Overcoming epithelial-mesenchymal transition-mediated drug resistance with monensin-based combined therapy in non-small cell lung cancer

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Abstract

Background: The epithelial-mesenchymal transition (EMT) is a key process in tumor progression and metastasis and is also associated with drug resistance. Thus, controlling EMT status is a research of interest to conquer the malignant tumors.

Materials and methods: A drug repositioning analysis of transcriptomic data from a public cell line database identified monensin, a widely used in veterinary medicine, as a candidate EMT inhibitor that suppresses the conversion of the EMT phenotype. Using TGF-β-induced EMT cell line models, the effects of monensin on the EMT status and EMT-mediated drug resistance were assessed.

Results: TGF-β treatment induced EMT in non-small cell lung cancer (NSCLC) cell lines and the *EGFR*-mutant NSCLC cell lines with TGF-β-induced EMT acquired resistance to EGFR-tyrosine kinase inhibitor. The addition of monensin effectively suppressed the TGF-β-induced-EMT conversion, and restored the growth inhibition and the induction of apoptosis by the EGFR-tyrosine kinase inhibitor.

Conclusion: Our data suggested that combined therapy with monensin might be

a useful strategy for preventing EMT-mediated acquired drug resistance.

Introduction

The epithelial-mesenchymal transition (EMT) is a phenotypic conversion of epithelial features into mesenchymal features: a decrease in intracellular adhesion and cell polarity, and an increase in matrix remodeling and motility. These changes confer the tumor metastatic behaviors of cancer cell invasion and migration. Several key signaling pathways, including transforming growth factor-β (TGF-β), Wnt, Notch and Hedgehog, are involved in this process. Of these, TGF-β is considered to be a pivotal mediator of the EMT during physiological processes [1-3], and TGF-β treatment is a well-established method of inducing the EMT *in vitro* in cell line models [4]. The EMT is also known to be involved in resistance to a variety of therapeutic modalities, including chemotherapy with cytotoxic drugs or molecularly targeted drugs, as well as radiation therapy in diverse cancer types [2, 5]. The mechanism of this EMT-mediated drug resistance has been studied for decades; so far, EMT-driven cancer stem cell (CSC)-like properties, such as an increase in drug efflux and anti-apoptotic effects, have

received attention for their pivotal roles in cancer drug resistance [1, 2, 6, 7]. The EMT is a promising target for novel therapeutic strategies targeting cancer progression and drug resistance; however, this approach is potentially challenging because the EMT is intricately regulated by numerous factors, such as extracellular matrix components, diverse signal pathways, soluble growth factors or cytokines, and microRNAs [1, 2, 8].

The process of finding new uses outside the scope of the original medical indications of existing drugs is known as drug repositioning. This approach represents an increasingly promising, cost-effective alternative strategy for accelerating the development of treatments for diseases, since previous development efforts and preclinical and clinical data, especially safety profiles, are usually available [9]. Several drugs have been successfully repositioned for new indications, with two of the most notable examples being sildenafil and thalidomide [10].

In this study, we conducted an *in silico* drug repositioning analysis of transcriptomic data from a cell line database and identified monensin as a candidate EMT inhibitor capable of reversing the EMT phenotype. Monensin is a polyether ionophore that is widely used as an antibiotic in livestock animals [11].

We then assessed the inhibitory effect of monensin on the EMT phenotype and its potential to overcome EMT-mediated drug resistance.

Materials and methods

Bioinformatics analysis

Comprehensive transcriptomic profiles of 28 NSCLC cell lines were obtained from the Cancer Cell Line Encyclopedia (CCLE) database (http://www.broadinstitute.org/ccle/). Gene set enrichment analysis (GSEA) was performed using GSEA_4.0.1 software and a database of gene sets obtained from Molecular Signatures Database v5.0 (https://www.gsea-msigdb.org/gsea/) [12]. A Connectivity Map (C-MAP) analysis was performed using the tool described by Lamb and colleagues [13]. We selected 164 probes that were positively correlated with the expression pattern of mir-200c and an additional 124 probes that were negatively correlated with the expression pattern of mir-200c in the CCLE database; we then used the selected probes as queries in comparisons with the rank-ordered lists for each drug treatment in the C-MAP database. Since C-MAP analysis is only applicable for Affymetrix type probe IDs of HG-U133A Gene Chips, we transformed the probe IDs from HG-U133 plus2

Gene Chips in the CCLE database into HG-U133A based on gene symbol comparisons using the GPL96 file from GEO (http://www.ncbi.nlm.nih.gov/geo/). As a result, 87 of the 164 positive-correlated probes and 52 of the 124 negative-correlated probes were used for C-MAP analysis.

Cell culture and reagents

Two EGFR-mutant NSCLC cell lines: HCC827 (EGFR exon19del E746-A750), H1975 (EGFR L858R and T790M), one KRAS mutant NSCLC cell line, A549 (KRAS G12S) and one pancreatic cancer cell line, PANC-1 (KRAS G12D), were used in this study. All cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). These cell lines were cultured in Roswell Park Memorial Institute-1640 medium supplemented with 10% fetal bovine serum at 37 °C in humidified incubator under 5% CO_2 gas. Osimertinib, EGFR-tyrosine kinase inhibitor, was purchased from ChemScene (Monmouth Junction, NJ, USA) and monensin was purchased from Cayman Chemical (Ann Arbor, MI, USA). TGF-β was purchased from Wako (Osaka, Japan).

Cell viability assay

The sensitivity to each drug was determined by a modified MTS assay. Cells were plated at a density of 3,000 cells per well in 96-well plates, and treated with the desired concentration of drugs. Cell viability was assayed after 96 hours of treatment using CellTiter 96 Aqueous bromide One Solution Reagent (Promega, Madison, WI, USA). For measurement of IC_{50} value, each condition was assayed in eight-replicate determinations in three independent experiments. Data was analyzed by non-linear regression using Graphpad Prism Ver. 6.0.3 (GraphPad Software, San Diego, CA).

Western blotting

Cells were treated with ethanol (Wako, Osaka, Japan) as a control, 2 ng/mL TGF-β and 100 nM monensin. The total cell lysate was extracted with lysis buffer, a mixture of RIPA buffer, phosphate inhibitor cocktails 2 and 3 (Sigma-Aldrich, St. Louis, MO, USA) and complete Mini Protease Inhibitor Cocktail (Roche, Switzerland). Western blot analysis was performed by using the following primary antibodies: E-cadherin, vimentin, poly ADP-ribose polymerase (PARP), GAPDH (Cell Signaling Technology, Danvers, MA, USA) and ZEB1 (Santa Cruz Biotechnology, Dallas, TX, USA). The secondary antibody was HRP-conjugated

anti-mouse or anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA). To

detect specific signals, the membrane was examined using the ECL Prime

Western Blotting Detection System (GE Healthcare, Amersham, UK) and LAS-

3000 (Fujifilm, Tokyo, Japan). The relative band intensities were quantified using

ImageJ software (National Institute of Health, Bethesda, MD, USA).

Results

Drug repositioning analysis for reversion of EMT signatures.

We previously reported the EMT status in 34 non-small cell lung cancer (NSCLC)

cell lines [14]. The EMT status was determined based on the protein expressions

of E-cadherin, vimentin, N-cadherin, ZEB1, and ZEB2 evaluated using western

blotting analyses and the expressions of miR-200s, which are microRNAs known

to be key players in the regulation of EMT features, evaluated using qPCR. For

this study, we extracted 28 cell lines for which comprehensive gene expression

data were available in the CCLE database (http://www.broadinstitute.org/ccle/),

and we stratified them into two groups: 12 cell lines with an epithelial-like phenotype, and 16 cell lines with a mesenchymal-like phenotype (Fig. 1A). We then performed GSEA to investigate the validity of this classification, and we confirmed that the Epithelial_Mesenchymal_Transition gene set was significantly enriched in the mesenchymal-like phenotype group (Fig.1B). Next, to identify reagents with the potential to reverse the EMT, we conducted C-MAP analysis. C-MAP is a widely used computational method for drug repositioning, containing a large transcriptomic compendium of drug-treated human cancer cells [13, 15]. By testing user-provided gene lists (query list) against a large number of transcriptional profiles, potentially bioactive small molecules can be screened [13, 15]. Based on the transcriptional profiles of 28 NSCLC cell lines, we selected 87 "epithelial-like" probes with positive correlations to mir-200c expression, including CDH1, as well as 52 "mesenchymal-like" probes with negative correlations to mir-200c expression, including ZEB1 (Supplementary Table 1). We then used these probes as a query list for comparisons with the rank-ordered lists for each drug treatment in the C-MAP database. We identified multiple Food and Drug Administration (FDA)-approved drugs with a significantly biased appearance of the query genes toward an epithelial-like feature (Table 1). The first ranked drug,

thioridazine, is an antipsychotic drug; however, it was withdrawn from the market worldwide in 2005 because of its effect on the central nervous system and cardiotoxicity. We then focused on the second-ranked drug monensin, which is a polyether ionophore that is currently used as an antibiotic in livestock animals. The results of Kolmogorov-Smirnov scanning for monensin are shown in Fig. 1C. Furthermore, all six independent experiments using different cell lines with different doses showed a positive effect on epithelial-like features (Fig. 1D). In summary, this bioinformatics approach identified monensin as a candidate anti-EMT drug.

TGF-β stimulation facilitates EMT in cancer cells, leading to resistance to molecularly targeted therapy

TGF-β is a critical effector facilitating EMT through the modulation of Smad signaling, and TGF-β treatment is a well-established method for inducing the EMT *in vitro* [4]. To assess the suppressive effect of monensin on the switch to EMT, we used TGF-β-induced EMT models *in vitro*. First, we examined the facilitating

EMT effect of TGF-β in three NSCLC cell lines (A549, HCC827 and H1975) and one pancreatic cancer cell line (PANC-1). As expected, following TGF-β stimulation for 72 hours, these cells showed an up-regulation of the mesenchymal markers vimentin and ZEB1 and/or a down-regulation of the epithelial marker E-cadherin (Fig. 2A). Morphological changes were also observed; some of the TGF-β-treated cells transformed to a spindle cell shape (Fig. 2B), indicating that these cell lines acquired an EMT phenotype as a result of TGF-β treatment. Next, to determine the drug sensitivities of TGF-β-induced EMT cell models, we compared the sensitivity to osimertinib, an EGFR tyrosine kinase inhibitor, in the EGFR-mutant NSCLC cell lines HCC827 and H1975 in the presence or absence of TGF-β. In the absence of TGF-β, cell proliferation was effectively suppressed in both cell lines, whereas cells with TGF-β-induced EMT in the presence of TGF-β were less sensitive to osimertinib (Fig. 2C). These results show that TGF-β stimulation induced EMT and that TGF-β-induced EMT NSCLC cell models are useful for investigating EMT-mediated drug resistance.

Combined therapy with monensin as a therapeutic strategy for preventing EMT-mediated resistance

To validate the *in silico* findings of monensin as a candidate anti-EMT drug, we tested the inhibitory effect of monensin on the EMT using TGF-β-induced EMT cell models. Cells were cultured in the presence or absence of monensin and/or TGF-β for 72 hours, and the protein expressions of EMT markers were then analyzed. As shown in Fig. 3A, the addition of monensin suppressed the up-regulation of vimentin by TGF-β stimulation in HCC827 and A549 cells; the up-regulation of ZEB1 in H1975, A549 and PANC-1 cells; and the down-regulation of E-cadherin in H827, H1975 and PAC-1 cells; although some of these changes were only slight. These results suggest that monensin has a partial inhibitory effect on TGF-β-induced EMT. We then examined the combinatorial effect on cell growth of osimertinib and monensin in cell viability assays to confirm whether acquired resistance to EGFR-TKI thorough TGF-β-induced EMT could be overcome by combination therapy with monensin. Treatment with monensin alone showed some effect on cell growth, with IC_{50} values of 1.06 μM for HCC827 cells, 1.76 μM for H1975 cells, 0.74 μM for A549 cells, and 0.29 μM for PANC-1 cells (Fig. 3B), and the addition of monensin restored the growth inhibitory effect of osimertinib in the two EGFR-mutant NSCLC cell lines with TGF-β-induced EMT

(Fig. 3C). To evaluate drug-induced apoptosis, cells were treated in the presence or absence of monensin and/or TGF-β for 72 hours, and the expression of the apoptosis marker cleaved-PARP was analyzed using western blotting. The expression of cleaved-PARP was suppressed by TGF-β treatment, while the addition of monensin restored the induction of apoptosis by osimertinib (Fig. 3D). Taken together, these results showed that the addition of monensin could restore the growth inhibitory effect of osimertinib by preventing TGF-β-induced EMT.

Discussion

Acquired drug resistance against molecularly targeted therapy has been intensely studied especially in EGFR-mutant NSCLCs, and the most prominent mechanism of the secondary EGFR T790M mutation can be targeted successfully using third-generation drugs such as osimertinib [16]. However, the long-term clinical benefits remain unsatisfactory because a diversity of other mechanisms also contribute to drug resistance. One of the key mechanisms of

resistance is the acquisition of an EMT signature, which is involved in not only molecularly targeted drugs, but also chemotherapy with cytotoxic drugs or radiation therapy in diverse cancer types [17-19]. Therefore, a novel therapeutic strategy to overcome this resistance is a promising approach to improving outcomes. In the present study, we used a bioinformatics approach using C-MAP analysis to search for drugs with the potential to revert the EMT, and we identified monensin as a candidate anti-EMT drug. *In vitro* validation analyses demonstrated that monensin inhibited EMT conversion and improved drug response.

Monensin is a polyether ionophore antibiotic that is known to be a promoter of muscle growth in veterinary medicine. Monensin has been added to cattle and poultry feed for years and has a positive safety profile [11]. Prior to this study, monensin had been reported to exhibit an anti-cancer effect by inhibiting cell proliferation, cell cycle progression and cell migration and by inducing cell apoptosis in diverse cancer types, such as lung cancer, pancreatic cancer, renal cancer, colon cancer, prostate cancer, and ovarian cancer [20-24]. Of note, malignant cells were 20-fold more sensitive to monensin than normal cells, indicating that monensin could be a cancer-specific therapeutic drug [22].

Mechanically, monensin has been shown to induce a Golgi apparatus stress response, mitochondrial damage, and oxidative stress, initiating signaling pathways to trigger apoptosis through the up-regulation of Bax, caspase-3 and caspase-8 [22, 24, 25]. Monensin has also been reported to target multiple signaling pathways, such as EGFR, Hedgehog, Wnt, CDK6, cyclin D1, and cyclin A, as well as multiple transcript factors, including E2F/DP1, STAT1/2, NFκB, AP-1 and Elk-1/SRF [20, 21, 23, 24, 26]. Of note, Vanneste et al. conducted a systematic high throughput screening for compounds with anti-EMT activity and demonstrated that EMT-like cells exhibited a greater uptake of monensin compared with cells with epithelial features, leading to a selective cytotoxic effect on an EMT phenotype [25]. However, our data using an isogenic NSCLC cell line model showed no relationships between the sensitivity to monensin and the EMT status (Supplementary Figure 1A and 1B). Specifically, we established a distinct isogenic cell model using HCC827 cells that had been chronically exposed to osimertinib, and the resistant cells acquired an EMT phenotype (Supplementary Figure 1A), leading to EMT-mediated drug resistance to EGFR-TKI and cytotoxic reagents [27]. In terms of sensitivity to monensin, unexpectedly, no significant differences were observed between the parental cells and the resistant cells

despite their different EMT statuses (Supplementary Figure 1B). Furthermore, monensin treatment did not restore the epithelial phenotype (Supplementary Figure 2A) or the sensitivity to EGFR-TKI in the resistant cells with EMT conversion (Supplementary Figure 2B). Considering these findings, monensin might not have a direct effect on the EMT signatures in our resistant cells, and the inhibition of EMT conversion might be obtained through the suppression of some EMT-facilitating factors, including TGF-β. Furthermore, its effect likely differs depending on the cancer type. Further study is warranted to elucidate this intriguing biological mechanism.

In the 1990s, the calcium channel blocker verapamil was tested in a clinical trial as a chemosensitizer to reverse drug resistance by inhibiting a drug efflux pump, which is the one of the key functions of EMT-mediated resistance. However, because of the dose-limiting toxicity, verapamil did not show any clear evidence of a beneficial effect on cytotoxic chemotherapy reagents [1]. Monensin has been approved by the FDA for veterinary use and has been shown to have a positive biosafety profile in veterinary medicine. In previous studies, the antitumor activity of monensin was demonstrated in an *in vivo* model, and no significant adverse effects were seen even at a much higher dose of monensin than the 100-nM dose

used in the present study[20, 23]. Nonetheless, in attempting to translate our findings clinically, careful assessment of any possible toxic effects of long-term use in humans is warranted prior to repositioning monensin as a combinatory therapy with standard antitumor reagents.

In conclusion, the current study demonstrated the possible utility of monensin as a prophylactic when co-administered with antitumor reagents to prevent the acquisition of an EMT signature, thereby prolonging the disease-control period.

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Figure Legends

Fig. 1. Identification of reagents with the potential to reverse the EMT using C-MAP analysis. (A) Classification of 28 NSCLC cell lines according to their EMT status. (B) Representative GSEA enrichment plots for the Epithelial Mesenchymal Transition gene sets for 28 NSCLC cell lines. (C) Representative results of Kolmogorov-Smirnov scanning showing an enrichment of outcome-associated probe sets for the 4th ranked monensin, indicating the up-regulation of 87 epithelial-like probes and the down-regulation of 52 mesenchymal-like

probes. (D) Summaries of all instances of monensin. The barview shows the instances of monensin in a total of 6100 instances.

Fig. 2. Establishment of TGF-β-induced EMT models using three NSCLC cell lines and one pancreatic cancer cell line. (A) Cells were treated with TGF-β (2 ng/mL) for 72 hours and then subjected to immunoblotting. The relative band intensity was quantified using a densitometric analysis with ImageJ software. (B) PANC-1 and A549 cells were treated with TGF-β (2 ng/mL) for 5 days; representative microscopy images are shown. The scale bar represents 100 nm. (C) HCC827 and H1975 cells were pretreated with TGF-β (2 ng/mL) for 24 hours and were then subsequently treated with osimertinib for 96 hours. Cell viability was determined using CellTiter 96 Aqueous bromide dye. Each experiment was assayed in eight replicate determinations, and the data are representative of three independent experiments (mean ± SE).

Fig. 3. Monensin restores the growth inhibitory effect of osimertinib through the prevention of TGF-β-induced EMT. (A) Cells were cultured in the presence or absence of monensin (100 nM) and/or TGF-β (2 ng/mL) for 72 hours, then

subjected to immunoblotting. The relative band intensity was quantified using a densitometric analysis with ImageJ software. (B) Cells were treated with monensin for 96 hours. Cell viability was determined using CellTiter 96 aqueous bromide dye. Each experiment was assayed in eight replicate determinations, and the data are representative of three independent experiments (mean ± SE). (C) HCC827 and H1975 cells were pretreated with TGF-β (2 ng/mL) in the presence or absence of monensin (100 nM) for 24 hours, then subsequently treated with osimertinib for 96 hours. Cell viability was determined using CellTiter 96 Aqueous bromide dye. Each experiment was assayed in eight replicate determinations, and data are representative of three independent experiments (mean ± SE). (D) HCC827 and H1975 cells were pretreated with TGF-β (2 ng/mL) in the presence or absence of monensin (100 nM) for 24 hours, then followed by osimertinib treatment for 72 hours.

Table 1 Permuted results of C-MAP analysis (top 20 list of reagent)

Rank	cmap name	n	Enrichment	P value
1	thioridazine	182	0.475	0.0001
2	monensin	20	0.731	0.00101
3	sulfamonomethoxine	12	0.806	0.00265
4	mebeverine	6	0.801	0.00298
5	perphenazine	4	0.726	0.00348
6	zuclopenthixol	4	0.782	0.0042
7	oxamniquine	4	0.782	0.00422
8	gibberellic acid	5	0.764	0.00585
9	phentolamine	4	0.599	0.00605
10	meclofenamic acid	4	0.695	0.00655