In silico identification of thiostrepton as an inhibitor of cancer stem cell growth and an enhancer for chemotherapy in non-small-cell lung cancer

Tse-Hung Huang1,2,3,4,5 | Alexander T. H. Wu6 | Tai-Shan Cheng7 | Kuan-Ting Lin8 | Chia-Jou Lai7 | Hao-Wen Hsieh9 | Peter Mu-Hsin Chang10,11 | Cheng-Wen Wu9,12 | Chi-Ying F. Huang7,9,13 | Kuan-Yu Chen14

1Department of Traditional Chinese Medicine, Chang Gung Memorial Hospital, Keelung, Taiwan
2School of Traditional Chinese Medicine, Chang Gung University, Taoyuan, Taiwan
3School of Nursing, National Taipei University of Nursing and Health Sciences, Taipei, Taiwan
4Graduate Institute of Health Industry Technology, Chang Gung University of Science and Technology, Taoyuan, Taiwan
5Research Center for Chinese Herbal Medicine, Chang Gung University of Science and Technology, Taoyuan, Taiwan
6The Ph.D. Program for Translational Medicine, College of Medical Science and Technology, Taipei Medical University, Taipei, Taiwan
7Institute of Biopharmaceutical Sciences, National Yang-Ming University, Taipei, Taiwan
8Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA
9Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan
10Department of Oncology, Taipei Veterans General Hospital, Taipei, Taiwan
11Faculty of Medicine, National Yang-Ming University, Taipei, Taiwan
12Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan
13Department of Biochemistry, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
14Division of Pulmonary Medicine, Department of Internal Medicine, National Taiwan University Hospital and College of Medicine, National Taiwan University, Taipei, Taiwan

Abstract
Cancer stem cells (CSCs) play an important role in cancer treatment resistance and disease progression. Identifying an effective anti-CSC agent may lead to improved disease control. We used CSC-associated gene signatures to identify drug candidates that may inhibit CSC growth by reversing the CSC gene signature. Thiostrepton, a natural cyclic oligopeptide antibiotic, was the top-ranked candidate. In non-small-cell lung cancer (NSCLC) cells, thiostrepton inhibited CSC growth in vitro and reduced protein expression of cancer stemness markers, including CD133, Nanog and Oct4A. In addition, metastasis-associated Src tyrosine kinase signalling, cell migration and epithelial-to-mesenchymal transition (EMT) were all inhibited by thiostrepton. Mechanistically, thiostrepton treatment led to elevated levels of tumour suppressor miR-98. Thiostrepton combined with gemcitabine synergistically suppressed NSCLC
1 | INTRODUCTION

Lung cancer is the leading cause of cancer-related death worldwide. The five-year survival rate is approximately 18% for patients with lung cancer and 3.9% for those with advanced stages of the disease, lower than the survival rates for colon (65%), female breast (90%) and prostate (99%) cancers. Non–small-cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers. Chemotherapies, including pemetrexed or gemcitabine in combination with platinum, are frequently used as the first-line therapy for advanced NSCLC. However, current chemotherapy regimens do not provide long-term disease control in most patients with NSCLC. Those who receive chemotherapy eventually encounter drug resistance and disease progression. Alternative therapeutic strategies are urgently needed.

Cancer stem cells (CSCs) play an important role in cancer recurrence, progression and drug resistance. Lung CSCs were first identified as CD133+ cells and isolated in established NSCLC cell lines, forming floating spheres in serum-free conditions. These CD133+ lung cancer spheres exhibited self-renewal abilities, stress/drug resistance, epithelial-to-mesenchymal (EMT) potential and the ability to recapitulate tumour heterogeneity in vivo. The drug resistance may be due to features related to the stem cell pathway, expression of high-level ATP-binding cassette transporters and specific surface biomarkers. Identifying agents to eliminate CSCs has become an important issue for anti-cancer drug development.

Developing an entirely new drug is expensive and time-consuming. Drug repurposing offers an alternative strategy for anti-cancer drug development, which requires analysing biological and medical information for a huge number of drugs. The Connectivity Map (CMap, http://www.broad.mit.edu/cmap/), which stores expression profiles of diseases, genes and chemicals, provides a tool for making inferences based on a query and the internal profiles. We hypothesized that drugs with the ability to reverse the expression of CSC-like gene signatures may inhibit CSC growth and could prove beneficial in the treatment of lung cancer. We tested the top-ranked agent identified by CMap for reversing potential CSC gene signatures for anti-CSC abilities in vitro and in vivo. The synergistic effect of identified agents and existing chemotherapies on tumour growth inhibition was also examined.

Thiostrepton, a macrocyclic thiopeptide antibiotic, belongs to the thiopptide family of highly modified macrocyclic peptides, which are produced as secondary metabolites by actinomycetes of the genus Streptomyces. Thiostrepton interacts directly with forkhead box M1 (FOXM1) and inhibits binding with genomic target sites. Thiostrepton also inhibits growth and induces apoptosis in human cancer cells by inhibiting FOXM1 expression. Based on CMap analysis, we identified thiostrepton as a candidate to be an anti-CSC agent. We also provided evidence that thiostrepton can suppress cancer cell proliferation, migration and CSC-like properties in vitro, as well as inhibit tumorigenesis in vivo. More importantly, thiostrepton combined with gemcitabine inhibited NSCLC cell growth synergistically. Collectively, our findings suggest thiostrepton can serve as an anti-CSC drug and play a beneficial role in lung cancer treatment.

2 | MATERIALS AND METHODS

2.1 | Cell lines and chemicals

A549, NCI-H441 and CL141 are lung adenocarcinoma cell lines with wild-type EGFR. CL152 is a lung squamous carcinoma cell line. H1299 is an NSCLC cell line, and H460 is large cell lung carcinoma cell line. A549-ON cells are A549 cells co-overexpressed Oct-4A and Nanog. H460 cells were infected by a CD133 P1 promoter-driven GFP reporter lentivirus and cultured in RPMI-1640 supplemented with 10% foetal bovine serum (FBS) (GIBCO). CD133+GFP+H460 cells were sorted by using a FACSArria cell sorter (BD Biosciences). The other cell lines were maintained in RPMI medium and supplemented with 10% FBS (GIBCO), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO). For cell culture experiments, a 10 mmol/L thiostrepton stock solution was dissolved in dimethyl sulfoxide (DMSO; Sigma). Pemetrexed, gefitinib and gemcitabine were purchased from LC Laboratories, while thiostrepton and penicillin and 100 μg/mL streptomycin were purchased from Sigma and Selleckchem, respectively.

2.2 | L1000 expression profiling

Gene expression profiles were obtained from cancer cells treated with perturbagens, including small molecules and Chinese herbal medicines, in triplicate for 6 hours, followed by L1000 expression profiling by Genometry Inc. In brief, cells were lysed after 6 hours of treatment in triplicate, and mRNA transcripts from whole cell lysates were rapidly captured by oligo-DT. The CDNAs generated by reverse transcription from mRNA were amplified through
ligation-mediated polymerase chain reaction (PCR). The PCR ampli-
con was then hybridized to bar-coded Lumixx beads to display the
expression values of specific genes. The cDNA was annealed with
specific probes for 978 landmark genes. A list of overexpressed and
underexpressed probe sets was obtained using a t test rank order.

2.3 Connectivity scoring by gene set enrichment analysis using CMap

Intensity values of gene expression profiles were first converted
to robust z-scores using the llktools downloaded from the
CMap/Library of Integrated Network-based Cellular Signatures
(LINCS) project of the NIH Common Fund programme. Integrated
Network-based Cellular Signatures has been replaced by the CLUE
platform as of February, 2017. The new analytical tool can be ac-
cessed from https://clue.io. A Perl script was used to calculate the
connectivity score for each gene expression profile against the
anti-CSC or CSC gene signature. The anti-CSC gene signature
was identified using GEO2R for differentially expressed genes in
the study of, for example, the Gene Expression Omnibus gene
signature GSE18150.

The 11,641 profiles in our collection were ranked by connectiv-
ity scores and used as the input of the ranking matrix for gene
set enrichment analysis (GSEA). Thiostrepton profiles (including
repeats and treatments in different cancer cells) were grouped to-
gether and used as the input gene set for GSEA to map the ranking
matrix and calculate the enrichment scores. Normalized enrichment
scores were obtained from 1000 permutations of gene sets. Some
of the gene signatures (eg GSE18931) were obtained as CSC gene
signatures. We scored the negative enrichment. Finally, for CMap/
LINCS analysis, the query-gene signature was uploaded to LINCS
Web Apps to obtain score_best4 scores of the perturbagens in the
database.

2.4 Colony formation assay

Non-small-cell lung cancer cells were seeded in 6-well plates at a
density of 600 cells per well and cultured for 14 days. Thiostrepton
was added 24 hours after seeding. The culture medium with thi-
ostrepton was renewed every 4 days. Following the treatments,
cells were washed with phosphate-buffered saline (PBS), and
the colonies were fixed in a methanol-acetic acid fixing solution
in methanol. After carefully removing the crystal violet solution
and rinsing with tap water, the colonies were counted manually.
Each experiment was performed independently, in triplicate, at least
twice.

2.5 Cytotoxicity assay and drug combination analysis

Cells were seeded in 96-well plates at a density of 2000 cells per
well in triplicate. The cells were treated with indicated agents for
48 hours on the second day to ensure adequate plating efficiency
and cell vitality. Cells were treated with different concentrations of
thiostrepton, pemetrexed, cisplatin, gemcitabine and gefitinib or a
non-fixed-ratio combination of thiostrepton and one of the anti-can-
cer agents.

The cytotoxicity was assessed by using a sulforhodamine B (SRB)
assay. Briefly, the medium was discarded, and the adherent cells
were fixed with 100 μL of cold 10% trichloroacetic acid (w/v) in each
well for 1 hour at 4°C. Cells were stained after fixing with 100 μL
well of 0.4% (w/v, in 1% acetic acid) SRB solution for 30 minutes at
room temperature and then washed five times with 1% acetic acid.
After air-drying, 100 μL of 10 mmol/L Tris base was added to each
well and the absorbance was read at 546 nm. Cytotoxicity was de-
\[\text{standard deviation.}\]

The synergy associated with inducing cytotoxicity among dif-
ferent drug combinations was evaluated by analysis of the med-
ian-dose effect and calculation of the combination index (CI) using
commercially available software of Chou and Talalay software
(CompuSyn). According to the recommendations of this meth-
ology, CI values of less or greater than 1 indicated synergism or
antagonism, respectively. A value of 1 indicated an additive effect.

2.6 Cell migration assay

Cell culture inserts (Millipore) were placed in 24-well plates. Serum-
starved cells (2 × 10^5 cells) were seeded in the upper chambers of
the transwell with 200 μL of serum-free medium, in the presence
of the vehicle (DMSO) or thiostrepton (5 μmol/L). The lower cham-
bers were filled with 750 μL of medium containing 10% FBS as a
chemo-attractant. After incubation for 16 hours, cells were fixed in
4% formaldehyde and stained with GIEMSA (Sigma). After washing,
the non-penetrating cells on the inner surfaces of the upper cham-
bers were wiped off with cotton swabs. The penetrated cells were
photographed and counted using a light microscope. Each assay was
performed in triplicate.

2.7 Immunoblotting

After treatments, cells were lysed in a lysis buffer. Total pro-
tein contents were isolated and subjected to SDS polyacrylamide
gel electrophoresis and electro-transfered onto polyvinylidene
fluoride membranes (Millipore). Immunoblotting was performed
using primary antibodies, including β-catenin (Cell Signaling
Technology), c-Myc (Cell Signaling Technology), CD44 (Cell Signaling
Technology), CD133 (GeneTex), Oct-4A (GeneTex), Sox2 (Cell
Signaling Technology), FOXM1 (Abcam), E-cadherin (Cell Signaling
Technology), c-Myc (Cell Signaling Technology), CD44 (Cell Signaling
Technology), CD133 (GeneTex), Oct-4A (GeneTex), Sox2 (Cell
Signaling Technology), FOXM1 (Abcam), E-cadherin (Cell Signaling
Technology), Src (Cell Signaling Technology), phosphorylated
Src (Tyr416, Cell Signaling Technology), caspase-8 (Cell Signaling
Technology), caspase-9 (Cell Signaling Technology), caspase-3 (Cell
Signaling Technology) and cleaved PARP (Cell Signaling Technology), GAPDH (Cell Signaling Technology), α-tubulin (Cell Signaling Technology), or β-actin (GeneTex) acted as an internal control. Protein detection was performed by using an enhanced chemiluminescence (ECL™) method and the Luminescence Imaging System (LAS-4000™, Fuji Photo Film Co., Ltd).

2.8 | Assays for cancer stemness characteristics

The assay of ALDH1 activity is frequently used to define lung cancer stem cell populations. Aldefluor assays were performed according to the manufacturer’s guidelines (StemCell Technologies). Briefly, a single cell suspension obtained from cell cultures was incubated in Aldefluor assay buffer containing an ALDH substrate (bodipy-aminoacetaldehyde, BAAA) for 50 minutes at 37°C. As a negative control, a fraction of cells from each sample was incubated under identical conditions in the presence of an ALDH inhibitor (diethylaminobenzaldehyde, DEAB). Flow cytometry was used to detect the ALDH-positive cell population.

A tumour sphere formation assay was carried out to evaluate cancer stemness. Lung cancer cells were seeded in 6-well ultralow-attachment plates (Corning Inc) at a density of 2000 cells/mL in a serum-deprived culture medium consisting of DMEM/F12 supplemented with 1% N2 Supplement (GIBCO), 20 ng/mL basic fibroblast growth factor (Sigma), 20 ng/mL epidermal growth factor (GIBCO), 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO) at 37°C in a humidified atmosphere of 95% air and 5% CO2. Tumour spheres were counted after harvest using a Countess™ (GIBCO) automated cell counter.

2.9 | Quantitative PCR analyses and microRNA assays

All RNA-related experiments were performed by using kits purchased from QIAGEN (Taiwan) and following the instructions provided by the vendor. Total RNA was isolated, quantified and reverse transcribed into cDNA. The primers of different microRNAs tested in this study for microRNA quantitative real-time PCR assays were also purchased from QIAGEN. Up-regulation and down-regulation of miRNAs were performed by transfecting cells with miRNA precursors or anti-miRNAs, respectively. Fifty nanomoles of miScript mimic and inhibitors were transfected into lung cancer cells by using the lipofectamine 2000 reagent (Thermo Fisher Scientific). Total RNA and protein were isolated 48 hours after treatments to determine the effects on target-protein expression profiles.

2.10 | Examination of anti-lung cancer effects mediated by thiostrepton in vivo

The inhibitory effect of thiostrepton on tumour growth was evaluated by using a mouse subcutaneous tumour xenograft model. Human lung adenocarcinoma cells (NCI-H441, purchased from ATCC) were injected subcutaneously in the right flank of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (female, 4-6 weeks old) at 105 cells/injection. Once the tumour became palpable, the tumour-bearing mice were randomly assigned to a thiostrepton group (5 mg/kg, 5 days/wk, intraperitoneal injection) or a control group (DMSO vehicle). Over a period of 4 weeks, the tumour sizes in both groups were measured weekly with standard calipers. The in vivo tracking of tumour growth was then determined and presented as the fold change in tumour volume over time. All animal experiments were approved by the Taipei Medical University Animal Center (Protocol number: LAC-2013-0086). The animals were humanely killed by cervical dislocation to minimize the suffering. All tumour samples were harvested for further analyses.

3 | RESULTS

3.1 | Identification of thiostrepton as a potential anti-CSC agent using the Connectivity Map database

We compared 20 different published data sets (Table S1) and employed CSC-related gene expression profiles as inputs to query 11 641 L1000-based gene expression profiles. Thiostrepton was identified as a candidate that could significantly reverse lung cancer gene signatures (Figure 1A). As an example, GSE18150 was originally generated from cells treated with DZNep by disrupting EZH2 and impairing CSC self-renewal. Thiostrepton profiles, including treatments in different cancer cells in triplicate, were grouped together and used as the input gene set for GSEA to map the ranking matrix and calculate an enrichment score. The anti-CSC gene signature from GSE18150 and cancer cells treated with thiostrepton had a strong enrichment score in our 11 641 L1000 assays (normalized enrichment score = 1.914, P-value <.0001, Figure 1B). The majority of thiostrepton profiles had a positive connectivity score, and only a few had a score of zero. This indicated a strong positive connectivity between thiostrepton and the anti-CSC gene signature, meaning thiostrepton is a potential anti-CSC agent.

When the anti-CSC gene signature GSE18150 determined by GEO2R was used to query the CMap 2.0 (a database containing more than 1.3 million gene expression profiles), thiostrepton was ranked as the top candidate (enrichment score = 0.797, P-value <.001; Figure 1B). In results from the query of the LINCS database, which contained 6100 gene expression profiles, thiostrepton was ranked as the 22nd among drugs with positive similarity scores (score_best4 = 97.368).

The human normal mammary stem cell (hNMSC) gene expression signature GSE18931, a CSC gene signature, can stratify biological and molecular features in tumours (Table S2). The degree of cells expressing hNMSC markers may reflect the CSC content of a tumour, L1000 profiles with a negative connectivity score (normalized enrichment score = −2.0146; P-value <.0001; false discover rate <.00001) indicated that thiostrepton treatment may reverse gene signatures of GSE18931. Similarly, when GSE18931 was used to query the LINCS database, the results showed that thiostrepton had a score_best4 of −93.176 among the compounds with negative
similarity scores. This strong concordance across different pertur‐bagen databases indicated that thiostrepton is likely to have anti‐CSC abilities.

In addition, our GSEA showed that thiostrepton has significant connectivity with the anti‐EMT gene signature of salinomycin (GSE17215) with specific toxicity for epithelial CSCs\(^\text{35}\) (normalized enrichment score = 1.436; \(P\)-value = .005, Figure 1C). Further validation was demonstrated by the up-regulated expression of E‐cadherin and down-regulated expression of Slug and vimentin in thiostrepton‐treated CL141 cells (Figure 1D). Moreover, treatment with thiostrepton not only distinctly reduced FOXM1 expression but also reduced vimentin and Slug expression and inhibited Src and AKT activation in CL141 cells in a dose‐dependent manner (Figure 1E).

### 3.2 Thiostrepton suppresses NSCLC cell growth, clonogenicity and migration

Non‐small‐cell lung cancer cells were treated with thiostrepton. Figure 2A,B shows that thiostrepton suppressed cellular viability and colony‐forming ability in a dose‐dependent manner. The half‐maximal inhibitory concentration (IC\(_{50}\)) of thiostrepton...
in the clonogenic assay was approximately 0.05 μmol/L, which was distinctly lower than that of cytotoxic effects (Table S3). In addition, treatment with thiostrepton significantly reduced the migration capability of A549, CL141, CL152 and H1299 cells (Figure 2C).

A previous study demonstrated enhanced sphere-forming ability, cisplatin resistance and migration ability in CD133+ H460 cells.26 To verify the potential anti-CSC effect of thiostrepton, we used H460 cells expressing GFP driven by a CD133 promoter for cytotoxicity analysis. Both parental H460 and CD133+ H460 cells were then subjected to an SRB assay. We found that the CD133+ H460 cells (IC50 = 1.7 μmol/L) were more sensitive to thiostrepton than the parental H460 cells (IC50 = 6.9 μmol/L; Table S3).

3.3 Thiostrepton suppresses CSC properties in NSCLC cells

For in vitro validation, tumour spheres of CL141 cells were generated under serum-free conditions and CSC properties were examined in these tumour spheres. By using an Aldefluor assay, we found CL141 tumour spheres were enriched with ALDH1+ cells (S: 1.2%) compared with their parental counterparts (P: 0.5%, Figure 3A), and the relative ALDH activity was significantly increased (Figure 3B). The expression of stem cell-related markers, including CD44, Oct-4A, Sox2 and c-Myc, was also up-regulated in CL141 tumour sphere cells (Figure 3C).

Thiostrepton treatment prominently suppressed CL141 tumour spheres formation in a dose-dependent manner (Figure 3D). This observation was supported by thiostrepton treatment in both parental cells and spheres. Treatment with thiostrepton reduced the expression of CSC markers, including CD133, Oct-4A and Nanog, as well as suppressed metastasis-associated Src signalling (Figure 3E).

Furthermore, a stable CSC-like A549-ON cells was used to validate the anti-CSC and anti-EMT ability of thiostrepton. Thiostrepton treatment significantly decreased expression of Oct-4A and Nanog and up-regulated expression of E-cadherin (Figure 3F), supporting the hypothesis that thiostrepton is a potential anti-CSC agent.

3.4 Thiostrepton and gemcitabine synergistically suppresses NSCLC tumour sphere formation

Thiostrepton in combination with gemcitabine, cisplatin, pemetrexed or gefitinib reduced NSCLC cell viabilities synergistically (Figure 4A). The obtained average CI values from thiostrepton in combination with gemcitabine, gefitinib, cisplatin and pemetrexed were 0.41 ± 0.16, 0.80 ± 0.19, 0.91 ± 0.25 and 1.04 ± 0.30, respectively. This indicates that thiostrepton plus gemcitabine showed the best synergistic effect among these combinations. In addition, the CI values in different ratios (1:1, 1:2, 1:4 and 1:8) all showed synergistic effects in the CL141, CL152 and H1299 cells (Figure 4B).

CD133 and Oct-4A, the CSC markers, have been suggested as protectors for cancer cells from apoptosis induced by chemotherapeutic agents.25,26,36 Significantly increased apoptosis-related proteins–cleaved caspase-3 and cleaved PARP—were observed in cells treated with gemcitabine plus thiostrepton (Figure 4C). Apoptosis induced by gemcitabine plus thiostrepton was more significant compared with that induced by gemcitabine or thiostrepton alone (Figure 4C).
Furthermore, the combination of thiostrepton and gemcitabine suppressed expression of stemness markers, such as Sox2, β-catenin and EMT transcription factor Slug in both CL141 and CL152 cells (Figure 4D), indicating that the combination of thiostrepton and gemcitabine suppresses stemness- and EMT-related profiles in both adenocarcinoma and squamous cell carcinoma cells. This finding suggests thiostrepton could reduce cancer stem-like cell populations and decrease the risk of distant metastasis.

We also characterized the combination treatment on CL141 parental cells and tumour spheres. Thiostrepton, with or without gemcitabine, was able to suppress CD133 expression, whereas gemcitabine treatment did not (Figure 4E). Importantly, CL141 spheres responded to a lower concentration of thiostrepton (1 µmol/L), as reflected by significantly decreased CD133 levels (Figure 4E). In the presence of thiostrepton, CL141 spheres also responded to gemcitabine at a lower concentration and underwent apoptotic cell death as reflected by the elevated level of cleavage of PARP.

### 3.5 Thiostrepton inhibits NSCLC tumorigenesis and decreases the proportion of CSC in vivo

NOD/SCID mice that received daily thiostrepton treatment (n = 5) exhibited a significantly slower tumour growth rate than mice receiving the vehicle treatment (n = 5) at the fifth week,
as demonstrated by approximately 2-fold smaller in tumour size (Figure 5A). The body weight of the mice did not change significantly during the treatment period (Figure 5B). Photographs of harvested tumour samples showed that thiostrepton treatment significantly suppressed tumour growth compared with control counterparts (Figure 5C). Further analysis of the tumour samples revealed that thiostrepton-treated tumours contained a significantly lower proportion of CD133+ cells (5.6%) compared with those of the control group (16.1%) (Figure 5D).

3.6 Thiostrepton treatment was associated with an increase in tumour suppressor miR-98

MicroRNAs have been extensively characterized and identified in lung cancer and reportedly to play an important promoter or suppressor role, depending on their target genes.37-39 We examined a panel of microRNAs in response to thiostrepton treatment in CL141 cells, and the miR-98 level appeared to be elevated by approximately 1.5-fold (Figure 6A). By comparison, CL141 parental cells contained a significantly higher level of intrinsic miR-98 than their sphere counterparts (Figure 6B). The level of miR-98 rose in the wake of thiostrepton treatment in both CL141 parental cells and spheres (Figure 6B).

By increasing miR-98 (mimic molecules) levels, Sox2, Oct-4A and β-catenin were suppressed in both CL141 and CL152 cells (Figure 6C). This was similar to the response to thiostrepton treatment in CL141 cells. Based on the decreased expression of Slug, EMT was considered reversed (Figure 6C). These findings suggest that the suppression of CSC characteristics and EMT by thiostrepton is through up-regulation of tumour suppressor miR-98.

4 DISCUSSION

In this study, thiostrepton was identified as a potential drug for converting embryonic stem cell-like gene signatures to those of adult stem cell gene signatures. Through in vitro and in vivo experiments, we demonstrated that thiostrepton effectively inhibited lung CSC growth by the suppressing cancer stemness and expression of EMT genes. A combination of thiostrepton and gemcitabine synergistically
suppressed NSCLC cell growth. This exploratory methodology connecting disease, genes and chemical profiles represents an alternative approach to the development of novel therapeutic agents.

In tumour sphere assays, increased EMT potential and expression of CSC markers were showed in NSCLC cells. This finding is in agreement with the previously established hypothesis that enhanced EMT potential is positively associated with the generation of CSCs. In addition, the well-established CSC marker, CD133, was shown to be associated with up-regulated expression of EMT-related genes, including Src and Slug. A recent study reported that FOXM1 directly and constitutively activates Snail in lung adenocarcinoma, thereby promoting cancer metastasis. Knockdown FOXM1 significantly suppressed EMT progression as well as tumour growth and metastasis. However, we demonstrated that thioestrepton treatment did not inhibit Snail expression but strongly inhibited Slug expression in NSCLC cells. These findings indicate that the CD133/Src axis, in addition to FOXM1, is a potential therapeutic target for thioestrepton.

Ectopic expression of Oct4 and Nanog in lung cancer cells was associated with a significant increase in the percentage of CD133+ cell and mesenchymal cell populations, the ability to form tumour spheres and enhanced drug resistance. To provide further support for the contention that thioestrepton is a potential anti-CSC agent, we utilized a cell model that A549 cells with overexpression of Oct-4A/Nanog exhibits enhanced CSC characteristics and EMT potential. Thioestrepton treatment not only significantly suppressed ectopic expression of both Nanog and Oct-4A in the A549-ON cells, but also markedly induced epithelial marker E-cadherin. These findings verify our bioinformatical prediction that thioestrepton treatment is associated with the reversal of CSC gene signatures, EMT status and CSC growth inhibition.

Thioestrepton reportedly enhances sensitivity to platinum in vitro and in vivo. It has also been reported that cisplatin treatment significantly increases the proportion of CD133+ cells through the Notch signal pathway, and CD133+ cells are related to an increase in cross-drug resistance to paclitaxel and doxorubicin. Therefore, the reduction in CD133+/Oc4-4A+ cell populations in NSCLC by thioestrepton treatment (Figure 3) provides further support for a role for thioestrepton in combination with chemo- and/or target therapeutic agents in preventing drug resistance. Thioestrepton acted synergistically with gemcitabine to inhibit lung cancer cellular viability in this study.

The GSEA results of thioestrepton and thioestrepton plus gemcitabine with concomitant enrichment scores were related to GSE18931 (Table S2), which was evaluated for CSC gene signatures with impacts on clinical outcomes and the pathological features of cancer. The gene signature of thioestrepton treatment with a positive enrichment score was also significantly related to GSE17215, an anti-CSC gene signature associated with EMT. Decreased CSCs
after drug treatment may prevent tumours from EMT. This finding provides evidence to link the CSC state with EMT. Thiostrepton may follow the same path as salinomycin in reverting M-type cells back to E-type cells. Chemotherapy combined with thiostrepton may therefore be an alternative regimen to improve treatment effectiveness and prolong survival in patients with NSCLC.

Chemotherapy is still a major treatment option for lung squamous cell carcinoma. Thiostrepton in combination with gemcitabine significantly inhibited lung squamous cell carcinoma growth. However, cellular responses towards different treatment regimens varied between adenocarcinoma and squamous cell carcinoma cells. As shown in Figure 4D, thiostrepton alone significantly suppressed Sox2, β-catenin and Slug expression to a greater extent in CL141 cells than in CL152 cells. While gemcitabine treatment alone appeared to suppress Sox2 expression more significantly in CL152 cells, the suppressive effect of stemness and EMT was more pronounced (Figure 4D). Our previous study showed that thiostrepton can suppress colony and sphere formation and trigger apoptosis of colorectal cancer stem cells in HCT-15 and HT-29 cells as well as EMT and chemo-resistant clones derived from them. These findings indicate that thiostrepton in combination with chemotherapies may improve treatment effectiveness in lung squamous cell carcinoma.

In this study, an increased level of miR-98 (a tumour suppressor) was observed in response to thiostrepton treatment. Because miR-98 is reportedly down-regulated in many cancer types, its targets, such as Myc, Kras and Wnt signalling, are generally oncogenic. The exact targets for thiostrepton-induced miR-98 induction in NSCLC warrant further investigation. In conclusion, thiostrepton was identified as a potential CSC inhibitor for lung cancer using an integrative bioinformatics and pre-clinical approach. In combination with chemotherapeutic agents, especially gemcitabine, thiostrepton synergistically suppressed NSCLC growth and tumour spheres formation. An elevated miR-98 level was associated with thiostrepton treatment. These pre-clinical evidence warrants further clinical studies for thiostrepton in patients with NSCLC.

ACKNOWLEDGEMENTS
This study was partially supported by grants from the Ministry of Science and Technology of Taiwan (MOST107-2320-B-182A-007-) and Chang-Gung Memorial Hospital Research Foundation (CMRPG2G0331 and CMRPG2G0332) to Tse-Hung Huang, by grant from 102CM-TMU-03 to Alexander T. H. Wu, by grants from the Ministry of Science and Technology of Taiwan (MOST107-2320-B-010-040-MY3), the Veterans General Hospitals and University System of Taiwan Joint Research Program (VGHUST108-G1-4-1) and NYMU-FEMH Joint Research Program (106DN20) to Chi-Ying F. Huang, and by grants from the Ministry of Science and Technology of Taiwan (MOST106-2314-B-002-102-MY3) to Kuan-Yu Chen.

CONFLICT OF INTEREST
The authors have no conflict of interest.

AUTHOR CONTRIBUTIONS
Tse-Hung Huang, Chi-Ying F. Huang and Kuan-Yu Chen designed research; Alexander T. H. Wu, Tai-Shan Cheng, Kuan-Ting Lin, Chia-Jou...
Lai, Hao-Wen Hsieh and Peter Mu-Hsin Chang analysed data; Tse-Hung Huang, M. Alexander T. H. Wu, Tai-Shan Cheng, Kuan-Ting Lin, Chia-Jou Lai, Hao-Wen Hsieh and Peter Mu-Hsin Chang performed research; Tse-Hung Huang, Alexander T. H. Wu, Tai-Shan Cheng, Chi-Ying F. Huang and Kuan-Yu Chen wrote the paper; Tse-Hung Huang, Peter Mu-Hsin Chang and Cheng-Wen Wu contributed new reagents or analytic tools.

DATA AVAILABILITY STATEMENT

The data sets generated and analysed during this study are available from the corresponding author on reasonable request.

ORCID

Chi-Ying F. Huang https://orcid.org/0000-0003-4898-4937

REFERENCES

1. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA: Cancer J Clin. 2014;64(1):9-29.
2. DeSantis CE, Lin CC, Mariotto AB, et al. Cancer treatment and survivorship statistics, 2014. CA: Cancer J Clin. 2014;64(4):252-271.
3. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin. 2018;68:7-30.
4. Stinchcombe TE, Socinski MA. Current treatments for advanced stage non-small cell lung cancer. Proc Am Thorac Soc. 2009;6:233-241.
5. Gronberg BH, Brennes RM, Flotten O, et al. Phase III study by the Norwegian lung cancer study group: pemetrexed plus carboplatin compared with gemcitabine plus carboplatin as first-line chemotherapy in advanced non-small-cell lung cancer. J Clin Oncol. 2009;27:3217-3224.
6. Sadowska AM, Nowe V, Janssens A, Boeykens E, De Backer WA, Geronpre PR. Customizing systemic therapy in patients with advanced non-small cell lung cancer. Ther Adv Med Oncol. 2011;3:207-218.
7. Schiller JH, Harrington D, Belani CP, et al. Eastern cooperative oncology G. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med. 2002;346:92-98.
8. Scagliotti GV, Parikh P, von Pawel J, et al. Phase III study comparing cisplatin plus gemcitabine with cisplatin plus pemetrexed in chemotherapy-naive patients with advanced-stage non-small-cell lung cancer. J Clin Oncol. 2008;26:3543-3551.
9. Galluzzi L, Vitale I, Michels J, et al. Systems biology of cisplatin resistance: past, present and future. Cell Death Dis. 2014;5:e1257.
10. Shanker M, Willcutts D, Roth JA, Ramesh R. Drug resistance in lung cancer. Lung Cancer (Auckl). 2010;1:23-36.
11. Bao S, Wu Q, Mclendon RE, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature. 2006;444:756-760.
12. Diehn M, Cho RW, Lobo NA, et al. Association of reactive oxygen species levels and radioresistance in cancer stem cells. Nature. 2009;458:780-783.
13. Clevens H. The cancer stem cell: premises, promises and challenges. Nat Med. 2011;17:313-319.
14. Eramo A, Lotti F, Sette G, et al. Identification and expansion of the tumorigenic lung cancer stem cell population. Cell Death Differ. 2008;15:504-514.
15. Pirozzi G, Tirino V, Camerlingo R, et al. Epithelial to mesenchymal transition by TGFbeta-1 induction increases stemness characteristics in primary non small cell lung cancer cell line. PLoS ONE. 2011;6:e21548.
16. Leung EL, Fiscus RR, Tung JW, et al. Non-small cell lung cancer cells expressing CD44 are enriched for stem cell-like properties. PLoS ONE. 2010;5:e14062.
17. Wang P, Gao Q, Suo Z, et al. Identification and characterization of cells with cancer stem cell properties in human primary lung cancer cell lines. PLoS ONE. 2013;8:e57520.
18. Wu Y, Wu PY. CD133 as a marker for cancer stem cells: progresses and concerns. Stem Cells Dev. 2009;18:1127-1134.
19. Chen K, Huang YH, Chen JI. Understanding and targeting cancer stem cells: therapeutic implications and challenges. Acta Pharmacol Sin. 2013;34:732-740.
20. Chen PC, Liu X, Lin Y. Drug repurposing in anticancer agent development. Comb Chem High Throughput Screening. 2017;20(5):395-402.
21. Lamb J, Crawford ED, Peck D, et al. The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. Science. 2006;313:1929-1935.
22. Bagley MC, Jaye DW, Merritt EA, Xiong X. Thiopeptide antibiotics. Chem Rev. 2005;105:685-714.
23. Hegde NS, Sanders DA, Rodriguez R, Balasubramanian S. The transcription factor FOXM1 is a cellular target of the natural product thiostrepton. Nature chemistry. 2011;3:725-731.
24. Bhat UG, Halasi M, Cartel AL. Thiazole antibiotics target FoxM1 and induce apoptosis in human cancer cells. PLoS ONE. 2009;4:e5592.
25. Chou SH, Wang ML, Chou YT, et al. Coexpression of Oct4 and Nanog enhances malignancy in lung adenocarcinoma by inducing cancer stem cell-like properties and epithelial-mesenchymal transdifferentiation. Cancer Res. 2010;70:10433-10444.
26. Liu YP, Yang CJ, Huang MS, et al. Cisplatin selects for multidrug-resistant CD133+ cells in lung adenocarcinoma by activating Notch signaling. Cancer Res. 2013;73:406-416.
27. Wawer MJ, Li K, Gustafsdottir SM, et al. Toward performance-diverse small-molecule libraries for cell-based phenotypic screening using multiplexed high-dimensional profiling. Proc Natl Acad Sci USA. 2014;111:10911-10916.
28. George J, Lim JS, Jang SJ, et al. Comprehensive genomic profiles of small cell lung cancer. Nature. 2015;524:47-53.
29. Suva ML, Riggi N, Janiszewska M, et al. EZH2 is essential for glioblastoma cancer stem cell maintenance. Can Res. 2009;69:9211-9218.
30. Pece S, Tosoni D, Confalonieri S, et al. Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. Cell. 2010;140(1):62-73.
31. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity testing. Nat Protoc. 2006;1:1112-1116.
32. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul. 1984;22:27-55.
33. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev. 2006;58:621-681.
34. Ginestier C, Hur MH, Charafe-Jauffret E, et al. ALDH1 is a marker of breast cancer stem cells: therapeutic implications and challenges. Cell Stem Cell. 2009;4:555-567.
35. Gupta PB, Onder TT, Jiang G, et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell. 2009;138:645-659.
36. Safa AR. Resistance to cell death and its modulation in cancer stem cells: therapeutic implications and challenges. Crit Rev Oncog. 2016;21:203-219.
37. Inamura K, Ishikawa Y. MicroRNA in lung cancer: novel biomarkers and concerns. Adv Enzyme Regul. 2006;46:41-50.
38. Levy B, Hu Z, Cordova KN, Close S, Lee K, Becker D. Clinical utility of liquid diagnostic platforms in non-small cell lung cancer. Oncologist. 2016;21:1121-1130.
39. Maltby S, Plank M, Tay HL, Collison A, Foster PS. Targeting MicroRNA function in respiratory diseases: mini-review. Front Physiol. 2016;7:21.

40. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008;133:704-715.

41. Chen YS, Wu MJ, Huang CY, et al. CD133/Src axis mediates tumor initiating property and epithelial-mesenchymal transition of head and neck cancer. PLoS ONE. 2011;6:e28053.

42. Ding Q, Miyazaki Y, Tsukasa K, Matsumura S, Yoshimitsu M, Takao S. CD133 facilitates epithelial-mesenchymal transition through interaction with the ERK pathway in pancreatic cancer metastasis. Mol Cancer. 2014;13:15.

43. Wei P, Zhang N, Wang Y, et al. FOXM1 promotes lung adenocarcinoma invasion and metastasis by upregulating SNAIL. Int J Biol Sci. 2015;11:186-198.

44. Zhang X, Cheng L, Minn K, et al. Targeting of mutant p53-induced FoxM1 with thiostrepton induces cytotoxicity and enhances carboplatin sensitivity in cancer cells. Oncotarget. 2014;5:11365-11380.

45. Ju SY, Huang CY, Huang WC, Su Y. Identification of thiostrepton as a novel therapeutic agent that targets human colon cancer stem cells. Cell Death Dis. 2015;6:e1801.

46. Ni R, Huang Y, Wang J. miR-98 targets ITGB3 to inhibit proliferation, migration, and invasion of non-small-cell lung cancer. Onco Targets Ther. 2015;8:2689-2697.

47. Yang G, Zhang X, Shi J. MiR-98 inhibits cell proliferation and invasion of non-small cell carcinoma lung cancer by targeting PAK1. Int J Clin Exp Med. 2015;8:20135-20145.

48. Jiang P, Wu X, Wang X, Huang W, Feng Q. NEAT1 upregulates EGCG-induced CTR1 to enhance cisplatin sensitivity in lung cancer cells. Oncotarget. 2016;7(28):43337-43351.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Huang T-H, Wu ATH, Cheng T-S, et al. In silico identification of thiostrepton as an inhibitor of cancer stem cell growth and an enhancer for chemotherapy in non–small-cell lung cancer. J Cell Mol Med. 2019;23:8184-8195. https://doi.org/10.1111/jcmm.14689