10 days in the presence

Fig. 2. Treatment of thiostrepton on the stem cell marker expression of triple-negative breast cancer cell lines. Flow cytometric analysis for cell surface phenotypes (CD44/CD24) was done in SUM149 and MDA-MB-231 cells following treatment with 0, 10, 20 μmol/L. (A) Graphs show percentage of CD44+/CD24- and CD44-/CD24+ cells in two cell lines. Columns, mean; bars, ±SD. * $P < 0.05$, *** $P < 0.001$. (B) Representative flow cytometric images were shown.

or absence of thiostrepton. As shown in Fig. 3A and 3B, Thiostrepton treatment caused a dramatic reduction in the number and size of mammospheres (Fig. 3C). As mammospheres are enriched for cancer stem cells, these results suggest that thiostrepton inhibits the growth of BCSC which results in significantly repressed mammosphere formation in TNBC cell lines.

Thiostrepton inhibits sonic hedgehog signaling pathway in triple-negative breast cancer cell lines

Previous studies have reported that SHH signaling plays a critical role in the regulation of CSC [16, 17]. In breast cancer, SHH ligand activation or Gli-1 expression promotes self-renewal of mammary stem cells [10]. Given the phenomenon that thiostrepton significantly reduced BCSC population, we hypothesized that SHH signaling pathway might be regulated by thiostrepton. To test this hypothesis, we analyzed SHH ligand and Gli-1 expression in different breast cancer cell lines. SHH ligand was highly expressed in two TNBC cell lines except for BT549, reflecting a different activation mode of SHH signaling (Fig. 4A). SUM149

Fig. 3. Effects of thiostrepton on secondary mammosphere formation of TNBC cell lines. Monolayer cells were enzymatically disaggregated to a single-cell suspension and plated in nonadherent culture medium. Primary mammospheres were grown in suspension for 10 days, after which primary mammospheres were dissociated into single cells and seeded in nonadherent culture system for more 10 days in the presence or absence of thiostrepton. (A) The box plot graph shows the mammospheres size data from three independent experiments. (B) The number of mammospheres was counted and percentage of MFU (mammosphere forming unit) number was calculated. Columns, mean; bars, $\pm$ SD. *** $P < 0.001$. (C) Representative images were shown, Bar: 100 μ m.

and MDA-MB-231 cells were treated with increasing doses of thiostrepton from 0 – 20 μ M for 24 h. As shown in Fig. 4B and 4C, FOXM1, a known thiostrepton target, was down-regulated upon treatment, indicative of active pharmacological action of thiostrepton. There was a pronounced decrease in SHH level after thiostrepton treatment (Fig. 4B and 4C). A significant SHH reduction was observed at a concentration of 10 μ M, suggesting that SHH could be one of the primary cellular targets of thiostrepton. Moreover, we found that the level of nanog, a key regulator of stem cell, was obviously down-regulated by thiostrepton treatment. It is notable that there were no significant changes in the level of Gli-1, a downstream transcription factor of Hh signaling. To further validate the inhibition effects on the SHH signaling by thiostrepton, the expression of SHH signaling targeted gene was measured in MDA-MB-231 cells. Cells were treated with increasing doses of thiostrepton from 0 to 20 μ M for 24h before harvesting for Real-time PCR analysis (Fig. 4D). The expression of FOXM1, N-MYC, and CCND1, all well-known downstream target of Hh signaling, significantly decreased in a dose dependent manner upon treatment. Previous study reported that Gli-1 nuclear location was considered a valuable marker of Hh pathway activation. We were curious to determine if Gli-1 localization was influenced by thiostrepton. Gli-1 was mainly nuclear before treatment and became cytoplasmic after thiostrepton treatment (Fig. 4E). This result further validated the inhibition effect on SHH signaling pathway by thiostrepton. Together these results suggest

Fig. 4. Thiostrepton inhibits SHH signaling pathway in triple-negative breast cancer cell lines. (A) Protein lysates were prepared from breast cancer cell lines grown at 80% confluency in plate. Expression levels of indicated proteins were analyzed by western blotting. Densitometric evaluation was performed by image J software and represented as a histogram. (B, C) Breast cancer cell lines were treated with thiostrepton at the indicated concentration for 24 h, after which protein lysates were prepared and analyzed by western blotting using specific antibodies. Densitometric evaluation was performed by image J software and represented as a histogram. (D) MDA-MB-231 cells were treated with indicated concentration of thiostrepton (0 - 20 μmol/L; 24 h), after which mRNA was isolated and determined by RTQ-PCR with specific primer. (E) MDA-MB-231 cells were treated with 5 μM or 10 μM of thiostrepton, after which immunofluorescence assay was applied to detect Gli-1 staining with specific antibody. Propidium iodide was used to detect nuclear DNA. Merged images of Gli-1 and nuclear staining were also shown.

inhibition of SHH signaling pathway is at least one of the important pharmacological actions of thiostrepton.

Thiostrepton decreases CD44+/CD24- stem-like population via down-regulation of SHH ligand

In the above experiment, we found that thiostrepton inhibited SHH signaling pathway and reduced CD44+/CD24- stem-like population in TNBC. Hitherto, the role of SHH ligand in maintaining CD44+/CD24- stem-like population of TNBC cells has not yet been established, we then sought to assess the effect of silencing of SHH ligand on CD44 and CD24 expression and mammosphere forming ability. Double strand SHH-antisense siRNA and control siRNA were transfected in MDA-MB-231 cells. As anticipated, SHH ligand level was decreased in antisense-expressing cells compared to negative control. Western blot analysis showed that SHH knockdown reduced the level of nanog (Fig. 5A), a well-known genes involved in CSC self-renewal, indicating that SHH ligand might modulate BCSC self-renewal through the regulation of key factors responsible for the function of CSC. To validate the functional role of SHH ligand in BCSC, we performed *in vitro* mammosphere formation assay. The result showed that siRNA of SHH resulted in a significant reduction in mammosphere forming unit (MFU) (Fig. 5B, $P < 0.01$). Moreover the stem cell marker analysis showed that the CD24+ cell population were increased to 14.1% (si-SHH-1) and 15.9% (si-SHH-2) in siRNA cell line compared with control cell line (Fig. 5C). To further examine the potential role of SHH ligand in the pharmacological action of thiostrepton, we over-expressed SHH ligand in MDA-MB-231 cells. The expression level of the ectopic SHH ligand remained relatively constant in the overexpression cell line compared to parental cell line in which SHH ligand was down-regulated after 10 μ M thiostrepton treatment (Fig. 5D). Over-expression of SHH ligand significantly prevented thiostrepton-mediated loss of cell viability by 33.7% (5 μ M) and 27.4% (10 μ M) respectively (Fig. 5E). We also observed that the effect of thiostrepton on CD44+/CD24- stem-like population was partly rescued in the SHH ligand overexpression cells (Fig. 5F). In conclusion, these results indicate that SHH pathway plays an important role in the maintenance of CD44+/CD24- stem-like population in TNBC cell lines. The down-regulation of SHH ligand is at least one of the important mechanism that leads to attenuated CD44+/CD24- stem-like population.

Discussion

In this study we reported for the first time that thiazole antibiotic thiostrepton suppressed CD44+/CD24- stem-like population and sphere-forming capacity through targeting SHH signaling pathway in TNBC cell lines. Notably, we found that thiostrepton inhibited SHH signaling pathway through down-regulation of SHH ligand and reduction of nuclear Gli-1. Furthermore, we showed that SHH ligand was important for maintaining CD44+/CD24- stem-like population in TNBC cell lines.

TNBC is reported to have characteristics of more frequent recurrence, chemoresistance and shorter survival than ER+ breast cancer [18, 19]. As a result, TNBC patients are unlikely to benefit from currently available targeted systemic therapy [20]. Strikingly, we found that the thiazole antibiotic thiostrepton effectively inhibited the cell growth of TNBC cell lines. After 24h exposure to thiostrepton, all three TNBC cell lines undergone sharply reduced cell viability compared to two ER+ cell lines. When cells were treated for 7 days the growth of TNBC and ER+ cell lines were both greatly inhibited. These results indicate that thiostrepton induces faster cell death in TNBC cell line and thiostrepton is an effective agent that targeted TNBC cells.

Cancer cells from TNBC often display a profile of cell surface markers that similar to that of BCSC, characterized by the phenotype CD44+/CD24- in which CD44 is expressed at high levels but levels of CD24 are low or undetectable [21, 22]. The unsatisfactory response of the TNBC to current chemotherapy and their intimate link with the CD44+/CD24- phenotype

Fig. 5. Gene silencing and overexpression of SHH ligand in MDA-MB-231. (A) SHH ligand was silenced by two target RNA sequences and one negative control sequence in MDA-MB-231 cells. Western blot was used to confirm silencing effects. (B) After treated by two siRNA sequences or negative control, MDA-MB-231 cells were tested by sphere forming assay. (C) After treatment as B, MDA-MB-231 cells were analyzed for cell surface marker (CD44/CD24) using Flow cytometric analysis. (D) MDA-MB-231 cells were transfected with SHH ligand overexpression (+) or control vector (-). Both cells were treated with thioestrepton for 24h at the dose of 5μM or 10μM. The expression of SHH ligand was confirmed by western blot. GAPDH was used as loading control. (E) After treatment as D, cell viability was determined by MTT assay. (F) After treatment as D, cell surface marker (CD24/CD44) was analyzed by Flow cytometric analysis. Representative flow cytometric images are shown. Each experiment was carried out in triplicate. Columns, mean; bars, ±SD. * P < 0.05, ** P < 0.01.

make CD44/CD24 targeting an attractive therapeutic alternative for TNBC patients. Consistent with earlier reports, we find that TNBC cell lines: SUM149 and MDA-MB-231 show more than 70% of cells expressing the CD44+/CD24- phenotype. Strikingly, *in vitro* thioistrepton (20 µmol/L) treatment of TNBC cells caused > 20% decrease in the proportion of CD44+/CD24- cells (Fig. 2A). In breast cancer, CD44 is positively associated with stem cell-like characteristics and CD24 expression is related to differentiated epithelial features. The decrease of CD24- cells indicates that CD24- cells were sensitive to thioistrepton treatment. In cancer stem cell model, therapies that promote differentiation significantly improve survival [23, 24]. It was reported that some infant tumors underwent spontaneous differentiation that leads to a favorable outcome even without therapy [25]. The observations from our group suggest that targeting differentiation marker CD24 may be an effective strategy to treat TNBC. Furthermore, thioistrepton remarkably suppressed mammosphere formation of TNBC cell lines *in vitro* suspension culture system, indicating that thioistrepton effectively inhibited BCSC number and activity of TNBC *in vitro*. Our observation that thiazole antibiotic thioistrepton effectively inhibited BCSC activity also consistent with a recent intriguing findings in which antibiotics were found to selectively target CSC in multiple tumor types [26].

Identification of specific target for TNBC patients remains an important clinical challenge. Evidence has revealed the significant roles of SHH signaling in TNBC [9, 27-29]. In a clinical sample immunohistochemistry analysis the expression of SHH ligand and Gli-1 in TNBC was significantly increased in comparison to non-triple-negative breast cancer (nTNBC) [9]. Another group recapitulated the finding that SHH ligand over-expression in a mouse model of basal breast cancer increased growth and induced a poorly differentiated phenotype [30]. We analyzed SHH ligand and Gli-1 expression in different breast cancer cell lines. SHH ligand was highly expressed in two TNBC cell lines except for BT549, reflecting a different activation mode of SHH signaling. Indeed, except for autocrine, a paracrine mode of SHH signaling has also been described in the basal-like subtype of breast cancer: the non-malignant stromal secrete SHH ligand, leading to SHH pathway activation in tumour cells. This communication between epithelial and stromal compartments plays an important role in breast cancer progression [31]. Interestingly, we found that SHH ligand protein level underwent dramatic decrease at the dose of 10 µM thioistrepton treatment, suggesting that SHH ligand may be a key target of thioistrepton. Previous study reported that thioistrepton inhibited proteasome which results in the stabilization of certain proteins that proves fatal to cancer cells [32]. This is unlikely to be the global action of thioistrepton because in our study we observed not only SHH ligand but also nanog, a key regulator of stem cell, were obviously down-regulated rather than stabilized by thioistrepton. It is intriguing to investigate if SHH ligand could be also suppressed by other proteasome inhibitors, such as Siomycin A, MG132 and bortezomib, etc. in the future. The inhibition effect of thioistrepton on SHH signaling pathway was further validated by the dose-dependent, rapid, and sustained loss of mRNA transcript level of certain target genes of SHH pathway, including CCND1 and N-Myc. Hitherto, the pharmacological action of thioistrepton is not well understood. Evidence has shown that thioistrepton affects the expression of FOXM1 at the gene promoter level and not through de novo protein synthesis because thioistrepton fails to repress an ectopic FOXM1 driven by a CMV promoter [5, 33]. It is interesting to note that FOXM1 has been shown to be an important downstream transcriptional target of Gli-1 [34]. Given the observation in our study, it raises the possibility that thioistrepton suppresses SHH signaling pathway which leads to reduced FOXM1 mRNA level. Although we cannot exclude the possibility that other mechanisms have involved, our study shed light on the potential mechanism of thioistrepton on FOXM1 expression. Moreover, we find that the SHH transcription factor Gli-1 nuclear localization is reduced following thioistrepton treatment. Since Gli-1 nuclear location is considered the activation of SHH pathway [35-37], this phenomenon further validated the inhibition effect of thioistrepton on SHH signaling pathway. Taken together, our study suggested that the inhibition of SHH signaling by thioistrepton might contribute to its anticancer activity.

Targeting stem cell related signaling pathway is supposed to be an effective therapeutic strategy for treatment of TNBC. SHH signal pathway has been shown to play a pivotal role in the maintenance of self-renewal of mammary stem cells [10]. Activation of hedgehog signaling increases breast cancer mammosphere initiating cell number, whereas inhibition of the pathway results in a reduction of these effects. Consistent with the inhibition effect of SHH signaling pathway by thioistrepton, we find that RNA silencing of SHH ligand also attenuates the CD44+/CD24- stem-like population and sphere-forming ability (Fig. 5B and 5C). In addition, over-expression of SHH ligand partly rescues the thioistrepton-mediated loss of CD44+/CD24- population. These results suggest that SHH ligand plays a pivotal role in the maintenance of CD44+/CD24- population. Although we cannot exclude the possibility that thioistrepton suppresses CD44+/CD24- stem-like population and sphere-forming ability through an as yet unknown molecular mechanism, our results indicate that down-regulation of SHH ligand is an important mechanism that leads to decreased CD44+/CD24- population by thioistrepton treatment.

Our findings that SHH signaling pathway is targeted by thioistrepton has important therapeutic implications. To date, there are already a number of clinical trials employing Hh inhibitor, including natural compound cyclopamine and synthetic small molecules in pancreatic, colorectal and breast cancer [38]. However, possible concerns have derived from the poor knowledge of tolerance and efficacy of inhibiting these mechanisms without inducing severe side effects. Some clinical trials have been reported to be suspended owing to side effects of Hh inhibitor [28]. As a natural antibiotic, thioistrepton has already used in veterinary medicine and exhibits no toxicity on normal breast cancer cell line MCF-10A [5, 39]. Thus the toxic side effects of thioistrepton-based therapy would be minimized. Moreover, thioistrepton has been reported recently to be successful delivered by nanoparticles in human breast cancer xenografts models to significantly reduce tumor burden [6]. Our findings suggest that thioistrepton is efficacious for targeting TNBC cell lines through the inhibition of SHH signaling pathway. Further work is required to assess whether this is true *in vivo*.

Overall, we report that thioistrepton effectively suppresses cell growth and CD44+/CD24- stem-like population of TNBC cell lines via inhibition of SHH signaling pathway. Down-regulation of SHH ligand protein level might be one of the mechanisms whereby thioistrepton exerts its cytotoxic effect on TNBC cells. We also show that SHH ligand plays an important role in the maintenance of CD44+/CD24- stem-like population in TNBC cell lines. Our results give a new insight into the mechanism of thioistrepton anti-tumor activity and provide evidence for using thioistrepton as an effective therapeutic method to treat SHH over-expression TNBC.

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Disclosure Statement

The authors declare no competing financial interest.

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