A novel cell-based assay for the high-throughput screening of epithelial–mesenchymal transition inhibitors: Identification of approved and investigational drugs that inhibit epithelial–mesenchymal transition

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ABSTRACT

Objectives: Lung cancer with distant metastases is associated with a very poor prognosis, and epithelial-mesenchymal transition (EMT) contributes to cancer metastasis. Therefore, elucidation and inhibition of EMT signaling in lung cancer may be a new therapeutic strategy for improving the prognosis of patients. We constructed a high-throughput screening system for EMT inhibitors. Using this system, we aimed to identify compounds that indeed inhibit EMT.

Materials and methods: We generated a luciferase reporter cell line using A549 human lung cancer cells and E-cadherin or vimentin as EMT markers. EMT was induced by transforming growth factor β1 (TGF-β1), and candidate EMT inhibitors were screened from a library of 2,350 compounds. The selected compounds were further tested using secondary assays to verify the inhibition of EMT and invasive capacity of cells.

Results: Values obtained by the assay were adjusted for the number of viable cells and scored by determining the difference between mean values of the positive and negative control groups. Four compounds were identified as novel candidate drugs. Among those, one (avagacestat) and two compounds (GDC-0879 and levothyroxine) improved the expression of E-cadherin and vimentin, respectively, in epithelial cells. GDC-0879 and levothyroxine also significantly inhibited the invasive capacity of cells.

Conclusion: We systematically screened approved, investigational, and druggable compounds with inhibitory effects using a reporter assay, and identified candidate drugs for EMT inhibition.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths in the United States of America (USA). In 2021, approximately 235,760 new cases and 131,880 lung cancer-related deaths were reported in the USA. Non-small cell lung cancer (NSCLC) accounts for approximately 80–85 % of lung cancer cases [1]. More than half (55 %) of patients with NSCLC in the USA have advanced (stage IV) disease and distant metastases outside the pleura and lungs [2]. Lung cancer with distant metastases is associated with a very poor prognosis versus disease without distant metastases (5-year survival rate: 6 % vs 59 %, respectively) [1].

Therefore, the prevention and treatment of metastasis is an unmet clinical need.

Epithelial–mesenchymal transition (EMT) is induced during cancer progression and contributes to the formation of metastatic colonies. It is the process by which epithelial cells abolish their differentiating characteristics and acquire mesenchymal traits. In this process, cancer cells acquire metastatic properties by increasing their mobility, invasiveness, and resistance to apoptosis. Furthermore, EMT-derived tumor cells acquire stem cell characteristics and become resistant to therapy. Thus, lung cancer therapy targeting the EMT pathway is a potentially promising strategy [3].

We previously conducted a comparative study of postoperative lung cancer resection specimens, using E-cadherin and vimentin as markers of EMT. The results showed that the null EMT conversion group (positive for E-cadherin and negative for vimentin) had the best prognosis, while patients with EMT progression were linked to a worse prognosis [4]. In our lung cancer resection specimens, both microscopic vessel invasion and visceral pleural invasion, which are established prognostic factors after lung cancer resection, were associated with poor prognosis and EMT [5]. In addition, we showed that drugs for other diseases, such as statins for p53-positive lung cancer [6] and tranilast for transforming growth factor-β1 (TGF-β1)-induced EMT in lung cancer [7], were effective as EMT inhibitors. Based on the various mechanisms involved in EMT in lung cancer, we also suggested that EMT inhibitors may be among currently existing drugs [8].

TGF-β1 has been used in numerous studies as a major activator that can induce EMT in NSCLC cells [9]. In the tumor microenvironment, TGF-β is produced and secreted by cancer cells and various other types of cells, such as fibroblasts, macrophages, leukocytes, and endothelial cells [10].

Since the toxicity, pharmacokinetics, and drug-drug interactions of clinically approved and investigational drugs have been previously established, such compounds are often re-evaluated for the discovery of new therapeutic effects. There is a need to develop an infrastructure for physiologically relevant high-throughput screening (HTS) to ensure that re-evaluations are performed with high certainty and reliability. In recent years, HTS has become a popular method for drug discovery, drug development, and target identification because it can measure thousands of compounds in a single experiment and select those with useful biological activities and high efficiency [11]. However, HTS can produce non-physiological results because it often uses target proteins expressed from exogenously introduced promoters or measures actual activity with surrogate markers [12].

In this study, we generated a luciferase reporter cell line from A549 human lung cancer cells, using E-cadherin or vimentin as EMT markers. This reporter cell line was used in the HTS platform to evaluate the effect of treatment with TGF-β1 on E-cadherin and vimentin luciferase proteins.

2. Materials and methods

2.1. Development of reporter constructs

The pNL2.2[NlucP/Hygro] Vector (Promega Corporation, Madison, WI, USA) is a 5.0 kb plasmid vector containing an SV40 minimal virus promoter linked to a luciferase reporter gene. NanoLuc™ is a luminescent enzyme with very low molecular weight (19.1 kDa); however, this luciferase is 100-fold brighter than conventional ones and has high thermal stability. The luminescence reaction is adenosine triphosphate-independent and measured using a novel substrate (furimazine) to maintain a high level of luminescence for a prolonged period of time. Cells transfected with this pNL2.2 vector express high levels of luciferase, and this vector was used for HTS in this experiment.

The E-cadherin and vimentin promoters were separated by restriction enzymes (*Nhe*I *Xho*I and *Nhe*I BamhI, respectively; TaKaRa Bio, Shiga, Japan) from a TCGP-Puro lentiviral EMT reporter system. This system contained E-cadherin promoter-driven enhanced green fluorescent protein (eGFP) and vimentin promoter-driven mCherry (a kind gift from Dr. Kiyotsugu Yoshikawa).

The E-cadherin luciferase reporter construct was genetically engineered by inserting the excised E-cadherin promoter into the 5-terminal side of the luciferase reporter vector pNL2.2. A vimentin luciferase reporter construct was also generated using the same method. These constructs were confirmed by direct sequencing. In the E-cadherin promoter sequence, the region from 5 to 232 bases upstream of the first ATG in the E-cadherin gene (available in the Genome Data Viewer [GDV]: https://www.ncbi.nlm.nih.gov/genome/gdv/) was cloned and

connected in tandem. In the vimentin promoter sequence, the region from 70 to 1,601 bases upstream of the first ATG in the vimentin gene (available in the GDV: https://www.ncbi.nlm.nih.gov/genome/gdv/) was cloned.

2.2. Cell culture and transfection

A549 NSCLC cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum (FBS; HyClone; Thermo Fisher Scientific K.K., Kanagawa, Japan) and 100 U/ml penicillin/streptomycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in Dulbecco's modified Eagle's medium at 37 °C and 5 % CO$_2$ under humidified conditions. The cell lines were utilized within 20 passages.

The NanoLuc reporter cell line was created by introducing the E-cadherin and vimentin gene promoters into the upstream of the first coding exon of the NanoLuc reporter sequence in the pNL2.2 vector. This was obtained by co-transfecting A549 lung cancer cells with the luminescence vector pNL2.2 incorporating the E-cadherin or vimentin promoter using Viafect reagent (Promega Corporation). Transient transfection of A549 lung cancer cells was performed to confirm TGF-β reactivity. This process produced a stably transfected cell line.

2.3. Compound screening

The 2,350 compounds registered in the Chemical Library (2020) were provided by the Center for Drug Discovery Research, Kyoto University (Kyoto, Japan), diluted to 10 mM in dimethyl sulfoxide, and dispensed into 96-well plates (31 plates; 80 compounds/plate). This experiment was performed in duplicate.

In a plate, cells transfected with the E-cadherin luciferase reporter construct were seeded at 5,000 cells/ml and incubated for 24 h. As a negative control, only cells were seeded in four wells of row 1. Subsequently, as a positive control, cells with a low concentration (1 ng/ml) of TGF-β1 were seeded in the four wells of row 12. For the remaining 80 wells in rows 2 through 11, cells were seeded with a low concentration (1 ng/ml) of TGF-β1 and each of the 80 compounds. The compounds were diluted in culture medium to a final concentration of 10 μM, and these cell plates were incubated for 48 h. In another plate, cells transfected with the vimentin luciferase reporter construct were seeded at 8,000 cells/ml. The cells were treated in the same manner as that for the first plate.

Subsequently, the luciferase activity was measured using the Steady-Glo luciferase substrate (Promega Corporation) in a Glomax-Multi + detection System (Promega Corporation) photodetector.

For the cell viability assay, the non-cell-destructive CellTiter-Fluor (Promega Corporation) was used. Immediately prior to the measurement of luciferase activity, fluorescence was measured with a Glomax-Multi + detection System (Promega Corporation) photodetector to determine alterations in the number of viable cells induced by each compound. All luciferase activity measurements per chemical, obtained in each plate-by-plate experiment, were adjusted with the fluorescence measurements reflecting the number of viable cells obtained by the cell viability assay.

Compounds whose adjusted measurements of luciferase activity were close to those of the negative control (cells only) were defined as effective EMT inhibitor compounds.

The difference between the adjusted measurements of the positive control (cells treated with TGF-β1 only) and those of cells treated with low concentrations of TGF-β1 and each compound was determined. The difference between the adjusted measurements of the negative control and the adjusted measurements of the positive control was determined; also, the ratio of these differences was quantified to evaluate the effect of each chemical. The promising compounds with high scores were confirmed as EMT inhibitors at the protein level through western blotting. Furthermore, these findings were functionally confirmed using the
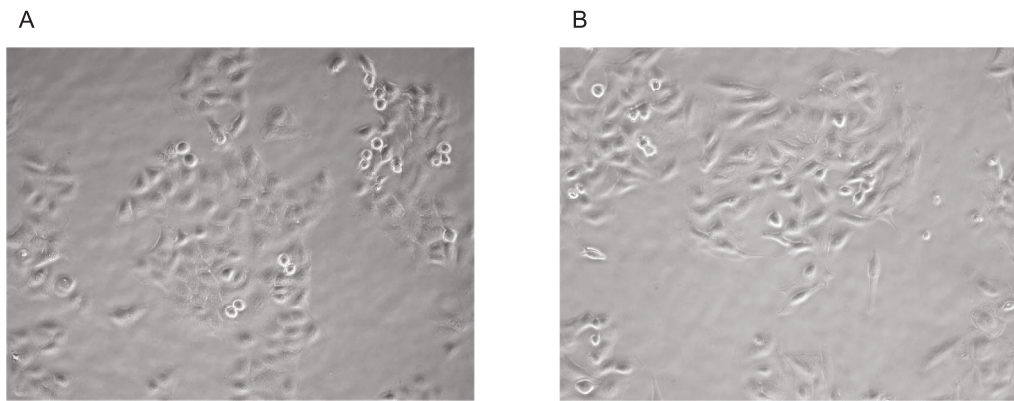
A

B

Fig. 1. A549 cells were cultured normally with or without transforming growth factor-β1 (TGF-β1) for 48 h. Normally cultured cells exhibited an epithelial phenotype (A), whereas those treated with TGF-β1 (1 ng/ml) presented a fibroblastic spindle shape (B).

invasion assay method.

2.4. Western blotting assay

Following incubation of A549 cells for 24 h, medium containing TGF-β1 (1 ng/ml) alone, or the screened candidate drugs (concentration: 0, 0.1, 1.0 and 10 μM) plus TGF-β1 (1 ng/ml) was added to the cultured cells, which were incubated for another 48 h.

Cells were incubated until they reached sub-confluency. Total cells were lysed using a radioimmunoprecipitation assay buffer containing protease inhibitor (25955-24; Nacalai Tesque, Kyoto, Japan), 0.1 % sodium dodecyl sulfate (#08714-04; Nacalai Tesque), and phosphatase inhibitor cocktail (1:100, #07575-51; Nacalai Tesque). Cell lysates were resolved by 20 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and proteins were transferred to polyvinylidene difluoride membranes.

The following primary antibodies were used: anti-E-cadherin (M106; TaKaRa Bio); anti-vimentin (#610921; BD Transduction Laboratories, Franklin Lakes, NJ, USA); anti-β-actin (#A5441; Sigma–Aldrich, St. Louis, MO, USA); Snail (#3879; Cell Signaling Technology Danvers, MA, USA); Slug (#mAb51772; Abcam plc Cambridge, UK); ZEB1 (#ab203829; Abcam plc); TWIST1(#46702; Cell Signaling Technology).

The membranes were blocked with 5 % FBS for 1 h at room temperature and incubated with primary antibodies at 4 °C overnight. Next, the membranes were washed with Tris-buffered saline and 0.1 % Tween 20 (Bio-Rad Laboratories, Hercules, CA, USA) and incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. The EzWestLumi Plus detection kit (ATTO, Tokyo, Japan) was used for protein visualization, and the LuminoGraph II imaging system (ATTO) was used for the detection of luminescence.

2.5. Immunofluorescence (IF) assay of EMT markers

The cells were seeded at 20,000 cells/well in a two-chamber slide, fixed in 4 % paraformaldehyde for 10 min at 37 °C, permeabilized with 0.1 % Triton X-100 for 15 min at room temperature (20–22 °C), and blocked in 2 % bovine serum albumin (Biosera, NUAILLE, France) in phosphate-buffered saline for 60 min at room temperature (20–22 °C). IF assays of E-cadherin and vimentin were performed manually using a mouse monoclonal primary antibody, namely anti-E-cadherin (#14472 diluted 1:400, Cell Signaling Technology Danvers, MA, USA), and a rabbit monoclonal primary antibody, namely anti-vimentin (#5741 D21H3 diluted 1:200, cell Signaling Technology), overnight at 4 °C. This was followed by incubation with Alexa Fluor 568 (#A10037 diluted 1:1000, Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 488 (#A21411 diluted 1:1000, Invitrogen; Thermo Fisher Scientific, Inc.) conjugated secondary antibodies. Fluorescent images were

captured using a BZ-X800 microscope (Keyence, Osaka, Japan).

2.6. Drug sensitivity assay

Cells were seeded in 96-well microplates at 5,000 cells/well and cultured at 37 °C for 24 h. Subsequently, the cells were incubated with increasing concentrations of each candidate drug from 0.01 μmol/l to 10 μmol/l for 48 h, followed by incubation with Cell Counting Kit-8 reagent (Dojindo Laboratories, Inc., Kumamoto, Japan) (10 μl) for 2 h. The absorbance was measured at 450 nm wavelength using Microplate Manager 6 (Bio-Rad Laboratories). The half-maximal inhibitory concentration (IC50) was calculated using the Prism 7 software (GraphPad Software, Inc. San Diego, CA, USA) with a three-parameter sigmoidal curve fit.

2.7. Cell invasion assay

A549 cells were incubated in medium containing TGF-β1 (1 ng/ml). This was followed by treatment with or without target drug (10 nM) and incubation for 48 h. Thereafter, cells were harvested for use in subsequent experiments. The harvested cells (100,000 cells/well) were suspended in 0.1 % FBS-containing medium and seeded onto Matrigel-coated membranes with 8-μm holes in the bottom of the upper chamber of the Matrigel Invasion Chamber (#354480;

Corning Incorporated, Corning, NY, USA). Medium containing FBS was added to the lower chamber as a chemoattractant. Candidate drugs were added to each chamber at the indicated concentrations, and the cells were incubated for 20 h at 37 °C in a 5 % CO_2 atmosphere. Subsequently, the non-invading cells were removed from the upper surface of the membrane using cotton-tipped swabs. The invading cells attached to the lower surface of the membrane were subsequently stained with Diff-Quick stain. The invasiveness of cancer cells was evaluated using a BZ-9000 microscope (Keyence). The number of infiltrated cells was counted by magnification in six random fields.

2.8. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA from A549 cells treated with or without TGF-β1 and with or without target drugs was extracted and reverse transcribed to cDNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Life Sciences, Pittsburgh, PA, USA) according to the instructions provided by the manufacturer. Each cDNA sample was mixed with THUNDERBIRD Probe qPCR Mix (Toyobo, Osaka, Japan) and a TaqMan Gene Expression Assay probe/primer set: E-cadherin (Hs01023895_m1; Thermo Fisher Scientific K.K.) and vimentin (Hs00958111 _m1; Thermo Fisher Scientific K.K.). These reactions were performed using a StepOnePlus Real Time IQ System (Thermo Fisher Scientific K.K.). The comparative cycle
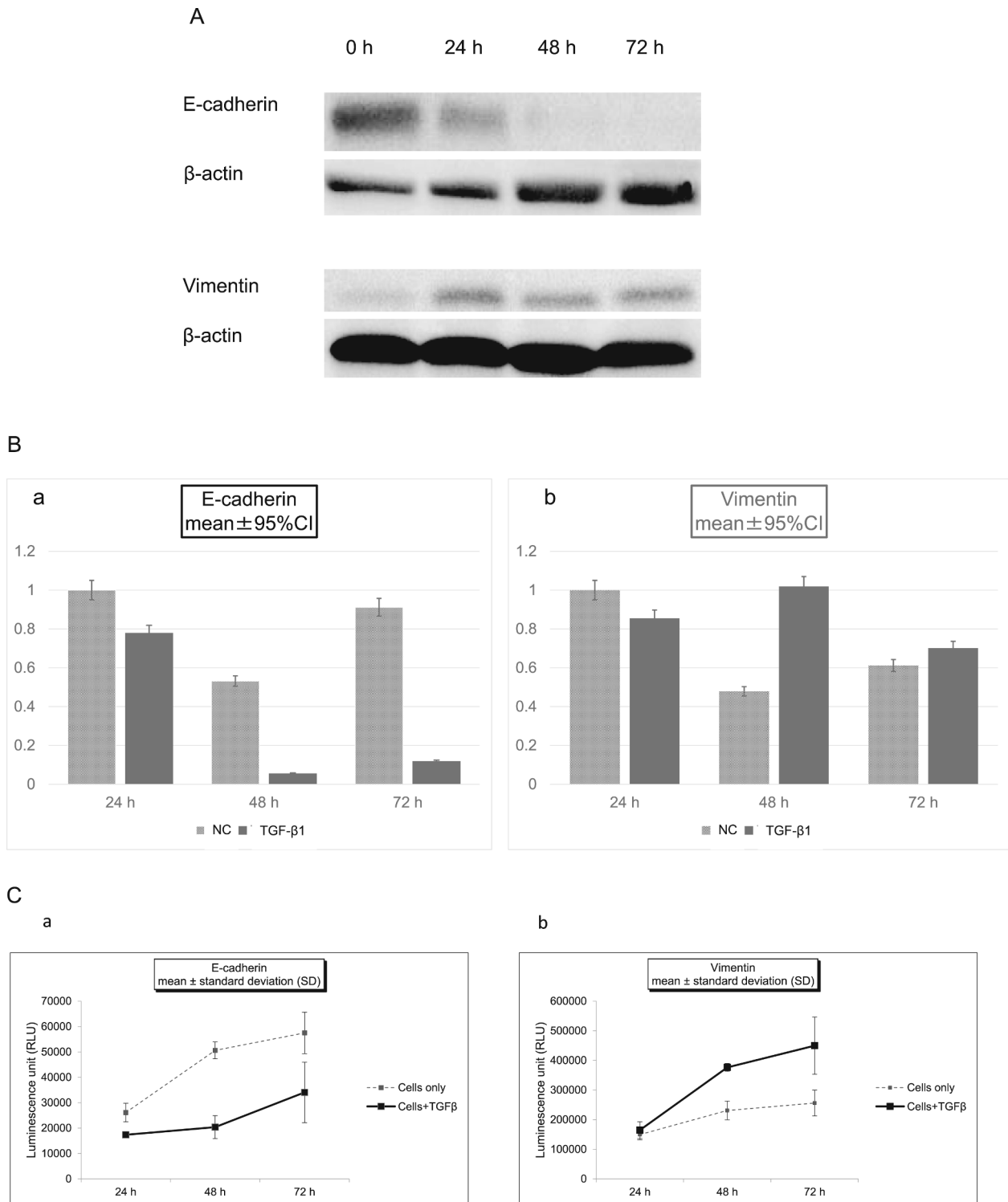
A

B

C

Fig. 2. (A) Western blotting analysis of E-cadherin for epithelial markers and vimentin for mesenchymal markers in A549 lung cancer cell lines cultured for 72 h. E-cadherin expression was decreased, whereas vimentin expression was increased. (B) Time-dependent changes in the mRNA expression levels of E-cadherin (a) and vimentin (b) induced by transforming growth factor-β1 (TGF-β1) in A549 cells. E-cadherin transcription was suppressed, whereas vimentin transcription was promoted. Data are expressed as the mean ± 95 % confidence interval (95 % CI) of values obtained from triplicate samples. (C) NanoLuc-A549 cells were transfected with the E-cadherin gene promoter or vimentin gene promoter and seeded into 96-well plates. The luminescence signals from cells were measured at 24, 48, and 72 h after seeding. Cell lines transfected with the E-cadherin gene promoter showed decreased signals (a). Cell lines transfected with the vimentin gene promoter showed increased signals (b). Data are expressed as the mean ± standard deviation (SD) of values obtained from four wells. NC, negative control; RLU, relative luminescence unit.
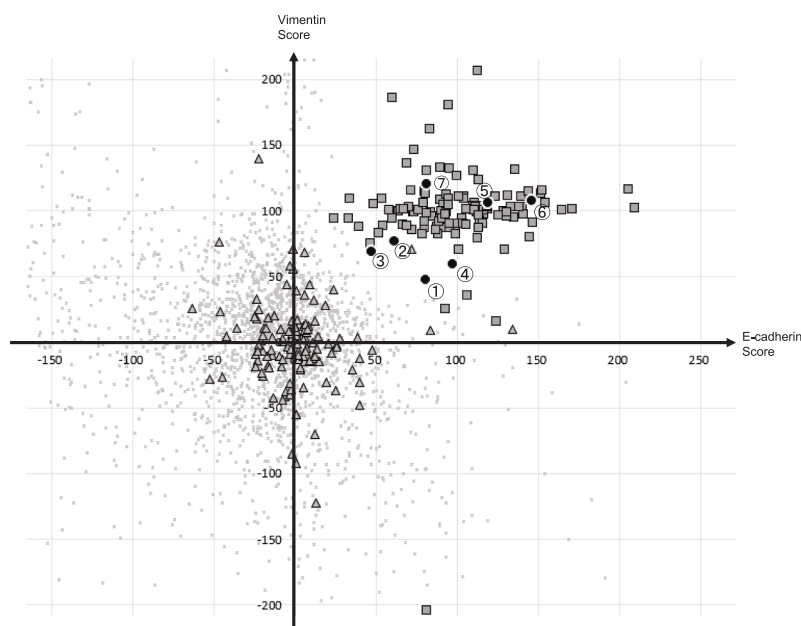
	Compound name	Pharmacological classification	Ecad score	Vim score
①	L-thyroxine	Thyroid hormone (T4)	80.5	47.8
②	Cladribine	Nucleic acid metabolism antagonist drug	61.1	76.6
③	GDC-0879	B-Raf inhibitor	47.6	68.5
④	BMS-708163 (Avagacestat)	γ-secretase inhibitor	97.3	58.9
⑤	SB-505124	TGF-β/Smad inhibitor	118.9	106.0
⑥	SB 431542	TGF-β/Smad inhibitor	146.3	107.8
⑦	GW788388	TGF-β/Smad inhibitor	81.3	120.0

Fig. 3. Compounds screened using NanoLuc-A549 lung cancer cells were scored and plotted. Four compounds and three transforming growth factor-β (TGF-β) inhibitors were identified. Scoring: (Data (DrugX) − TGF-β1(+) Ave.)/(TGF-β1(−) Ave. − TGF-β1(+) Ave.) × 100. Table 1 shows the names, score values, and pharmacological classification of these compounds. ● Compounds identified as hits. × Compounds not identified as hits. ▓ Positive control group. ▲ Negative control group.

threshold (Ct) (ΔΔCt) method was used to determine relative expression levels using β-actin as the internal control.

2.9. Statistical analyses

Data obtained from the cell invasion assay were expressed as the mean ± standard deviation of values derived from six random fields. Statistical analysis of the data was performed through one-way analysis of variance (ANOVA) using the JMP Pro 13 software (SAS Institute, Cary, NC, USA). The p-values < 0.05 denoted statistically significant differences.

3. Results

3.1. Induction of EMT in A549 cells by TGF-β1

EMT is a multifaceted system of phenotypic changes through which epithelial cells acquire mesenchymal features, such as alterations in polarity and cytoskeletal structure [13]. Inflammatory stimuli, including growth factors (e.g., TGF-β, hepatocyte growth factor [HGF], epidermal growth factor [EGF], WNT), hypoxia, and extracellular matrix components (e.g., collagen I), activate the EMT. It has been reported that EMT acts through developmental transcription factors (e.g., SNAIL, SLUG, TWIST, zinc finger E-box binding homeobox 1/2 [ZEB1/2], E2A protein, E12/E47) [14].

We compared the morphological changes of A549 lung cancer cells before and after treatment with TGF-β1. A549 lung cancer cells exhibited an epithelial phenotype in normal culture. However, after treatment with TGF-β1 (1 ng/ml) for 48 h, the cells maintained their spindle shape and grew in a scattered manner (Fig. 1). This morphological change is a phenotype of the EMT process in tumors [9,15].

Western blotting was performed to detect the expression levels of EMT-related proteins in A549 lung cancer cells transfected with E-cadherin or vimentin luciferase reporter constructs. The results showed that the expression levels of E-cadherin (an epithelial marker) were decreased, whereas those of vimentin (a mesenchymal marker) were

increased (Fig. 2A). We also assessed the mRNA levels of E-cadherin and vimentin in the presence and absence of TGF-β1. In A549 cells, the addition of TGF-β1 suppressed the transcription of E-cadherin and promoted that of vimentin (Fig. 2B). These results substantiate the EMT-inducing effect of TGF-β1 in A549 lung cancer cells transfected with the luciferase reporter construct.
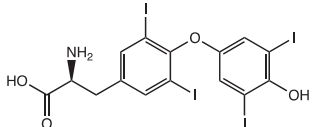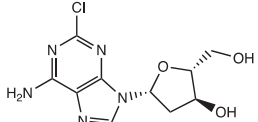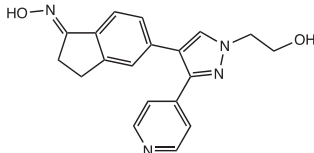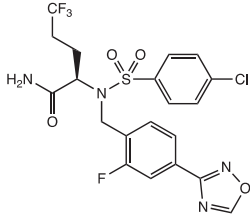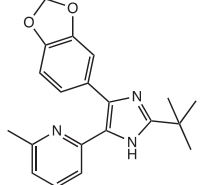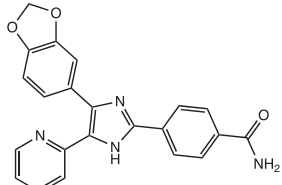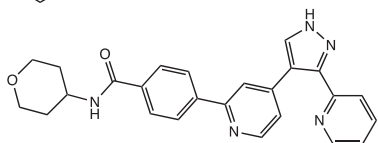
3.2. Reporter cell construction

NanoLuc-A549 cells transfected with the E-cadherin gene promoter (hereafter referred to as A549 type E) were treated with TGF-β1 (1 ng/ml). A decrease in luminescence compared with control was observed from 48 h. NanLluc-A549 cells transfected with the vimentin gene promoter (A549 type V) showed an increase in luminescence after 48 h of treatment (Fig. 2C).

3.3. Screening using a reporter assay system

In a 96-well plate containing 80 compounds, four wells were set with TGF-β1-untreated cells (negative control) and four wells were set with TGF-β1-treated cells (positive control). The mean values of the four negative and four positive control wells were set as score 100 and 0, respectively. The difference between each compound and the mean value of these controls was calculated for scoring.

Among all compounds, those with scores > 50 for both E-cadherin and vimentin were defined as candidate drugs (Supplementary Table). Exceptionally, one compound had an E-cadherin score of 47.6 and another compound had a vimentin score of 47.8. Seven compounds, which inhibited the attenuation of E-cadherin and the enhancement of vimentin, were identified (Fig. 3). Of those, three were established TGF-β inhibitors, while the remaining four (i.e., L-thyroxine, Cladribine, GDC-0879, and avagacestat) were novel candidate drugs as TGF-β-induced EMT inhibitors (Table 1).

Table 1
Summary of potency and efficacy values of the selected EMT inhibitors.

Compound name	Chemical structure	Pharmacological classification	Ecad score	Vim score
L-thyroxine		Thyroid hormone (T4)	80.5	47.8
Cladribine		Nucleic acid metabolism antagonist drug	61.1	76.6
GDC-0879		B-Raf inhibitor	47.6	68.5
BMS-708163 (Avagacestat)		γ-secretase inhibitor	97.3	58.9
SB-505124		TGF-β/Smad inhibitor	118.9	106.0
SB 431542		TGF-β/Smad inhibitor	146.3	107.8
GW788388		TGF-β/Smad inhibitor	81.3	120.0

Ecad, E-cadherin; EMT, epithelial–mesenchymal transition; L-thyroxine, levothyroxine; TGF-β, transforming growth factor-beta; Vim, vimentin.

3.4. EMT inhibition by each candidate drug

After culturing the cell lines in which EMT was induced by TGF-β1, we performed western blotting to evaluate whether these candidate drugs exhibit inhibitory ability for EMT. The results showed that only one drug, avagacestat, suppressed the decrease of E-cadherin. Moreover, GDC-0879 and levothyroxine (L-thyroxine) suppressed the increase of vimentin. None of the examined compounds suppressed both markers, and cladribine did not exert an effect on either of the markers (Fig. 4A). The expression levels of E-cadherin and vimentin in cultured cell lines in which EMT was induced by TGF-β1 were analyzed using IF. Addition of TGF-β alone was negative for E-cadherin and positive for vimentin. On the other hand, when each candidate drug was added together with TGF-β, E-cadherin was positive in avagacestat and weakly positive in L-thyroxine. Vimentin was negative in L-thyroxine and GDC and weakly negative in avagacestat (Fig. 4B). The influence of these candidate drugs on the viability of A549 lung cancer cells was examined using a drug sensitivity assay. The viability of A549 lung cancer cells was reduced by 10 μM cladribine, with a IC50 of 0.969 μM (Fig. 5A). Therefore, the expression levels of EMT-related proteins were re-evaluated by western blotting after treatment with different concentrations of candidate drugs. At 10 μM, levothyroxine (L-thyroxine) suppressed the increase of vimentin. Avagacestat suppressed both the decrease of E-cadherin and the increase of vimentin in a concentration-dependent manner. At 1.0 μM, cladribine suppressed both the decrease of E-cadherin and the increase of vimentin. GDC did not exert any inhibitory effect on EMT regardless of the concentration (Fig. 5B). E-cadherin plays an important role in the maintenance of the cell adhesion system, while vimentin acts as a cytoskeleton that changes into a spindle shape. Thus, following the occurrence of EMT, the invasive potential of tumor cells is enhanced. Therefore, compounds that control either of the two markers may functionally regulate cell invasion.
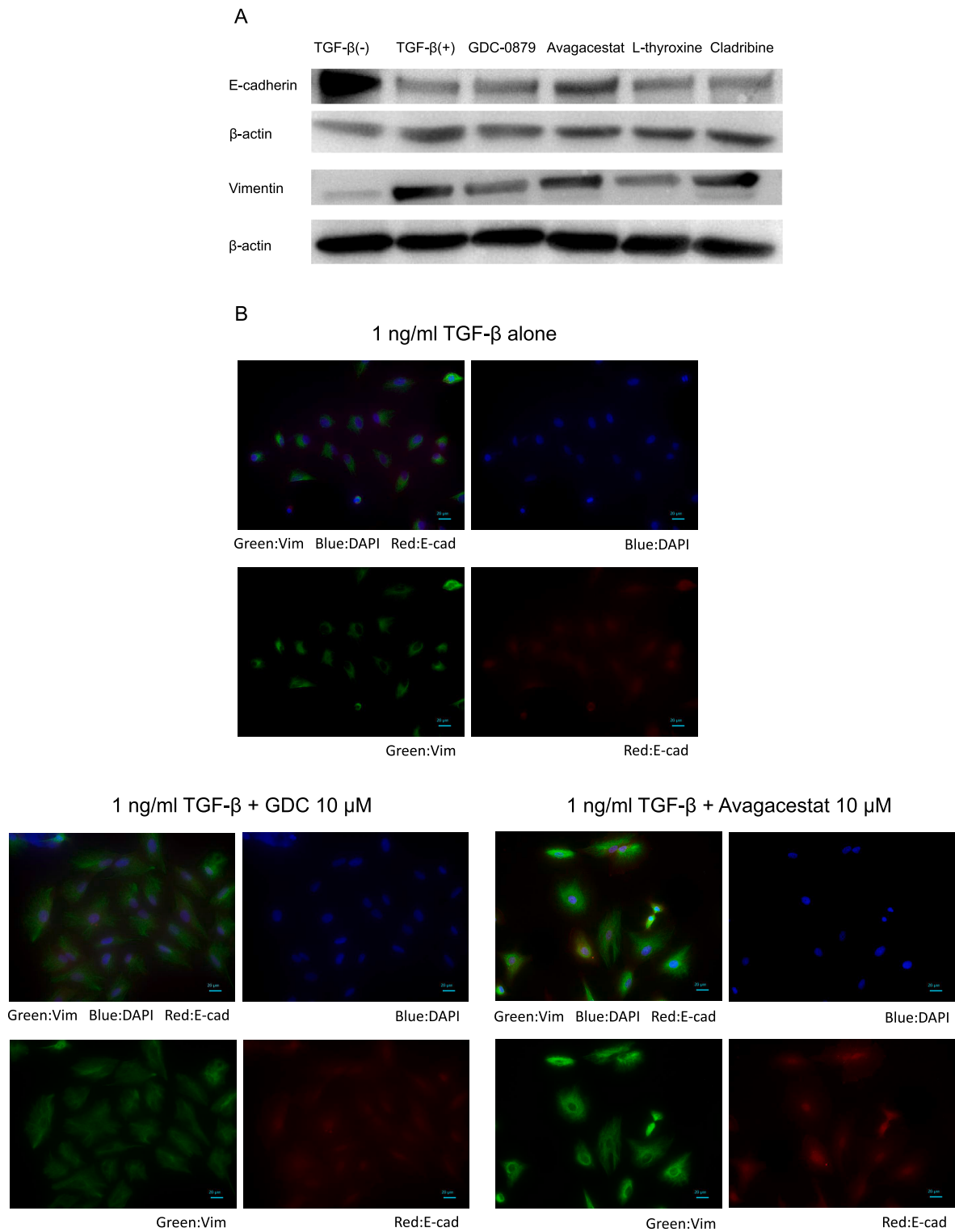
Fig. 4. (A) A549 lung cancer cell lines were cultured for 48 h and pretreated without transforming growth factor-β1 (TGF-β1), with TGF-β1 alone, or with TGF-β1 and each drug (10 μM). Cell lysates were immunoblotted to detect the indicated proteins. Avagacestat improved the decrease in E-cadherin expression, while GDC-0879 and levothyroxine (L-thyroxine) improved the increase in vimentin expression. (B) Immunofluorescence image illustrating E-cadherin and vimentin. Alexa 568 (red) staining indicates E-cadherin, Alexa 488(green) indicates vimentin, and DAPI (4′,6-diamidino-2-phenylindole) (blue) indicates the nucleus. Scale bar: 184.52 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Inhibition of invasion

Matrigel invasion assay was performed to evaluate the inhibitory effect of the candidate drugs on TGF-β1-mediated invasion. For comparison, A549 lung cancer cells were divided into the control group, TGF-β1 alone group, and TGF-β1 plus each candidate drug group. After 48 h of incubation with the candidate drug, the cells were collected, seeded onto Matrigel precoated upper chambers, and incubated for
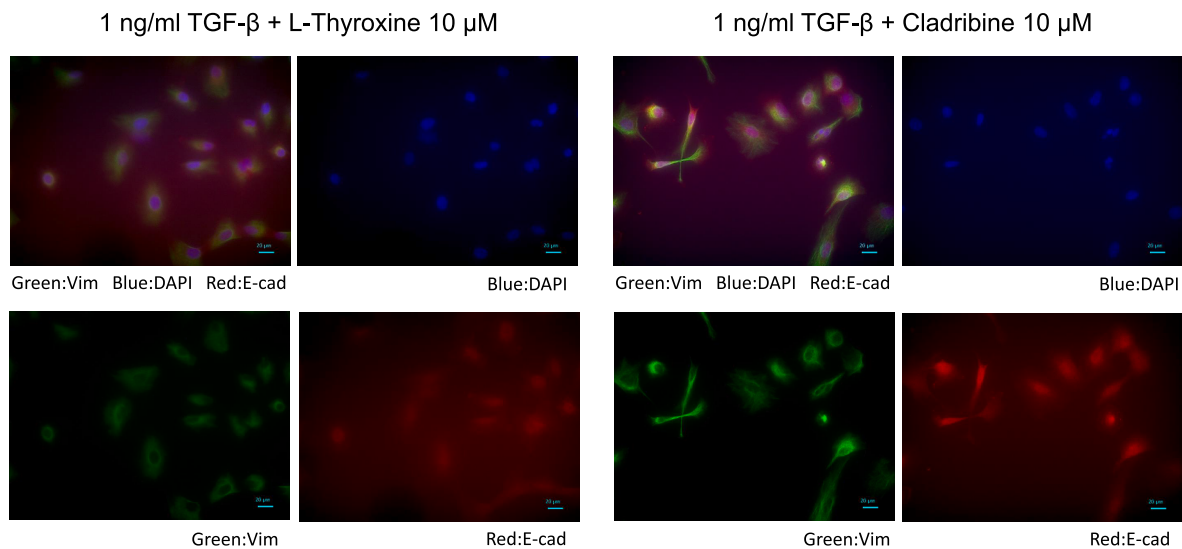
1 ng/ml TGF-β + L-Thyroxine 10 µM

Green:Vim Blue:DAPI Red:E-cad

Blue:DAPI

Green:Vim

Red:E-cad

1 ng/ml TGF-β + Cladribine 10 µM

Green:Vim Blue:DAPI Red:E-cad

Blue:DAPI

Green:Vim

Red:E-cad

Fig. 4. (*continued*).

another 22 h. Cell invasion was clearly enhanced in the presence of TGF-β1. In contract, the invasive ability was significantly inhibited in cells treated with GDC-0879 or L-thyroxine (Fig. 6).

4. Discussion

4.1. HTs

Historically, drug screening has relied extensively on animal models. However, although animal models provide useful and abundant information for drug screening, they are expensive, inefficient, and involve ethical concerns. Cell-based HTS platforms that can provide biological information *in vivo* have recently been developed to increase the effectiveness of drug screening and minimize animal testing. Such platforms are used in more than half of all drug screenings because they are biologically informative [16]. Moreover, such analyses are often conducted in two-dimensional culture in plates [17,18], and are increasingly being performed in three-dimensional (3D) cell culture models that can better reproduce tissue growth and differentiation *in vivo* [19]. In addition, new 3D model-based screening systems that can mimic the tumor microenvironment without the need for transfection or complex equipment are currently under development [20].

4.2. Drug repositioning

Molecules and their mechanisms of action are involved in numerous biological events. Hence, an existing drug may demonstrate efficacy against diseases other than those originally targeted. Drug repositioning takes advantage of this efficacy to expand the indications of drugs that are already in use as prescription medications or failed during the clinical development stage to new diseases [21]. Firstly, drug repositioning shortens the time required and reduces the cost of drug development. Secondly, it skips the steps of basic research on the pharmacodynamics and safety of a compound in humans, which are essential in the development of new drugs.

In this study, we created a screening system that does not require any special equipment or techniques, and screened a library of 2,350 compounds to evaluate its effectiveness. The results showed that three established TGF-β inhibitors and four compounds have potential inhibitory activity for EMT.

L-thyroxine is used in the treatment of hypothyroidism to replace thyroid hormones and maintain normal mental and physical activity. However, the relationship between thyroid hormones, particularly T3,

and EMT is under investigation [22]. Cladribine is an antineoplastic agent classified as a metabolic antagonist, which inhibits DNA synthesis or exerts a cytotoxic effect. Nevertheless, it is not indicated for the treatment of solid tumors [23]. GDC-0879 is a highly selective and potent RAF inhibitor. In lung cancer, the combination of dabrafenib and trametinib has been used to treat previously untreated BRAF V600E-positive NSCLC, and has shown satisfactory efficacy [24]. Gamma-secretase inhibitors, originally developed for the treatment of Alzheimer's disease, have been repurposed as anticancer agents by inhibiting Notch1 signaling [25]. Activation of Notch signaling promotes EMT caused by TGF-β via induction of SNAIL [26]. In addition, patients with NSCLC who overexpressed Notch1 and Notch3 had significantly shorter overall survival compared with those who did not [27]. Clinical trials investigating this mechanism have been conducted for various carcinomas, including lung cancer [28].

4.3. EMT in lung cancer

When tumor epithelial cells acquire a mesenchymal phenotype, they change their morphology, increase their migratory capacity, invade the surrounding stroma, and metastasize to other organs via the bloodstream and lymphatic system. The loss of cell-to-cell adhesion, development of resistance to apoptosis and chemotherapy, and acquisition of stem cell-like characteristics further promote this phenomenon. Cancer metastasis is a major cause of cancer-related death, and EMT is thought to play an important role in this process. Tumor cells with activated EMT signaling alter the tumor microenvironment in a manner that promotes metastasis to other organs [29]. In addition, it has been shown that overexpression of EMT-induced transcription factors contributes to the high malignant potential of lung cancer, including invasiveness, metastatic potential, and stem cell characteristics [30–32].

Our results obtained from the Matrigel invasion assay indicate that, among the four candidate drugs, GDC-0879 and L-thyroxine had inhibitory ability for cell invasion. The western blotting assay demonstrated that these two drugs inhibited the increase of vimentin. Through these analyses, we established an HTS experimental system for EMT inhibitors and confirmed their actual efficacy.

4.4. Therapeutic resistance

Recent studies have shown that resistance to chemotherapeutic agents in NSCLC is associated with EMT. Human NSCLC cells acquire resistance to chemotherapy when EMT is induced by TGF-β, and such
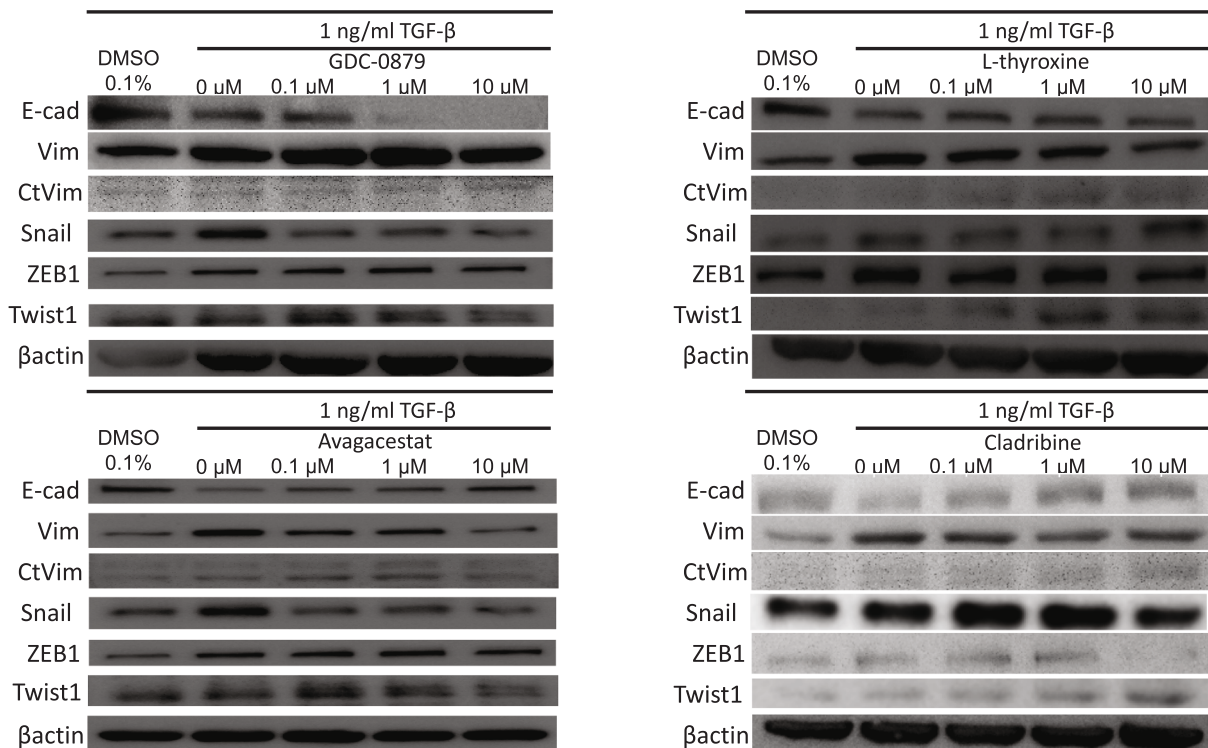
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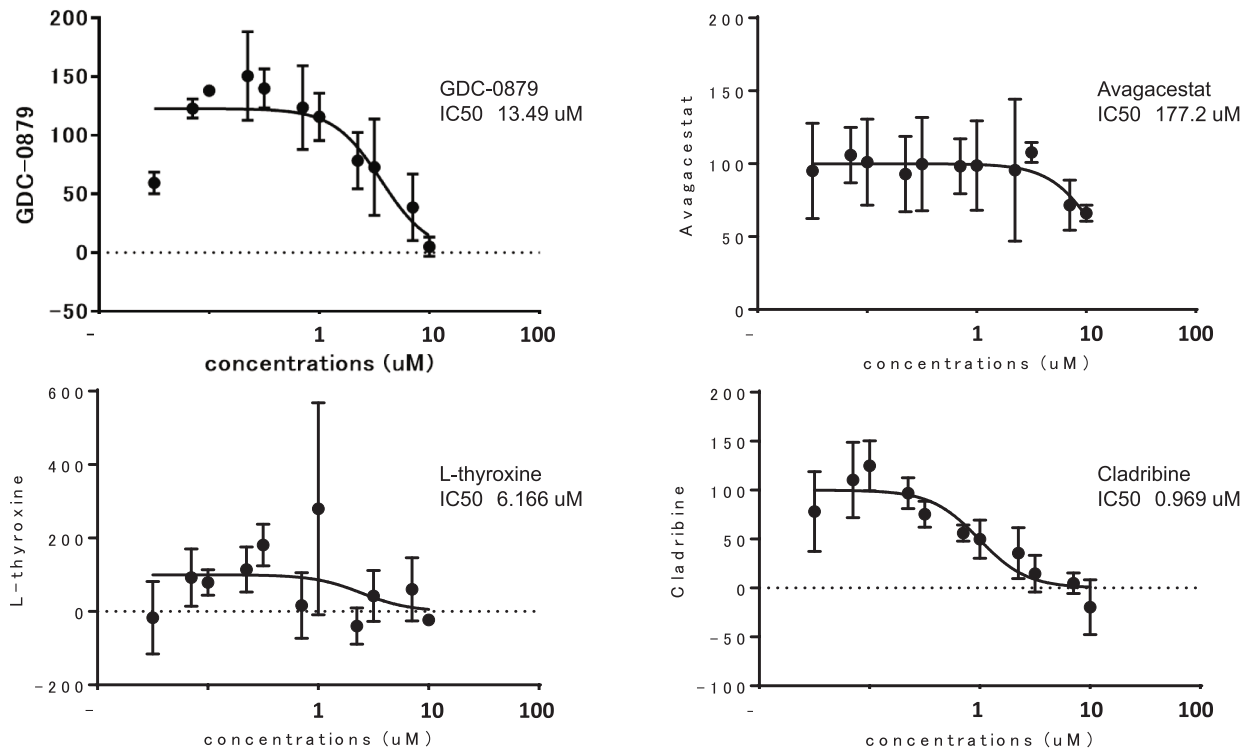
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Fig. 5. (A) Viability of A549 lung cancer cells incubated with candidate drugs (10 μM) for 48 h. Among the candidate drugs, cladribine and levothyroxine (L-thyroxine) had IC50 values < 10 μM. (B) Western blots of E-cadherin, vimentin, Ct vimentin, SNAIL (snail family zinc finger), ZEB1 (zinc finger E-box binding homeobox 1), TWIST1 (twist family bHLH transcription factor 1) and β-actin in A549 cells treated with TGF-β1 and different concentrations of target drugs. The addition of avagacestat suppressed EMT in a concentration-dependent manner.
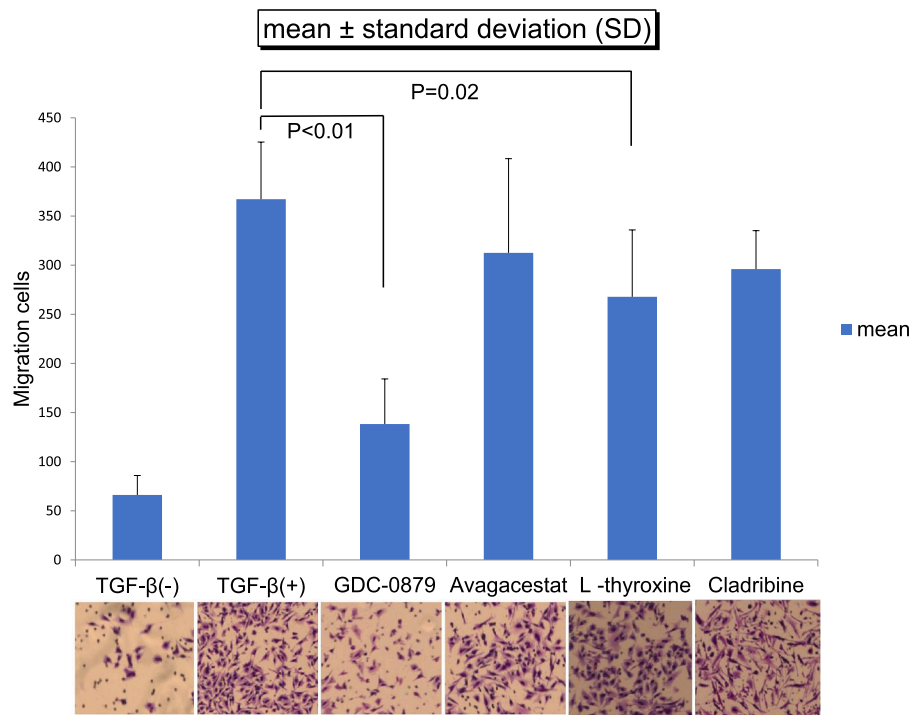
Fig. 6. Matrigel invasion assays were performed to examine the effects of each drug on transforming growth factor-β1 (TGF-β1)-mediated cell invasion. Treatment with GDC-0879 and levothyroxine (L-thyroxine) following exposure to TGF-β1 (1 ng/ml) significantly suppressed the invasive capacity of A549 cells.

EMT-associated resistance is dependent on the anti-apoptotic pathway [33].

Molecularly targeted agents, including epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs), have been introduced into the treatment of patients with NSCLC and are highly effective in patients with mutation-positive NSCLC. Although the secondary T790M mutation in EGFR has been identified as a mechanism of acquired resistance to EGFR-TKIs [34], transient stimulation with TGF-β has been shown to induce both EMT and resistance to EGFR-TKIs [35].

Following the induction of EMT, cancer cells exhibiting stem cell-like features (CSCs) emerge and can lead to the development of drug resistance. It has been demonstrated that agents (e.g., salinomycin) which induce differentiation of mesenchymal-like cancers promote the differentiation of poorly differentiated cancers (including numerous CSCs) to a more epithelial state. EMT inhibition is an effective anticancer approach [36]. In the present study, we screened candidate compounds for EMT inhibition from a collection of compounds with confirmed pharmacodynamics and safety in humans. Notably, some of those compounds may not exert complete inhibitory effects. However, it is hoped that these compounds could be used in combination with drugs for which resistance has been developed by cells, thereby potentially restoring their efficacy.

There are several limitations in this study. Firstly, we used one region of the promoter and assayed the promoter activity using the luciferase enzyme. Hence, the results may differ from those obtained through the assay performed with the actual endogenous promoter. Secondly, the screening of drugs was scored by correcting for the number of viable cells. However, because the concentration of all drugs was 10 μM, we could not correctly evaluate compounds that were highly cytotoxic.

5. Conclusions

We systematically screened approved, investigational, and druggable compounds that could inhibit EMT in TGF-β1-treated NSCLC cell lines. Using a reporter assay, we investigated changes in E-cadherin and vimentin, and identified candidate drugs for the inhibition of EMT. EMT

is involved in a wide variety of processes, such as invasion, metastasis, and drug resistance in cancer cells. Therefore, it is possible that some of the existing compounds can be reused as new anticancer agents in different settings. In addition, elucidation of the mechanisms and pathways through which compounds are involved in the regulation of EMT may promote clinical research. The methodology presented in this article may be adjusted according to specific assay systems and can be adapted to other cancer cell types. This approach can lead to the discovery of other therapeutic targets that regulate EMT and promote the development of new therapeutic agents.

CRediT authorship contribution statement

Hiroyuki Ishikawa: Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft. **Toshi Menju:** Conceptualization, Validation, Investigation, Data curation, Writing – review & editing, Funding acquisition. **Toshiya Toyazaki:** Validation, Resources. **Hideaki Miyamoto:** Validation, Resources. **Naohisa Chiba:** Validation, Resources. **Misa Noguchi:** Validation, Resources. **Shigeyuki Tamari:** Validation, Resources. **Ryo Miyata:** Validation, Resources. **Yojiro Yutaka:** Data curation, Writing – review & editing. **Satona Tanaka:** Data curation, Writing – review & editing. **Yoshito Yamada:** Data curation, Writing – review & editing. **Daisuke Nakajima:** Data curation, Writing – review & editing. **Akihiro Ohsumi:** Data curation, Writing – review & editing. **Masatsugu Hamaji:** Data curation, Writing – review & editing. **Yukiko Okuno:** Conceptualization, Methodology, Data curation, Visualization, Resources. **Hiroshi Date:** Supervision, Writing – review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lungcan.2022.11.015.

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